Regulation of soil organic matter dynamics and microbial activity by endogeic earthworms

Von dem Fachbereich Biologie der Technischen Universität Darmstadt

zur

Erlangung des akademischen Grades

eines Doctor rerum naturalium

genehmigte

Dissertation von

Dipl. –Biol.

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Tag der Einreichung: 24. Januar 2007

Tag der mündlichen Prüfung: 16. März 2007

Darmstadt 2007

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Denn das Leben ist wie das Wetter. Tausende von kleinen Fliegen, die von einem gewaltigen Tief nach unten gedrückt werden. Du gehst da durch, und sie verfangen sich in deinen Haaren, kleben in deinen Augenwinkeln, jucken in deinen Kleidern. So lange, bis es regnet. Dann sind sie für ein Weilchen fort.

A.S.

Wer seine Hände in den Schoß legt, muss deshalb nicht untätig sein.

Casanova

Contents

Chapter Five - Earthworms and the soil microbial food web - Insight into a `black box´ using RNA stable isotope probing

Summary

Earthworms are among the most important members of the soil invertebrate fauna in temperate ecosystems. Through burrow construction, mixing of organic matter and mineral soil, comminution of organic matter and casting they beneficially affect soil structure and chemical properties, and thus the biomass, activity and community composition of microorganisms. Microorganisms, particularly bacteria and fungi, are the primarily decomposers of organic matter in soils. Since earthworms control the microbial community they also affect the fate of soil organic matter which may lead either to its mobilization or stabilization. This study investigates the role of different factors presumably controlling the interaction of earthworms and microorganisms with regard to the stabilization and mobilization of soil organic matter.

The effect of endogeic earthworms (*Octolasion tyrtaeum* (Savigny)) and the availability of clay on the mobilization and stabilization of ^{14}C-labelled catechol mixed into an arable and a forest soil was investigated. Production of $^{14}CO_2$ and accumulation of ^{14}C in humic fractions of casts and the non-processed soils were determined. *Octolasion tyrtaeum* did not affect $^{14}CO_2$-C production in the forest soil, but increased it early in the arable soil; clay counteracted the effect of *O. tyrtaeum* in the arable soil. Clay and *O. tyrtaeum* did not affect integration of ^{14}C into the humic fractions of the forest soil. In contrast, in the arable soil *O. tyrtaeum* increased the amount of ^{14}C in the labile fractions, whereas clay increased it in the humin fraction, indicating that endogeic earthworms and clay are only of little importance in soils with high organic matter content, high clay content and high microbial biomass, but contrarily affect phenolic compounds in poor soils. Endogeic earthworms strongly increase microbial activity and thus mineralization of phenolic compounds, whereas clay decreases it by binding phenolic compounds when passing through the earthworm gut.

The effect of cutting of shoots of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) on the availability of soluble carbon in the rhizosphere, and on the biomass and activity of soil microorganisms and juveniles of the endogeic earthworm species *O. tyrtaeum* (Savigny) and *Aporrectodea caliginosa* (Savigny) were studied. Both plant species increased microbial biomass in the soil, but microbial activity remained unaffected. Although defoliation increased the availability of carbon in the rhizosphere as indicated by the increase in microbial biomass, the biomass of both earthworm species decreased during the experiment, suggesting that earthworms were unable to exploit the microbial carbon pool in soil, rather, rhizosphere microorganisms appeared to have effectively competed with endogeic earthworms and exploited root carbon exudates.

The interaction of saprophytic fungi and *O. tyrtaeum* on the translocation and stabilization of litter derived carbon ($^{13}C/^{15}N$ labelled rye leaves) into the upper layer of an arable soil was investigated in a microcosm experiment. Phospholipid fatty acids and ergosterol were used as marker molecules to determine the effects of earthworms on bacterial and fungal abundance and microbial community composition. The results indicate that saprophytic fungi translocate and stabilize litter derived carbon in their mycelial network in the upper mineral soil. Endogeic earthworms reduce fungal biomass by grazing and thereby counteract the fungal stabilization of carbon. In addition, earthworms reduce bacterial biomass in the soil while mainly competing with Gram-negative bacteria for labile carbon resources.

The flux of bacterial carbon (^{13}C labelled *Serratia marcescens* cells) through the soil microbial community as affected by the passage through the gut of endogeic earthworms *A. caliginosa* was investigated by means of terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments and rRNA stable isotope probing (SIP). Production of $^{13}CO_2$-C was strongly increased in presence of *A. caliginosa* and in casts compared to the soil. Microbial communities in casts and soil after one day of incubation were almost similar and consist of eleven different bacterial lineages, with *Proteobacteria* and *Actinobacteria* being the most abundant; however they differed in the abundance of *Acidobacteria*, *Firmicutes* and *Planctomycetes*. After six days microbial community in the soil was almost similar to that of casts at day one. The clone library of the casts, in contrast, consisted of only five different lineages and was dominated by members of the phylum *Firmicutes*, predominantly *Staphylococcus* spp., indicating that the passage through the gut of endogeic earthworms considerably changes the activity and composition of the microbial community, and the mineralization and incorporation of bacterial carbon.

The results of the present study document that endogeic earthworms increase the mineralization of soil organic matter by promoting, feeding on or competing with specific microorganisms in the soil. The effect, however, also depends on abiotic soil conditions such as soil texture.

Zusammenfassung

Regenwürmer zählen zu den bedeutendsten Organismen der Bodenfauna in temperierten Ökosystemen. Durch ihre Grabtätigkeit, die Durchmischung von organischer Substanz mit mineralischem Bodenbestandteilen und die Ablagerung von Kotaggregaten fördern sie die Struktur und chemischen Eigenschaften des Bodens, und auf diese Weise die Biomasse, Aktivität und Zusammensetzung der mikrobiellen Bodengemeinschaft. Mikroorganismen, vor allem Bakterien und Pilze, sind die Hauptzersetzer der organischen Substanz im Boden. Da Regenwürmer die mikrobielle Gemeinschaft kontrollieren, steuern sie die Stabilisierung oder Mobilisierung der organischen Substanz im Boden. In der vorliegende Arbeit wurden verschiedene Einflussfaktoren untersucht, welche die Interaktion zwischen Regenwürmern und Mikroorganismen im Hinblick auf die Stabilisierung und Mobilisierung organischer Substanz im Boden kontrolliert.

Der Einfluss der endogäischen Regenwurmart *Octolasion tyrtaeum* (Savigny) und der Verfügbarkeit von Ton auf die Mobilisierung und Stabilisierung von homogen in einen Acker- und einen Waldboden eingemischtem, ^{14}C markiertem Catechol, wurde in einem Mikrokosmosversuch untersucht. Die Produktion von $^{14}CO_2$ und die Anreicherung von ^{14}C in den unterschiedlichen Huminstoffen in Kotaggregaten und Boden wurden bestimmt. *Octolasion tyrtaeum* hatte keinen Einfluss auf die Produktion von $^{14}CO_2$ im Waldboden aber erhöhte sie zu Beginn im Ackerboden. Die Verfügbarkeit von Ton wirkte dem mobilisierendem Effekt von *O. tyrtaeum* im Waldboden entgegen. Die Verfügbarkeit von Ton und *O. tyrtaeum* hatten keinen Einfluss auf die Anreicherung von ^{14}C in den unterschiedlichen Huminstoffen des Waldbodens. Im Ackerboden hingegen erhöhte *O. tyrtaeum* die Anreicherung von ^{14}C in den leicht abbaubaren Huminstoffen, während die Verfügbarkeit von Ton die Anreicherung von ^{14}C in den schwer abbaubaren Huminstoffen erhöhte. Die Ergebnisse zeigen, dass endogäische Regenwürmer und die Verfügbarkeit von Ton nur einen geringen Einfluss auf die Festlegung phenolischer Verbindungen in Böden mit hohem Gehalt an organischer Substanz, hohem Gehalt an Ton und hoher mikrobiellen Biomasse haben. In Böden mit geringem Gehalt an organischer Substanz, geringem Gehalt an Ton und geringer mikrobiellen Biomasse erhöhen endogäische Regenwürmer die Aktivität der mikrobiellen Gemeinschaft, und damit die Mobilisierung phenolischer Verbindungen, während Ton phenolische Verbindungen durch die Durchmischung im Verlauf der Darmpassage stabilisiert.

In einem weiteren Experiment wurde untersucht wie sich der Beschnitt der oberirdischen Biomasse einer Leguminose (*Trifolium repens*) und eines Grases (*Lolium perenne*) auf die Verfügbarkeit von Kohlenstoffexsudaten in der Rhizosphere, und auf die Biomasse und

Aktivität von Bodenmikroorganismen und juvenilen endogäischen Regenwürmern (*O. tyrtaeum, Aporrectodea caliginosa*) auswirkt. Beide Pflanzen erhöhten die Biomasse jedoch nicht die Aktivität der Mikroorganismen im Boden. Obwohl der Beschnitt die Kohlenstoffverfügbarkeit und die mikrobielle Biomasse im Boden erhöhte, nahm die Biomasse der Regenwürmer im Laufe des Experiments ab. Die Ergebnisse deuten darauf hin, dass Regenwürmer mikrobiellen Kohlenstoff in der Rhizosphere nicht nutzen können und weiterhin, dass Mikroorganismen in der Rhizosphere erfolgreich mit endogäischen Regenwürmern um Kohlenstoffressourcen konkurrieren.

Die Interaktion saprophytischer Pilze und *O. tyrtaeum* auf die Translokation und Stabilisierung streubürtigen Kohlenstoffs in die obere Schicht eines Ackerbodens wurde in einem Mikrokosmosversuch untersucht. Die Analysen des Phospholipid-Fettsäure-Muster (PLFA) und des Ergosterolgehaltes des Bodens dienten der Untersuchung des Einflusses von *O. tyrtaeum* auf die bakterielle und pilzliche Biomasse. Die Ergebnisse zeigen, dass saprophytische Pilze streubürtigen Kohlenstoff in den Boden eintragen und stabilisieren. Endogäische Regenwürmer fressen an den Pilzmyzelien und mobilisieren den darin enthaltenen Kohlenstoff. Weiterhin konkurrieren endogäische Regenwürmer mit Gram-negativen Bakterien im Boden um gelösten Kohlenstoff und reduzieren die bakterielle Biomasse.

In einem weiteren Experiment wurde der Effekt von *A. caliginosa* auf die Nutzung von ^{13}C markierten Bakterien (*Serratia marcescens*) im mikrobiellen Nahrungsnetz mit Hilfe der T-RFLP Analyse von 16S rRNA Fragmenten und des Stable Isotope Probing (SIP) untersucht. Aporrectodea caliginosa erhöhte die Produktion von $^{14}CO_2$. Die Zusammensetzung der bakteriellen Gemeinschaft im Kot und Boden unterschied sich nach einem Tag nur sehr gering und wurden von *Proteobacteria* und *Actinobacteria* dominiert, unterschieden sich jedoch in ihrem Gehalt an *Acidobacteria, Firmicutes* und *Planctomycetes*. Nach sechs Tagen war die Zusammensetzung der bakteriellen Gemeinschaft im Boden nahezu identisch zu der im Kot nach einem Tag. Die bakteriellen Gemeinschaft im Kot hingegen wurde durch *Firmicutes*, hauptsächlich *Staphylococcus* spp. Dominiert. Die Ergebnisse zeigen, dass die Passage durch den Darm von endogäischen Regenwürmern einen beträchtlichen Einfluss auf die Aktivität und Zusammensetzung der bakteriellen Gemeinschaft hat und das mikrobielle Nahrungsnetz und die Mineralisation beeinflusst.

Die Ergebnisse der Arbeit zeigen, dass endogäische Regenwürmer die organische Substanz im Boden durch Fraß von Mikroorganismen und Konkurrenz mit Mikroorganismen mobilisieren. Diese Effekte sind jedoch von abiotischen Bodenfaktoren wie Bodentextur abhängig.

Chapter

1

General Introduction

1. General Introduction

1.1 Carbon cycle and global change

The earth contains approximately 10^{23} g carbon with the oceans, comprising 38.000 PgC (PgC = 10^{15} g carbon) being the largest pool on the earth. The atmosphere harbours 730 PgC, whereas approximately 2000 PgC is stored in terrestrial ecosystems with 1500 PgC accumulated in the soil and 500 PgC in the vegetation (Schimel, 1995).

Carbon is continuously cycled between reservoirs in the ocean, on the land, and in the atmosphere, where it occurs primarily as carbon dioxide. For instance, terrestrial ecosystems withdraw carbon from the atmosphere through photosynthesis and add it again through respiration and decay. In addition, the annual carbon uptake of the oceans is estimated to be approximately 92 PgC, with 90 PgC return into the atmosphere (further details are given in: http://www.ipcc.ch). However, the current increase of CO_2 in the atmosphere is a non steady state condition, due to anthropogenic activities such as extensively forest cutting and burning, the disruption of soils and fossil fuel burning. The release of CO_2 is faster than the ability of soils, oceans and vegetation to take it up, resulting in a net transfer of carbon from biomass into the atmosphere.

The annual emission of carbon by anthropogenic activities is estimated to be approximately 7.1 ± 1.1 PgC yr^{-1}. During the same period around 3.3 ± 0.2 PgC yr^{-1} are stored in the atmosphere and 2.0 ± 0.8 PgC yr^{-1} in the oceans. Approximately 0.5 ± 0.5 PgC yr^{-1} is believed to be stored in forest regrowth in the northern hemisphere (Schimel, 1995). While the fluxes among the pools should balance, the average values lead to a 'missing sink' of 1.3 ± 1.5 PgC yr^{-1}. This reflects the primary problem in our understanding of the current state of the global carbon cycle. It is unclear where the anthropogenic CO_2 remains. Although it is possible that this 'missing sink' could be located in any of the major pools of carbon on earth, it is most likely that the pools with a shorter residence time such as vegetation, soils or the ocean are most important.

Several studies indicate that terrestrial ecosystems particularly soils, which contain the largest dynamic carbon pool on earth, are the most likely repository of this carbon and serve as substantial sinks for anthropogenic CO_2 (Cramer et al., 2001; Pacala et al., 2001; Schimel et al., 2001); however, they may also serve as source for CO_2 through soil respiration (Torbert et al., 1997; Swift, 2001; Six et al., 2002).

The stabilization and mobilization of carbon in the soil is inextricably linked to global climate change and the “greenhouse effect”. The “greenhouse effect” generally negatively used, is a naturally occurring process affecting the heating of the earth surface and atmosphere and is

essential for suitable temperature conditions and life on earth (Schlesinger, 1997). Briefly, atmospheric gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) absorb longwave radiation emitted from the surface of the earth to maintain the average temperature on the earth at 15°C. Without this process the average temperature would be rather -15°C and life on earth would be impossible. However, since the "industrial revolution" in the beginning of 1700 the activities of humans have increased the concentration of the major greenhouse gases (Schlesinger, 1997). Next to methane (CH_4), CO_2 is the most important atmospheric climate gas with the highest emission and concentration in the atmosphere. Before the "industrial revolution" levels of CO_2 in the atmosphere were approximately 280 ppm (parts per million). Since 1750 anthropogenic activities particularly fossil fuel combustion for industry, transportation, disruption of soils and destruction of vegetation caused a steady increase of atmospheric carbon dioxide reaching approximately 380 ppm at present. As a general prediction the greenhouse effect will enhance and the earth climate will become warmer.

Recent studies show that the accumulation of greenhouse relevant gases in the atmosphere has contributed to a temperature rise of 0.6 ± 0.2 °C since the end of nineteenth century (IPCC). It is supposed that increased temperature will significantly modify the capacity of terrestrial ecosystems to store carbon (Davidson et al., 2000; Davidson and Janssens, 2006). For instance, increased temperature and concentrations of CO_2 might result in an increased biomass production hence organic matter allocation into soils. In addition, environmental conditions for soil microorganisms and soil fauna key organisms in nutrient cycling processes are supposed to be improved by increased temperature and organic matter allocation. This may result either in increased stabilization or mobilization of organic matter in soils. However, due to the multitude of factors influencing the fate of carbon in soils there is still considerable uncertainty about the mechanisms controlling the stabilization and mobilization of carbon in terrestrial ecosystems, particularly in the soil.

1.2 Soil organic matter

The initial source of organic matter in soils is the fixation of atmospheric CO_2 by plant photosynthesis. When the plants die their organic matter enters the soil as plant residues, both above- and belowground, such as dead roots, litter and woody parts of plants. In addition, a considerable proportion of organic matter enters the soil as belowground input from living plants in the form of rhizodeposites. Although the amount of rhizodeposites is difficult to determine, it is estimated that up to 5-30% of total assimilated carbon becomes incorporated into the soil (Liljeroth et al., 1990; Johansson, 1992; Swinnen et al., 1995).

Soil organic matter is composed of different groups of constituents that vary considerably in their chemical composition. Generally, soil organic matter represents the organic constitutes of the soil including undecayed plant and animal tissue, but commonly referred to non living components resulting from biological and chemical transformation as fragmented plant litter, labile organic materials such as carbohydrates, lipids, amino acids and recalcitrant polymorph organic compounds termed as humus (Sollins et al., 1996; Scheffer and Schachtschabel, 2002). The turnover time of soil organic matter vary widely and ranges from hours for highly decomposable sugars exuded by roots, to more than 100.000 years for carbon associated with noncrystalline minerals (Torn et al., 1997).

Carbohydrates, structural components of plants, such as cellulose and hemicellulose or simple sugars, are utilized by microorganisms and released as mono-, oligo- or polysaccharides. They increase the stability of soil aggregates, contribute to cation exchange capacity and serve as energy source for microorganisms and soil fauna. Soil lipids are a diverse group of materials ranging from simple compounds as fatty acids to complex substances as waxes, sterols or terpens, affecting plant growth and soil physical conditions, such as water holding capacity. Amino acids enter the soil in the form of proteins and peptides, being either rapidly utilized by microorganisms and or may be protected from degradation by encapsulation into the network of refractory biopolymers (Knicker and Hatcher, 1997).

Humus consists of non-cellular organic matter accumulated in the soil. Although humic substances possess typical physical and chemical properties they do not belong to well defined compounds, such as lipids, carbohydrates or amino acids. Humic substances are of relatively high molecular weight containing an aromatic or numerous aromatic rings with phenolic or organic acid groups and several attached organic and inorganic compounds (Baldock and Skjemstad, 2000). They form recalcitrant complexes with clay particles responsible for cation exchange and water holding capacity in the soil. However, on the basis of its solubility humic substances are divided in three different groups. Fulvic acids are of light brown to yellow colour and soluble in water. Humic acids are of dark brown to black colour and not soluble in water under acid conditions, whereas humin consists of dark brown to black insoluble substances in the soil (Scheffer and Schachtschabel, 2002). Humic substances are ubiquitous in the soil and are the most important carbon reservoir in terrestrial ecosystems. They are highly recalcitrant, protecting organic matter from microbial mineralization (Baldock and Skjemstad, 2000).

Organic matter is the most important component of soils, playing a key role in determining physical, chemical and biological processes, such as the binding of soil particles, aeration, soil temperature and water holding capacity, soil pH and cation exchange capacity. In addition, soil organic matter forms the nutritional basis for soil macrofauna and microorganisms (Oades,

1988). On the other hand the fate of organic matter in soils is affected by climate, the quality of the organic matter, the physical soil environment and certainly the activity of soil invertebrates and soil microbial biomass.

Once entered the soil, organic matter is subject to decomposition and mineralization by soil invertebrates and microorganisms. Soil microorganisms, primarily bacteria and fungi, contribute to the mineralization of organic matter in soil leading to the release of CO_2, H_2O and plant available nutrients. However, they may also transform organic matter into more resistant organic compounds, referred to as humus, thereby contribute to the stabilization of carbon in the soil matrix. Soil invertebrates such as earthworms are only of minor importance for the mineralization of organic matter. They considerable contribute to the decomposition of organic matter in soils, and play an essential role in controlling the mineralization through affecting chemical, physical and microbial processes in soils, thereby leading either to the stabilization or mobilization of organic matter.

Whether soil can act as a source or sink for carbon strongly depend on the stabilization and mobilization of soil organic matter by soil macrofauna and microorganisms and their interaction. Among others, earthworms are supposed to be key species controlling the decomposition of soil organic matter in temperate ecosystems (Edwards and Bohlen, 1996). Beside their direct contribution by feeding and digestion they strongly affect the biomass, activity and community composition of microorganisms, the main drivers of organic matter mineralization and CO_2 production in terrestrial ecosystems. Hence, knowledge about the interaction of earthworms and microorganisms in decomposition and mineralization is required to determine the fate of organic matter in soil.

1.3 Earthworms

1.3.1 Earthworms - Biology and Systematic

Earthworms form the dominant component in the soil animal biomass and are regarded to be the most important soil animals participating in nutrient cycling and soil structure modification (Edwards and Bohlen, 1996; Spehn et al., 2000). The importance of earthworms was first recognized by Aristotle who termed them “the intestines of the earth“, and Charles Darwin even stated in his 1881 book entitled „The Formation of Vegetable Mould through the Action of Worms“ that „It may be doubted whether there are many other animals which have played so important a part in the history of the world, as have these lowly, organized creatures”.

Earthworms belong to the order Oligochaeta, class Chaetopoda, phylum Annelida with actually over 5.500 named species known worldwide. They are grouped into eleven main families (Lumbricidae, Hormogastridae, Sparganophilidae, Almidae, Megascolecidae, Acanthodrilidae, Ocnerodrilidae, Octochaetidae, Exxidae, Glossoscolecidae and Eudrilidae). The family Lumbricidae pools approximately 600 species and is the most common family in temperate areas of the northern hemisphere. Generally, earthworms are found all over the world, expect in arid regions and deserts, areas under constant snow and ice sheets, mountain ranges, and areas almost entirely lacking soil and vegetation. Their size ranges from a few millimetres to as much as two meters, but most common species in temperate areas of the northern hemisphere are only a few centimetres in length. However, in the southern hemisphere, such as South Africa and America, Australia and New Zealand some larger earthworm species are described. For example, in the family Megascolecidae, a large family restricted to the tropics, some species of *Digaster* found near Kyogle in New South Wales are reported to grow to a length of more than 1.5 meters, and the well known "Gipsland Giant", *Megascolides australis*, may even reach two meters in length (Lee, 1985).

Earthworms are hermaphrodites with both male and female reproductive organs but they usually cross-fertilize. When two individuals copulate they exchange sperm, store it and fertilize their own eggs in a sheath, the so called cocoon, secreted by the clitellum, a thicker ring visible around the front of the earthworm. In addition, some earthworm species are parthenogenetic, such as *Octolasion tyrtaeum* (Savigny), reproducing without mating (Sims and Gerard, 1999). The cocoons are deposited into the soil, the embryo worms develop and young worms emerge when temperature and moisture conditions are suitable. Earthworms may reach an age of up to 10 to 12 years, but in nature earthworms generally survive only about 2 to 4 years (Lee, 1985).

Although, the name earthworm suggests that their distribution is restricted to the soil, some species also live in aboveground habitats including litter layers, the bark of standing trees, in organic material accumulated at the base of epiphytes and even in the intertidal zone (Lee, 1985). However, indeed, the main habitat of earthworms is the soil, with different earthworm species preferring different habitats and depend on different food resources. Hence earthworms were classified into different categories depending on the soil horizons they were most commonly found (Lee, 1985). In 1972, Bouche separated the dominant European family Lumbricidae into three major morpho-ecological groups, the epigeic, endogeic and anecic species.

Epigeic species, so called litter-dwelling species, live above the mineral soil surface, for instance in the litter layer of forests, and are generally absent from agricultural ecosystems. They form no burrows, feed on decomposing litter and are dorsally and ventrally dark

pigmented. Epigeic species have relatively high reproductive rates and grow rapidly. Common species are *Lumbricus rubellus* (Hoffmeister), *Lumbricus castaneus* (Savigny) and *Dendrobena octaedra* (Savigny).

Anecic species live in permanent, vertical burrows that may extend three meters depending on soil texture. The burrows are open at the soil surface and surrounded by middens, a mixture of plant residues and casts, serving as protection and food resource. Anecic species pull dead leaves and other decaying organic materials down into their burrow, where they are rapidly colonized by microorganisms and consumed at later time. Generally, anecic species are little pigmented dorsally and unpigmented ventrally, with *Lumbricus terrestris* (Linnaeus) and *Aporrectodea longa* (Ude) being the major representatives of this group.

Endogeic species do not create permanent burrows; rather randomly burrow through the upper layer of the mineral soil. They primarily consume soil and associated humified organic residues and only little amounts of plant residues at the soil surface. In Europe, among others, this generally unpigmented or lightly pigmented group includes the species *Octolasion tyrtaeum* (Savigny) and *Aporrectodea caliginosa* (Savigny).

Both anecic and epigeic species primarily feed on plant residues aboveground, and are summarized as “litter-feeders”, whereas endogeic species primarily feed on soil and organic matter belowground are characterized as geophagous species.

1.3.2 Earthworms - Abundance and Habitat

Earthworm biomass, abundance and community composition is affected by various environmental and management factors, i.e. abiotic factors, such as climate, soil, vegetation and litter supply, and biotic factors, such as competition, predation, parasitism, disease and food relationship (Curry, 2004).

Climate, predominantly temperature and moisture conditions, is regarded to be the most important factor controlling earthworm biomass and abundance in the soil. The temperature tolerance of earthworms is narrow, generally ranging from 0-30°C with the optimum for temperate species typically being in the range of 10-20°C. However, some tropical and subtropical species are adapted to temperatures above 30°C and there are some species reported to tolerate temperatures below 0°C. Soil temperature also determines soil moisture conditions. High temperature is often correlated with moisture shortage and moisture stress for soil animals. Earthworms do not have specialized respiratory organs and take up oxygen through their cuticule which has to be moist.

Earthworms depend on the chemical and physical properties of the soil, such as pH, texture, and organic matter supply. The concentration of hydrogen ions (pH) is supposed to be an important environmental factor limiting earthworm abundance; earthworm species vary in their pH tolerance and preference. Most earthworm species favour neutral to slightly acid soils with pH ranging from 5.0-7.4. The earthworm species *Arctiostrotus* spp., however, was found in organic soil horizons with pH ranges from 2.6-6.2 on Vancouver Island in Canada (Spiers et al., 1986), and *Helodrilus africanus* is reported to tolerate pH values up to 9.2 (Madge, 1969). Since the soil pH also affects soil environmental factors, such as the concentration of calcium ions and is simultaneously affected by environmental factors, such as clay content, the direct effect of pH on earthworm abundance is difficult to prove. However, neurophysiological tests by Laverack (1961) support the opinion that the pH itself determines the abundance of earthworms in the soil.

Soil texture, the proportion of clay, silt and sand in the soil matrix, generally correlates with moisture conditions and affects the abundance of earthworms. Coarse textured sandy soils restrict the abundance due to low water holding capacity as a result of fast water drainage, whereas fine clay and silt textured soils with high water holding capacity may limit earthworm survival due to oxygen depletion. In addition, earthworms may be absent in coarse textured soils, due to physical abrasion of their body surface.

Soil organic matter content is determined by the supply of aboveground and belowground plant residues, such as litter, roots and root exudates, and affects the biomass, abundance and community composition of earthworms. Earthworms are saprophagous animals mainly feeding on dead and decaying plant residues below- and aboveground according to the ecological group they belong to. Hence, litter feeding anecic and epigeic earthworm species as *L. terrestris* and *L. rubellus* only rarely depend on organic matter in the soil, whereas geophageous earthworm species, such as *A. caliginosa*, primarily require humified organic residues and other organic matter in the soil.

Due to a protein content of 60-90% of its dry weight earthworms are a favoured component of the diet of a wide range of predators as birds, amphibians, reptiles, mammals and even insects including ants, beetles and centipedes. However, the amount of earthworms in the diet of predators in the field and the effect of predation on the biomass and abundance of different earthworm species is difficult to detect. Presumably, the use of stable isotope techniques and the extraction of DNA from the gut of predators may shed more light into that black box in the near future.

1.3.3 Earthworms - "Ecosystem engineers"

The biomass, abundance and community composition of earthworms is not only affected by the physical and chemical properties of the soil, rather, earthworms themselves modify the chemical, physical and biological properties of the soil. Earthworms are regarded to function as "ecosystem engineers" directly or indirectly affecting below- and aboveground ecosystem processes (Jones et al., 1997; Lavelle, 1997).

By burrowing particularly anecic earthworm species are of great importance in keeping the soil structure open and allowing drainage and aeration of the soil. Furthermore, earthworms are responsible for the formation of soil and it has been estimated that the top centimetres of ecosystems inhabiting earthworm communities consists almost entirely of earthworm casts (Bal, 1982; Scheu, 1987a; McInerney and Bolger, 2000). Anecic earthworm species collecting plant residues on the surface next to their burrows and deposit their casts on the surface are of great importance in the development of the soil profile and the structure of soils, whereas endogeic earthworms depositing their cast below the soil surface, are more important for pedogenesis (Lee, 1985).

Earthworms modify the chemical composition of the soil and affect the distribution and availability of carbon, nitrogen, phosphorous and other nutrients in the soil (Lee, 1985; Edwards and Bohlen, 1996). However, due to their feeding and casting activity earthworms of different ecological groups are of importance for nutrient cycling.

The main effect of epigeic earthworms on nutrient cycling is the comminution of organic matter, thereby providing habitats and nutrient resources for other invertebrates and microorganisms. Fragmented organic material on the soil surface becomes more exposed to precipitation, resulting in increased leaching of nutrients into the soil matrix were they may be used by soil microorganisms and plants.

Anecic earthworms are most important for the incorporation of aboveground organic material into the mineral soil. They remove large amounts of surface litter and it has been documented that they are able to consume the total annual litter fall in a mixed forest in about three months (Satchell, 1967). The removal of surface litter does not necessarily imply ingestion of the litter. Anecic earthworms generally collect surface plant residues in their burrows where they are stored until they are partially decomposed by microorganisms.

Living in the upper mineral soil and consuming large amounts of soil endogeic earthworms are able to detect and ingest nutrient hot spots (Wolter and Scheu, 1999; Marhan and Scheu, 2006). The casts containing more available nutrients than the surrounding soil and affect nutrient availability for microorganisms and plants.

Along with cycling of nutrients and the control of soil physical properties, earthworms significantly affect plant growth (Brown et al., 1999; Scheu, 2003). Generally, the digestion efficiency of earthworms is very low and only little of the organic material ingested is assimilated and incorporated into the biomass of earthworms (Wahlen and Parmelee, 1999). The main part is deposited in the cast containing beside particulate organic material, nutrients excreted such as urine or mucoproteins from the gut wall. Hence, the carbon and nitrogen content of earthworm casts is approximately two and the phosphorous content up to ten times that of surrounding soil (Lee, 1985). These nutrient enriched cast aggregates are of great importance in the soil and function as preferred habitats for microorganisms and contribute to the nutrient supply of plants. In addition to plant nutrient effects, earthworm change the density and activity of microorganisms and invertebrates beneficial or harmful to plants, produce plant growth-regulating substances, consume living plant parts, such as roots and seedlings, and distribute seeds in the soil matrix, resulting in changes in plant competition and community composition (Cortez and Bouche, 1992; Shumway and Koide, 1994; Kreuzer et al., 2004, Milcu et al., 2006).

1.4 Microorganisms

The soil microbial community comprises of bacteria, fungi, actinomycetes and algae. Bacteria and fungi forming up to 90% of the soil microbial biomass and are responsible for the mineralization of organic matter in the soil and central to the cycling of carbon and nitrogen (Swift et al., 1979; Paul and Clark, 1989). However, the proportion of fungi and bacteria in the soil microbial community varies with environmental conditions such as soil depth, moisture, aeration and pH.

Bacteria are prokaryotic organisms, ubiquitous distributed in all types of soil due to the ability to withstand extreme climate conditions. The diversity and biomass of bacteria in soils is enormous with estimates up to 4×10^6 taxa (Curtis et al., 2002) and up to 10^{11} bacteria in one gram of soil (Horner-Devine et al., 2004). The distribution of bacteria is restricted by the availability of energy resources. Beside few autotrophic, photoautotrophic and chemoautotrophic, the bulk of the bacterial community in the soil consists of heterotrophic bacteria depending on organic matter to sustain their nutrition and energy supply. Organic matter in the soil is heterogeneously distributed with the rhizo-, drilo-, and detritusphere are the most important microenvironments supporting the main part of the bacterial biomass, with decreasing biomass and activity with increasing distance from these hot-spots (Beare et al.,

1995; Brown et al., 2000; Furlong et al., 2002). Bacteria are able to move through the soil and exploit these microenvironments, but rely on water films.

Fungi are heterotrophic organisms relying on the availability of organic matter as energy resource. In contrast to bacteria, fungi are able to actively colonize and exploit patchy distributed microenvironments. They form widespread mycelia composed of branched hyphae with estimates up to 250 kg ha^{-1} dry weight in the upper 5 cm of a grassland (Bardgett et al., 1993) and even up to ten times more was found in a deciduous woodland soil (Frankland, 1982). Hyphae penetrate organic residues such as litter and absorb nutrients within and between the plant cells. These nutrients can be translocated from this nutrient rich source to parts of the mycelia which grow in distance, thereby improving nutrient supply of the whole mycelia. Translocation of nutrients through fungal mycelia is well documented (Lindahl et al., 1999; 2001). For instance, decomposing fungi are reported to translocate huge amounts of soil nitrogen into aboveground litter layers, thereby speeding up decomposition, generally supposed to be nitrogen limited (Hart et al., 1993; Frey et al., 2000). Frey et al. (2003) demonstrated that translocation of nutrients also occurs from aboveground plant residues into the soil matrix.

Fungi as well as bacteria produce various enzymes which act externally. However, fungi produce a wide range of enzymes enabling them to degrade complex organic compounds as cellulose and lignin, the main structural component of plant cell walls. By breaking up cell walls fungi get access to more easily available labile nutrients inside the cell, making them generally superior competitors for the decomposition of plant residues (Harley, 1971).

In addition to heterotrophic fungi, some fungi actively capture prey, such as *Arthrobotrys* feeding on nematodes (Farrell et al., 2006), and are parasitic, feeding on living organisms without killing them. Mycorrhiza are important plant associated fungi, increasing the root surface area to aid plants in acquiring water and nutrients from the soil and getting energy rich sugars in return. However, depending on nutrient availability in the soil, mycorrhizal fungi may act as parasitic or symbiotic.

1.5 The interaction of earthworms and microorganisms

The most important loss mechanism for carbon that becomes incorporated into soils is the mineralization of organic matter by microorganisms, primarily bacteria and fungi. However, microbial activity, hence mineralization efficiency and release of CO_2 from soil, vary with temperature, moisture conditions, the chemical composition of the organic matter entering the soil and certainly with the composition and activity of soil fauna, particularly earthworms. In addition, the activity and biomass of earthworms also varies with these environmental factors

and the activity and biomass of the soil microbial community. However, earthworms dominate this dependency as "ecosystem engineers" and control directly or indirectly the biomass, activity and community composition of microorganisms, hence, decomposition processes, mineralization of organic matter and the release of CO_2 from soils (Jones et al., 1997; Lavelle, 1997).

Although the effect of earthworms and microorganisms on soil organic matter decomposition and mineralization is well investigated (Lee, 1985; Edward and Bohlen, 1996), there is still uncertainty about the effect of earthworms on soil microorganisms with regard to the stabilization and mobilization of soil organic matter.

Modification of the chemical and physical properties of the soil by earthworms generally alters the habitat structure and nutrient availability for soil microorganisms; hence, indirectly affect their biomass and activity. As stated before, earthworms incorporate significant amounts of organic material and mix it with the mineral soil with the incorporation of organic matter into the soil and the formation of cast aggregates, burrows, deposition of plant litter in the burrows and middens are generally considered to be beneficial for microorganisms and stimulate their activity (Subler and Kirsch, 1998).

Cast aggregates contain higher concentrations of nutrients and increased microbial biomass than the surrounding soil (Blair et al., 1995, Parmelee et al., 1998). However, it has been demonstrated that the microbial biomass and activity in casts changes in time and the mineralization of soil organic matter incorporated into casts aggregates occurred in three different phases (Wolters, 2000). In fresh casts the mineralization increased compared to the uningested soil, due to the relative enrichment of available nutrients, particularly on the surface of cast aggregates. When nutrients become limited on the surface of casts the mineralization declines due to protection of nutrients enclosed inside (Scheu, 1987b; Scheu and Wolters, 1991; Tiunov and Scheu, 2000). The protection of nutrients enclosed inside the cast aggregates generally lasts until casts are broken up for example by precipitation or coprophagy and nutrients are exposed again. The length of the different phases depend on quality and quantity of the organic matter incorporated and earthworm species. However, the protection of organic matter is reported to be the longest phase, and therefore earthworms are regarded to protect soil organic matter in their casts (Wolters, 2000).

Earthworm burrow walls are lined with casts and mucus, and are generally increased in pH and the concentration of organic matter (Schrader, 1994; Amador et al., 2003). Burrow walls are preferred habitats of soil microorganisms and these microenvironments are quickly colonized by microorganisms. For example, Tiunov and Scheu (1999) analysed the burrow wall of *L. terrestris* and detected an increased microbial activity and biomass, due to increased nitrogen,

phosphorous and organic matter content. These results are in line with other experiments documenting a significant increase in microbial biomass, activity and basal respiration in the burrow walls of earthworms compared to the surrounding soil (Parkin and Berry, 1999; Tiunov and Dobrovolskaya, 2002). However, Görres et al. (1997) showed that the microbial biomass in the burrow linings of *L. terrestris* significantly decreased compared to the surrounding soil. Presumably, the response of the microbial biomass and microbial activity in the lining of earthworm burrows depends on the quality and quantity of the organic matter incorporated and the earthworm species.

Around the openings of their burrows anecic earthworms create middens, which are accumulations of soil, cast and organic residues serving as protection and repository. Middens bear higher moisture content and pH and lower C/N ratio than the surrounding soil and are microbial hotspots with increased microbial biomass and activity (Tiunov and Kuznetsova, 2000). They are regarded to function as external rumens in which microorganisms decompose plant residues and thereby increase the palatability of the organic material for earthworms (Swift et al., 1979; Lavelle et al., 1995).

In addition to indirect effects on microorganisms, by modifying soil chemical and physical structure, earthworms directly affect the microbial biomass and community composition by grazing. Since microorganisms considerably differ in their mineralization rate and efficiency, the selective consumption of microorganisms and also the resistance of microorganisms to earthworm ingestion may alter the fate of organic matter in soils. Several studies investigated the effect of the earthworm gut passage on the biomass and activity and community composition of microorganisms, but the results are variable and it is still a matter of debate if microorganisms significantly contribute to earthworm nutrition.

Fungi are the most important microorganisms on aboveground organic residues and dominate the microbial biomass in the litter layer of forest ecosystems (Dix and Webster, 1995). Hence, it has been supposed that litter decomposing fungi may be an important food component for anecic and epigeic earthworm species (Edwards and Fletcher, 1988; Brown, 1995). The palatability of organic matter increases with the stage of decomposition, with litter colonized by microorganisms preferentially ingested by earthworms (Moody et al., 1996). For instance, Bonkowski et al. (2000) observed in a food choice experiment with nine fungal species and five different earthworm species that earthworms preferentially feed on early litter colonizers, such as *Fusarium nivale* or *Mucor sp.*, whereas basidomycetes, generally regarded to be later colonizers, were only little consumed. They concluded that litter feeding earthworms are able to detect these microsites indicated by initial colonizers that primarily exploit easily available

soluble organic compounds as major carbon source, such as amino acids and carbohydrates. The fate of fungi during the gut passage significantly varies with the species of earthworm and fungi. For instance, Moody et al. (1996) detected no viable spores of *Fusarium lateritium* after the passage through the gut of *L. terrestris* and *A. longa*, whereas the spores of *Chaetomium globosum* were not affected. In addition, the spores of *Mucor hiemalis* were significantly more reduced by the gut passage of *L. terrestris* than of *A. longa*.

Data on the fate of bacteria during earthworm gut passage are variable too, but Parle (1963) documented that the biomass of bacteria generally increases, indicating that bacteria are only of minor importance for the nutrition of earthworms (Edwards and Fletcher, 1988). Furthermore, Horn et al. (2003) reported that N_2O producing bacteria are favoured in the gut of *L. terrestris*. In contrast, Flack and Hartenstein (1984), investigate the effect of bacteria on the growth of *Eisenia fetida*, showed that some bacteria did not survive the gut passage, suggesting digestion by the earthworms. In addition, it has been demonstrated that different earthworm species affect bacteria in different ways. For example, Kristufek et al. (1992) reported that the number of bacteria significantly decrease in the gut of *A. caliginosa*, whereas it significantly increase in the gut of *L. rubellus*.

Although less investigated, protozoa are regarded to be an important component of the nutrition of earthworms (Edwards and Bohlen, 1996). For example, Miles (1963) investigated the growth and reproduction of *E. fetida* and demonstrated that they rely on protozoa in their food resource to reach sexual maturity. Furthermore, *A. caliginosa* and *O. lacteum* are reported to graze on naked amoebae (Bonkowski and Schaefer, 1997).

Overall, bacteria appear to be only of minor importance whereas protozoa and particularly fungi play a significant role in the nutrition of earthworms. However, the effect of earthworms on the microbial biomass, activity and on the microbial community composition varies greatly in time, with earthworm species and group (epigeic, endogeic and anecic), food resources (litter or soil) and environmental conditions.

1.6 Objectives

The aim of the present study was to investigate the effect of soil microorganisms on the stabilization and mobilization of soil organic matter as affected by the activity of earthworms. We restrict our investigation to endogeic earthworm species since they are primarily responsible for the formation of organo-mineral complexes in the soil, thereby strongly affecting microbial activity and decomposition of organic matter (Scheu, 1995). We deal with selective parameters, such as soil matrix, time of incubation and quality of organic matter, likely modifying the effect

of endogeic earthworms on the biomass and activity of microorganisms in soils. In addition, with regard to the mineralization of soil organic matter, direct effects on the microbial community composition in soil were investigated. I present four experiments which were conducted in microcosms under controlled temperature and light conditions in climate chambers of the Technical University of Darmstadt, the RWTH Aachen and the Max Planck Institute for Terrestrial Microbiology in Marburg.

In two microcosm experiments we studied the effect of the availability of clay and the activity of endogeic earthworms on the fate of phenolic compounds in a forest and an agricultural soil **(Chapter 2)**. Phenolic compounds are ubiquitously distributed in soils forming up to 10% of the total soluble soil organic carbon and are regarded to function as a precursor of humic substances. It is hypothesized that in the presence of clay phenolic compounds are stabilized in cast aggregates and become protected against microbial mineralization. Since both soils significantly differ in texture, nutrient saturation and microbial biomass and activity, we hypothesized that effects of clay and earthworms on the fate of phenolic compounds vary with soils. The use of carbon tracer (^{14}C-catechol) enabled us to quantify the stabilization in humic fractions (DOM, fulvic acid, humic acid and humin) and mobilization in CO_2 evolved in the microcosms. Two experiments were set up to investigate short- and long-term patterns of these processes.

The second experiment **(Chapter 3)** investigated the competition between endogeic earthworms and microorganisms for root exudates of a grass (*Lolium perenne* L.) and a legume (*Trifolium repens* L.). Due to the dependence of both on easily available carbon resources, we studied whether earthworms or microorganisms are the superior competitor for root exudates. We hypothesized that endogeic earthworms protect carbon rhizodeposites against microbial mineralization indirectly by grazing on microorganisms in the rhizosphere and directly by feeding on rhizodeposites and subsequent biomass incorporation and protection in the inner compartments of their cast aggregates.

In a third microcosm experiment we analysed the interaction of endogeic earthworms and fungi in the decomposition of ^{13}C-labelled rye litter provided on the soil surface **(Chapter 4)**. Since both primarily colonize the upper mineral soil layer we investigated if fungi translocate and stabilize litter derived carbon in the upper layer of the mineral soil, as well as if endogeic earthworms mobilize carbon when feeding on fungal hyphae. We hypothesised that endogeic earthworms decrease the fungal translocation of litter derived carbon. As a consequence, earthworms might contribute to the mobilization of litter derived carbon in the upper mineral soil by ingesting and disrupting mycelial networks of litter decomposing fungi. The analysis of

phospholipid acid profiles and ergosterol in the soil gained insight into the microbial community composition.

In the fourth experiment we investigated the influence of the passage through the gut of endogeic earthworms on microbial activity and the microbial food web by analysis of the microbial community composition in soil and cast **(Chapter 5)**. Arable soil was inoculated with ^{13}C-labelled *Serratia marcescens* and incubated with and without earthworms. Microbial community and activity was determined using RNA Stable Isotope Probing. Microbial communities were characterized by Terminal Restriction Fragment Length Polymorphism (T-RFLP) followed by cloning and sequencing. We hypothesized that the passage through the earthworm gut significantly alters the composition of microbial community by differently affecting microbial species. Since microorganisms differ in their mineralization rate and efficiency, this may alter the fate of organic matter in soils, thereby the release of CO_2 from soils.

The results of all experiments are summarized and discussed in the context of earthworms mediated effects on microbial biomass, activity and community composition with regard to the stabilization and mobilization of organic carbon in soils (**Chapter 6)**.

Chapter

2

The fate of catechol in soil as affected by earthworms and clay

Abstract

The effect of endogeic earthworms (*Octolasion tyrtaeum* (Savigny)) and the availability of clay on the mobilization and stabilization of uniformly ring ^{14}C-labelled catechol mixed into an arable and a forest soil was investigated in a short- and a long-term microcosm experiment. By using an arable and a forest soil the effect of earthworms and clay in soils differing in the saturation of the mineral matrix was investigated. In the short-term experiment microcosms were destructive sampled when the soil had been transformed into casts. In the long-term experiment earthworm casts produced during seven days and non-processed soil were incubated for three further months. Production of CO_2 and $^{14}CO_2$ were measured in regular intervals. Accumulation of ^{14}C in humic fractions of the casts and the non-processed soils and incorporation of ^{14}C into earthworm tissue were determined.

Incorporation of ^{14}C into the earthworm tissue was low, with 0.1% in the short-term and 0.44% in the long-term experiment. Cumulative production of CO_2-C was significantly increased in presence of *O. tyrtaeum* and in casts produced from the arable soil, but lower in casts produced from the forest soil; generally the production of CO_2-C was higher in forest than in arable soil. Both soils differed in the pattern of $^{14}CO_2$-C production; whereas it was initially higher in the forest soil than in the arable soil, it was contrary later. *Octolasion tyrtaeum* did not affect $^{14}CO_2$-C production in the forest soil, but increased it early in the arable soil; clay counteracted the effect of *O. tyrtaeum* in the arable soil. Clay and *O. tyrtaeum* did not affect integration of ^{14}C into the humic fractions of the forest soil. In contrast, in the arable soil *O. tyrtaeum* increased the amount of ^{14}C in the labile fractions, whereas clay increased it in the humin fraction.

The results indicate that endogeic earthworms and clay are only of little importance in soils with high organic matter content, high clay content and high microbial biomass, but contrarily affect phenolic compounds in poor soils; i.e. endogeic earthworms strongly increase microbial activity and thus mineralization of phenolic compounds, whereas clay decreases it by binding phenolic compounds when passing through the earthworm gut.

2.1. Introduction

Phenolic compounds are the most widespread secondary plant metabolites with several thousand different compounds being identified (Hättenschwiler and Vitousek, 2000). It is assumed that they comprise up to 60% of the plant dry mass (Northup et al., 1998) being responsible for UV protection, defence against pathogens and herbivores and contribute to the colouring of plant tissues (Hättenschwiler and Vitousek, 2000). Chemically phenolic compounds are composed of an aromatic ring with one (phenol) or more (polyphenol) hydroxyl substituents and functional derivates as esters, methyl esters or glycosides (Kuiters, 1990). They are ubiquitously distributed in the soil forming up to 10% of the total soluble organic carbon (Gallet and Keller, 1999) entering the soil on two different pathways: They are leached by rain from the tree canopy and are present in above- and belowground litter, which is the dominant pathway (Kuiters, 1990, Siqueira et al., 1991, Hättenschwiler and Vitousek, 2000). In the soil phenolic compounds undergo various transformations primarily due to biological activity. They may rapidly degrade and mineralize, dissolve or be leached, adsorb to clay particles or build chelates with aluminium or iron ions, or be transformed into insoluble recalcitrant humic substances. These transformations are mediated by heterotrophic microorganisms altering phenolic compounds together with amino acids and protein thereby forming stable humic substances (Martin and Haider, 1980). Hence, phenolic compounds are either stabilized into the soil matrix or mobilized as CO_2 or leachate depending on biotic and abiotic soil conditions. Haider and Martin (1975) stressed early that phenolic compounds are preferentially retained in the soil compared to other easily available carbon resources such as amino acids and glucose.

Phenolic compounds alter the composition and activity of the soil decomposer community. They are reported to restrict the activity and abundance of soil fauna and to stimulate or inhibit the activity and biomass of soil microflora and –fauna, thereby significantly affecting litter degradation and nutrient cycling in soil as reviewed by Kuiters (1990). A range of experiments demonstrated that the polyphenol concentration of litter is one of the most important driving forces determining litter palatability for decomposer soil fauna, with litter containing high polyphenol concentration being less palatable than litter with low concentration (Hendriksen, 1990, Tian et al., 2000).

Earthworms form a major part of the soil decomposer macrofauna and play an important role in organic matter processing and nutrient cycling in temperate ecosystems (Edwards and Bohlen, 1996). Endogeic earthworm species often dominate the earthworm community in forest (Scheu, 1992) and arable ecosystems (Curry and Byrne, 1992). They are very active species, living in non permanent burrows in the upper mineral soil ingesting two to five times their own weight of

soil and organic matter per day. Hence, endogeic earthworm species are responsible for the formation of organo-mineral complexes in the soil, thereby strongly affecting microbial activity and decomposition of organic matter (Scheu, 1995). Due to the low assimilation efficiency earthworm cast aggregates represent nutrient rich microsites in the soil providing favourable habitats for microorganisms (Tiwari et al., 1989; Devliegher and Verstraete, 1997; Tiunov and Scheu, 1999). Endogeic earthworms are regarded as ecosystem engineers (Lavelle, 1997), directly or indirectly controlling the availability of resources to other organisms by controlling physical state changes in biotic and abiotic materials (Jones et al., 1997). However, although the casts of endogeic earthworms are microbial activity hotspots they are generally regarded to protect carbon against microbial attack in inner compartments (Guggenberger et al., 1995; Edwards and Bohlen, 1996). It has been demonstrated that the mineralization of carbon incorporated into casts changes in time. In fresh casts the mineralization is enhanced; microorganisms utilise easily available nutrients such as sugars and proteins derived from the destruction of soil aggregates enriched in organic matter, the comminution of organic matter and the addition of mucus during the gut transition. In aging casts the availability of nutrients declines, nutrients become limited due to the reduced accessibility of microorganisms to nutrients enclosed inside cast aggregates, resulting in decreased carbon mineralization. When casts break up by biotic or abiotic processes such as precipitation or coprophagy, enclosed nutrients become exposed and mineralization increases again. Recent studies have shown that the composition of the soil matrix may alter the duration of these phases by affecting the stability of casts. For example, Marhan and Scheu (2006) demonstrated that the availability of an unsaturated mineral soil matrix increases the stabilization of carbon in earthworm casts. Further, clay protects soil organic matter against microbial degradation (Ladd et al., 1985, Feller and Beare, 1997) and increases the aggregate stability of casts, resulting in increased protection of carbon enclosed inside the casts (Wolters, 2000).

Despite the high annual input of polyphenols into the soil system via below- and aboveground plant litter and canopy leachates, and the relatively high persistence in the soil matrix, there has been little research into the interaction of soil fauna and soil mineral matrix on the fate of phenolic compounds in soils. Considering the growing interest in the stabilization of carbon in soils to counteract the global increase in atmospheric CO_2 concentration, knowledge on the biotic and abiotic mechanisms affecting the fate of phenolic compounds in soils is required.

We analysed the influence of the availability of clay and activity of the endogeic earthworm species *Octolasion tyrtaeum* (Savigny) on the fate of catechol in forest and agricultural soil. By using a forest and an agricultural soil the effect of clay and earthworms on the stabilization of catechol in soils differing in the saturation of the mineral matrix was investigated. Catechol was

used as a model monomeric phenolic substance being commonly formed during microbial degradation of many naturally occurring and anthropogenic aromatic substances and regarded as precursor of soil humic substances (Haider et al., 1975). Uniformly ring-^{14}C-labelled catechol was used enabling us to follow the fate of catechol in soils. Two experiments were set up to investigate long term and short term stabilization and mobilization processes.

2.2. Materials and Methods

2.2.1 Soils and earthworms

In September 2004 arable soil was sampled from the long-term fertilisation experiment in Bad Lauchstädt (Germany, Saxony-Anhalt). The long-term annual mean temperature in Bad Lauchstädt is 8.7°C and the annual precipitation is 484 mm. The soil is a Mollisol, Haplic Chernozem loam (FAO classification) with an average particle size distribution of 17.6% clay, 70.5% silt, 10.6% fine sand (63 – 250 µm) and 1.3% coarse sand (> 250 µm). The soil was of neutral pH (6.9; 0.01 M $CaCl_2$, 1/2.5 w/v) with 1.8% C and 0.2% N. Further details are given in Körschens (1994).

Forest soil was sampled in September 2004 in a 130-year old beech wood on limestone near Göttingen (Germany, Southern Lower Saxony). Long-term annual mean temperature in Göttingen is 7.9°C and annual rainfall is 720 mm. The soil is shallow and consists of redzina, terra fusca and brown earth with an average particle size distribution of 29.6% clay, 61.4% silt, 6.3% fine sand (63 – 250 µm) and 2.7% coarse sand (> 250 µm). Soil was of neutral pH (7.0; 0.01 M $CaCl_2$, 1/2.5 w/v) with 13.0% C and 1.0% N. Further details are given in Schaefer (1991).

Soil samples were taken from the upper 10 cm of the study sites, sieved (4 mm) to remove stones and large plant residues and defaunated by freezing at -28°C for 5 days. Two weeks before the experiments were set up the soil samples were kept at 4°C.

Adult specimens of *O. tyrtaeum* were sampled by hand in the beech wood described above. Earthworms were transferred to the laboratory, identified and kept in containers filled with soil from the beech wood for four weeks at 4°C. Two weeks before the experiments were set up earthworms for the arable soil treatments were incubated in containers with soil of the arable field to avoid contamination with forest soil.

2.2.2 Synthesis of ^{14}C-labelled catechol

^{14}C-labelled catechol was prepared by a modified and improved method of Loudon and Scott (1953) as described in detail in Ji and Schaeffer (2002).

2.2.3 Short-term experiment

In October 2004 the short-term experiment with a total of 36 microcosms was set up. The microcosms consisted of glass flasks (height 107 mm, diameter 100 mm), closed at the top by lids. Small plastic vessels filled with 3 ml alkali (1 N NaOH) were placed into the microcosms to absorb CO_2. The soils were filled in small plastic vessels (height 40 mm, diameter 30 mm) and placed into the microcosms. One half was filled with 5 g dry weight arable soil, the other with 5 g dry weight forest soil. Before adding the soil 0.5 g dry weight clay (Bentonite, Montmorillonite, Riedel-de Haën, Seelze, Germany) was mixed homogeneously by hand into half of the soil samples to establish the clay treatments. Due to differences in maximum water holding capacity of the soils different ^{14}C-catechol solutions in distilled water were prepared; the specific radioactivity was 975 kBq ml^{-1} for the arable and 370 kBq ml^{-1} for the forest soil. To establish a final water content of 60% of the maximum water holding capacity, 0.2 ml and 0.5 ml of the appropriate solutions were mixed homogeneously into the soils resulting in final specific radioactivity of 39 and 37 kBq g^{-1} dry weight soil in the arable and forest soil, respectively. One individual of *O. tyrtaeum* was added per microcosm to half of the soil and soil-clay treatments to establish the following treatments: arable (A) and forest soil (F), arable (AC) and forest soil with clay (FC), arable (AOt) and forest soil with *O. tyrtaeum* (FOt) and arable (ACOt) and forest soil with clay and *O. tyrtaeum* (FCOt). Four replicates per treatment were established; four empty microcosms served as control for quantification of mineralization of ^{14}C-catechol.

Microcosms were incubated in darkness in a climate chamber at 20°C until the soil in the earthworm treatments was transformed into casts.

2.2.4 Long-term experiment

In January 2005 the long-term experiment with a total of 40 microcosms was set up. Microcosms consisted of plastic tubes (height 135 mm, diameter 60 mm) fixed air tight on ceramic plates, which allowed drainage of the soil under semi-natural conditions by lowering the atmospheric pressure in a box below the ceramic plates. Leaching water of each microcosm

was sampled in separate vessels placed in the box. Microcosms were closed by lids at the top. Small vessels attached to the lids were filled with 3 ml alkali (1 N NaOH) to absorb CO_2. Treatments were established as described above but with the tenfold amount of soil, clay and earthworm individuals per microcosm. Ten ml of a ^{14}C-labelled catechol solution (specific radioactivity 4.6 kBq ml^{-1}) were mixed homogeneously into the soils to establish a final specific radioactivity of 0.93 kBq^{-1} g dry weight soil. Treatments with earthworms were replicated five times, those without four times; four empty microcosms served as control for quantification of mineralization of ^{14}C-catechol.
Microcosms were incubated in darkness in a climate chamber at 20°C and watered by hand once a week with 10 ml distilled water. After the soil had been transformed into casts, earthworms were collected and the soil respectively cast material were incubated for three further months.

2.2.5 Mineralization

During incubation CO_2 production was determined according to Macfadyen (1970). Briefly, CO_2 evolved in the microcosms was trapped in alkali (1 N NaOH), and an aliquot (0.5 ml) was measured titrimetrically with 0.1 N HCl after precipitation of carbonate with saturated $BaCl_2$ solution. Mineralization of ^{14}C-catechol was determined by liquid scintillation counting (LSC). An aliquot (0.5 ml) of the alkali was added into 5 ml vials containing 4.5 ml scintillation cocktail (Luma-Safe Plus, Lumac-LSC, Groningen, Netherlands). Vials were gently mixed and radioactivity in the alkali was measured immediately after sampling in a liquid scintillation counter (Tri-Carb 2800 TR, PerkinElmer Inc., Shelto, USA). Measurements were carried out daily in the short-term experiment and in the beginning of the long-term experiment, and twice a week in the long-term experiment after earthworm collection.

2.2.6 Leaching water

In the long-term experiment leaching water was sampled and the amount of dissolved ^{14}C was measured. A pooled sample from one week was analysed for dissolved ^{14}C. For determination an aliquot (0.5 ml) was added to 5 ml vials containing 4.5 ml scintillation cocktail and measured by LSC as described above.

2.2.7 Earthworms

When the soil had been transformed into casts, earthworms were extracted from microcosms, weighed and killed by freezing at -28°C for one day. Earthworm guts were cleared by dissection and the tissue was dried for two days at 65°C. For determination of the radioactivity approximately 20 mg dry weight earthworm tissue were weighed into cellulose capsules and combusted (Compact Automatic Oxidiser OX-500, Zinsser Analytic GmbH, Frankfurt, Germany). Evolved CO_2 was absorbed in 4 ml scintillation cocktail (Oxysolve C-400, Zinsser) and radioactivity was measured by LSC.

2.2.8 Humin fractionation of soil and casts

Fractionation of soil and casts in the short-term experiment was performed using the whole soil and cast material in the microcosms; in the long-term experiment an aliquot equivalent to 5 g dry weight soil and cast was fractionated.

Dissolved organic matter (DOM) was extracted from 1 g dry weight soil or casts with 4 ml distilled water for 3 h by horizontal shaking at 250 rev min^{-1}. After centrifugation at 3000 rpm for 20 min, 0.5 ml supernatant was gently mixed with 3 ml scintillation cocktail and radioactivity was measured by LSC.

For fractionation of water insoluble humin 4 g soil, respectively cast, were extracted with 16 ml 0.1 M NaOH by horizontal shaking at 250 rev min^{-1} and 30°C for 24 h under anoxic conditions. Solutions were centrifuged for 30 min at 14000 rpm for separation of the alkaline soluble and insoluble humin fraction. Approximately 50 mg of humin fractions were dried at 65°C for two days, ground to powder, weighed into cellulose capsules an analysed for specific radioactivity by combustion and subsequent LSC as described for earthworm tissue. An aliquot (4 ml) of the soluble alkaline extract was stored under anoxic conditions at -28°C until further analysis by high-performance gel permeation chromatography (HP-GPC, see below). For measurement of specific radioactivity in alkaline extract 0.05 ml were gently mixed with 5 ml scintillation cocktail and analysed by LSC.

Separation of soluble fulvic acids (FA) from insoluble humic acids (HA) was performed by acidification of 1.8 ml alkaline extract to pH<1 with 67 µl 6 M HCl. Humic acids were allowed to precipitate for 24 h at 4°C and separated by centrifugation at 14000 rpm for 10 min from soluble fulvic acids. For analysis of radioactivity 0.2 ml fulvic acid fraction was mixed with 3 ml scintillation cocktail and measured by LSC. Specific radioactivity in the humic acid fractions

was calculated by subtracting the radioactivity in the fulvic acid fraction from that in the alkaline extract fraction.

The alkaline insoluble humin fraction was further separated by silylation (Haider et al., 1992). Briefly, 0.5 g dry weight humin were ground to powder, added to 2 ml Eppendorf tubes containing 1 ml silylation reagent trimethylchlorosilane (Aldrich Chemical Company, Inc., Milwaukee, WI, USA) and extracted on a rotary shaker at 250 rev min^{-1} and 30°C for 12 h. The solution was centrifuged at 14000 rpm for 15 min and supernatants were analysed for radioactivity by LSC. Radioactivity in insoluble fractions was calculated by subtracting the radioactivity in the soluble humin fractions from that in the humin fractions before silylation.

2.2.9 Statistical analysis

Data on incorporation of ^{14}C into earthworm tissue, earthworm survival and changes in earthworm biomass were analysed by two-factor analysis of variance (ANOVA) with the factors Soil (arable soil, forest soil) and Clay (with and without). Cumulative respiration of CO_2 and $^{14}CO_2$, cumulative leaching of ^{14}C and distribution of ^{14}C in the humic fractions were analysed by three-factor ANOVA with the factors Soil (arable soil, forest soil), Clay (with and without) and *O. tyrtaeum* (with and without). Rates of $^{14}CO_2$ production were analysed by repeated measures analyses of variance (RM-ANOVA). In the second part of the long-term experiment rates of $^{14}CO_2$ production of three consecutive measurements were averaged and analysed by RM-ANOVA. Significant time x treatment interactions were investigated using ANOVA for each sampling event and significant treatment differences were investigated using Tukey´s HSD test.

Prior to ANOVAs data were inspected for homogeneity of variance (Levene test) and log-transformed if required. A statistical probability $P<0.05$ was considered significant. STATISTICA 7.0 (Statsoft, Tulsa, USA) and SAS 8.0 (Statistical Analysis System, SAS Institute Inc., Cary, USA) software packages were used for statistical analyses.

2.3. Results

2.3.1 Earthworms

2.3.1.1 Short-term experiment

In the short-term experiment the soil had been transformed into casts after eleven days and earthworms were collected. All introduced earthworms survived; earthworm body mass generally decreased (-14.3 ± 8.9%) but was not affected by Soil ($F_{1,11}$=0.09, P=0.77) and Clay ($F_{1,11}$=0.26, P=0.62).

Total incorporation of ^{14}C into earthworm tissue was low but significantly affected by Soil ($F_{1,12}$=51.4, P<0.0001) and Clay ($F_{1,12}$=9.8, P=0.009) with on average 0.05% of the amount of ^{14}C added recovered in the tissue of *O. tyrtaeum* in forest soil and 0.10% in arable soil, and 0.06% in treatments with and 0.08% without clay (Table 2.1).

2.3.1.2 Long-term experiment

After seven days the soil in the long-term experiment had been transformed into casts and earthworms were collected. In total 26 of 200 earthworms had died; survival was significantly affected by Soil ($F_{1,16}$=4.71, P=0.04) with 77.0 ± 28.7% and 97.0 ± 4.8% surviving in the arable and forest soil, respectively. Replicates in with less than nine individuals survived were excluded from further analysis. Earthworm body mass generally increased by 21.6 ± 12.3% and was not affected by Soil ($F_{1,8}$=0.0005, P=0.98) and Clay ($F_{1,8}$=2.42, P=0.16).

Total incorporation of ^{14}C into earthworm tissue was affected by Soil ($F_{1,8}$=36.6, P=0.0003) with significant higher incorporation in earthworms in arable soil (0.44 ± 0.14%) than in forest soil (0.05 ± 0.02%). The addition of clay did not affect incorporation of ^{14}C in earthworm tissue ($F_{1,8}$=0.07, P=0.79; Table 2.2).

2.3.2 Mineralization

2.3.2.1 Short-term experiment

Cumulative production of CO_2-C in the short-term experiment was significantly affected by Soil ($F_{1,24}$=292.5, P<0.0001) with on average 3.27 mg CO_2-C microcosm^{-1} in the arable and 6.77 mg CO_2-C microcosm^{-1} in the forest soil. *Octolasion tyrtaeum* significantly increased cumulative

production of CO_2-C ($F_{1,24}$=175.2, P<0.0001) from 3.67 mg CO_2-C microcosm^{-1} to 6.37 mg CO_2-C microcosm^{-1} without and with *O. tyrtaeum*, respectively. Clay did not affect cumulative production of CO_2-C ($F_{1,24}$=2.43, P=0.13; Fig. 2.1a)

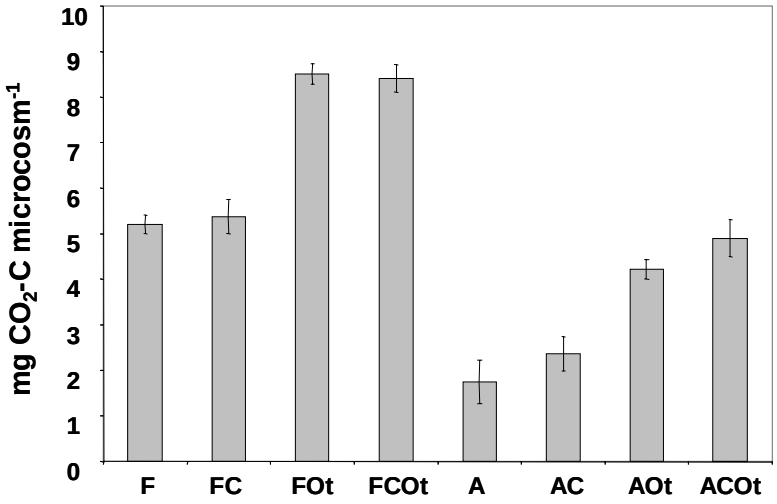

Figure 2.1a: Effect of the presence and absence of *Octolasion tyrtaeum* (Ot) and Clay (C) on the cumulative mineralization of carbon in Forest (F) and Arable soil (A) during short-term experiment. Means of three replicates with 1 S.D.

2.3.2.2 Long-term experiment

Cumulative production of CO_2-C in the first part of the long-term experiment was strongly affected by soil ($F_{1,16}$=689.5, P<0.0001) with significant higher cumulative production in the forest (48.62 mg CO_2-C microcosm^{-1}) than in the arable soil (16.35 mg CO_2-C microcosm^{-1}). In treatments with *O. tyrtaeum* cumulative production of CO_2-C was increased with the effect being more pronounced in the arable (from 3.14 to 29.92 mg CO_2-C microcosm^{-1}) than in the forest soil (from 32.00 to 65.23 mg CO_2-C microcosm^{-1}; Soil x *O. tyrtaeum*; $F_{1,16}$=6.97, P=0.02). Clay increased the cumulative production of CO_2-C only in the forest soil from 45.31 to 51.92 mg CO_2-C microcosm^{-1} (Soil x Clay; $F_{1,16}$=9.13, P=0.008), and in absence of *O. tyrtaeum* from 14.70 to 20.44 mg CO_2-C microcosm^{-1} (Clay x *O. tyrtaeum*; $F_{1,16}$=5.29, P=0.04; Fig. 2.1.b).

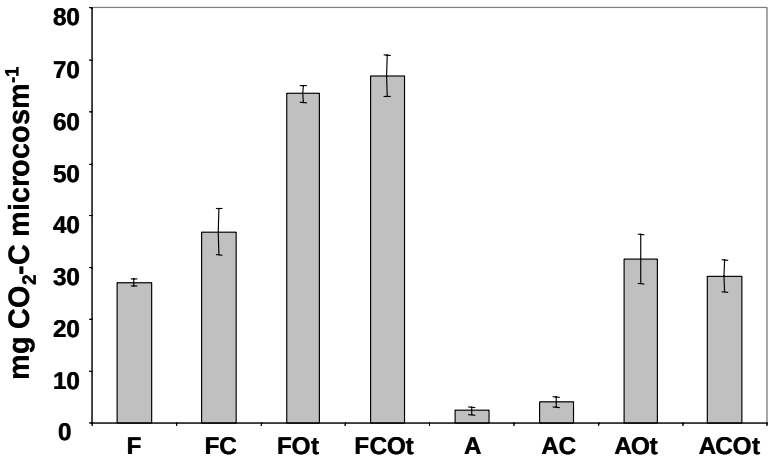

Figure 2.1.b: Effect of the presence and absence of *Octolasion tyrtaeum* (Ot) and Clay (C) on the cumulative mineralization of carbon in Forest (F) and Arable soil (A) during the long-term experiment in presence of *Octolasion tyrtaeum*. Means of three replicates with 1 S.D.

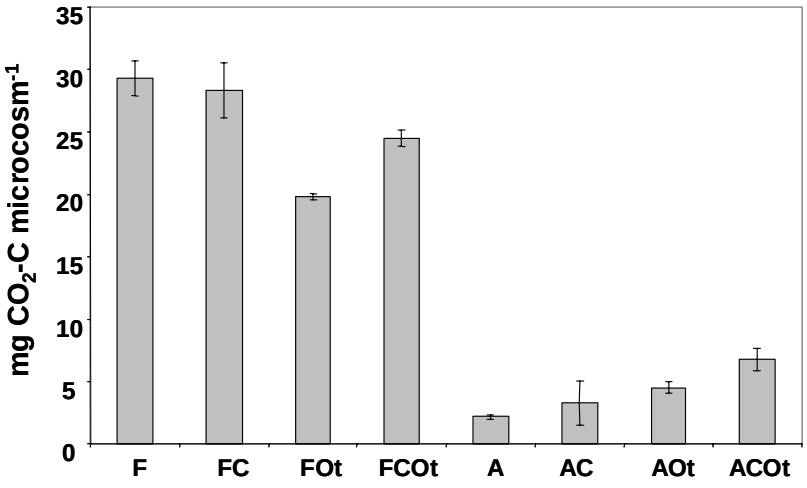

Figure 2.1.c: Effect of the presence and absence of *Octolasion tyrtaeum* (Ot) and Clay (C) on the cumulative mineralization of carbon in Forest (F) and Arable soil (A) during the long-term experiment after the extraction of *Octolasion tyrtaeum*. Means of three replicates with 1 S.D.

The effect of soil persisted in the second part of the experiment ($F_{1,16}$=1852.9, P<0.0001) with significant higher cumulative production of CO_2-C in the forest (25.45 mg CO_2-C microcosm^{-1}) than in the arable soil (4.36 mg CO_2-C microcosm^{-1}). *Octolasion tyrtaeum* also strongly affected cumulative production of CO_2-C but the effect depended on Soil (Soil x *O. tyrtaeum*; $F_{1,16}$=98.5, P<0,0001); whereas cumulative production of CO_2-C was higher in casts produced from arable

soil (5.40 mg CO_2-C microcosm^{-1}) than in the arable soil (3.33 mg CO_2-C microcosm^{-1}), it was significantly lower in casts produced from forest soil (21.62 mg CO_2-C microcosm^{-1}) than in forest soil (29.27 mg CO_2-C microcosm^{-1}; Fig. 2.1.c). Clay increased cumulative production of CO_2-C only in the forest soil from 23.63 to 27.27 mg CO_2-C microcosm^{-1} (Soil x Clay; $F_{1,16}$=5.53, P=0.03).

2.3.3 Mineralization of ^{14}C catechol

2.3.3.1 Short-term experiment

Cumulative production of $^{14}CO_2$-C in the short-term experiment was significantly higher in forest soil than in arable soil ($F_{1,24}$=158.8, P<0.0001). Generally, *O. tyrtaeum* increased cumulative production of $^{14}CO_2$-C, but the effect depended on Soil and Clay (Soil x Clay x *O. tyrtaeum*, $F_{1,24}$=39.1, P<0.0001). *Octolasion tyrtaeum* did not affect cumulative production of $^{14}CO_2$-C in the forest soil, but significantly increased it in the arable soil; clay increased cumulative production of $^{14}CO_2$-C in the arable soil in absence of *O. tyrtaeum*, but decreased it in presence of *O. tyrtaeum* (Table 2.1).

The rate of $^{14}CO_2$-C production ($^{14}CO_2$-C microcosm^{-1} h^{-1}) was high in the beginning, rapidly decreased until day four and slightly decreased to the end of the experiment (Fig. 2.2.a). Soil significantly affected the rate of $^{14}CO_2$-C production, but the effect varied with Time (Time x Soil; $F_{7,168}$=3801, P<0.0001). Early in the experiment the rate of $^{14}CO_2$-C production was significantly higher in the forest than in the arable soil, whereas it was the opposite later. *Octolasion tyrtaeum* significantly increased the rate of $^{14}CO_2$-C production ($F_{1,24}$=57.3, P<0.0001) but the effect depended on Soil and Clay (*O. tyrtaeum* x Soil; $F_{1,24}$=13.0, P=0.0014, *O. tyrtaeum* x Clay; $F_{1,24}$=22.7, P<0.0001). *Octolasion tyrtaeum* did not affect the rate of $^{14}CO_2$-C production in the forest soil, but significantly increased it in the arable soil late in the experiment (Time x Soil x *O. tyrtaeum*; $F_{1,168}$=5.5, P<0.0001), with the increase being more pronounced without Clay (Time x Clay x *O. tyrtaeum*; $F_{1,168}$=7.1, P<0.0001; Fig. 2.2.a).

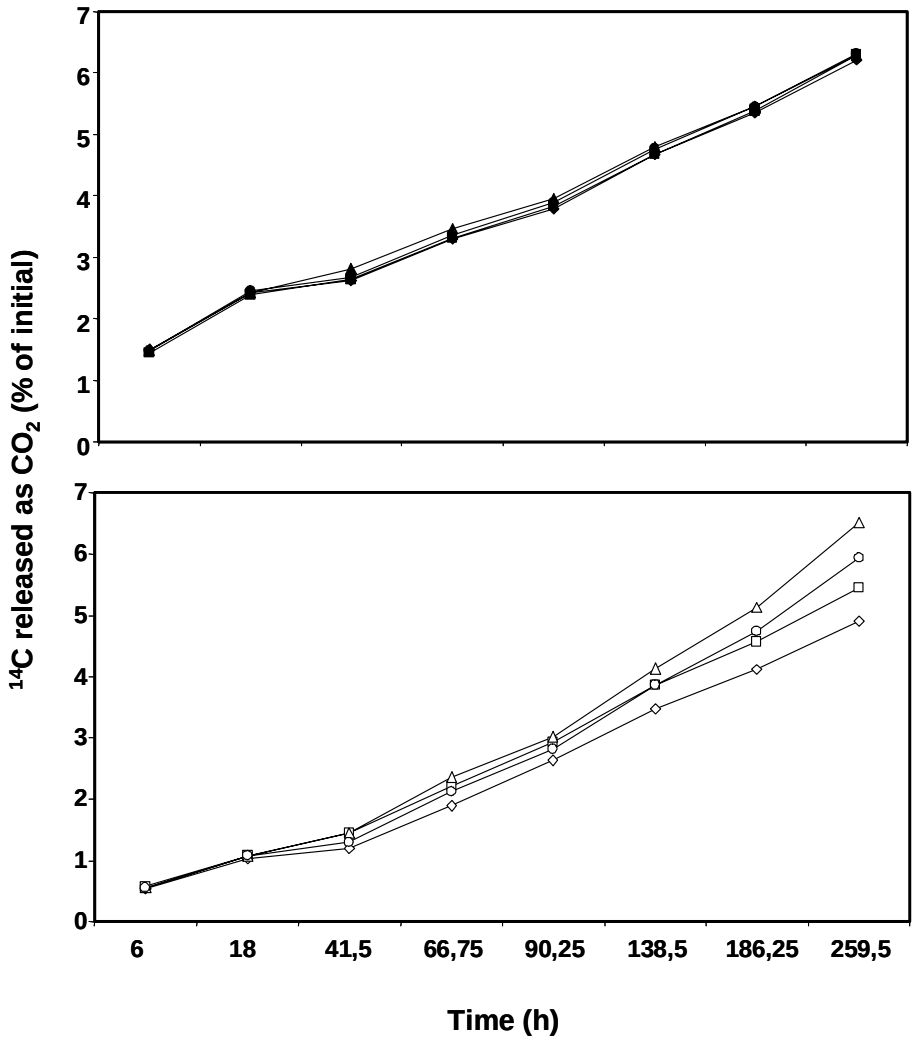

Figure 2.2.a: Production of $^{14}CO_2$ in pure Soil (rhombi), with addition of Clay (squares), in presence of *Octolasion tyrtaeum* (triangles) and with addition of Clay in presence of *Octolasion tyrtaeum* (circles) in Forest (closed symbols) and Arable soil (open symbols) during short-term experiment. Means of three replicates.

Table 2.1: Distribution and recovery (%) of radioactivity from ^{14}C-labelled catechol in Arable (A) and Forest soil (F) with and without Clay (C) and in presence and absence of *Octolasion tyrtaeum* (Ot) in the short-term experiment. Means of three replicates.

	O.t.	CO_2	DOM	FA	HA	Humin s.	Humin is.	total recovery
F		6.21 ± 0.04	0.17 ± 0.01	3.45 ± 2.51	2.51 ± 0.12	19.30 ± 0.64	78.28 ± 1.08	**109.92 ± 0.07**
A		4.95 ± 0.05	1.12 ± 0.53	6.01 ± 0.17	6.10 ± 0.19	36.27 ± 4.73	33.12 ± 0.63	**87.58 ± 4.69**
FC		6.29 ± 0.06	0.18 ± 0.01	3.01 ± 0.10	2.31 ± 0.06	17.66 ± 0.88	68.89 ± 4.21	**98.34 ± 5.09**
AC		5.49 ± 0.20	0.84 ± 0.01	5.94 ± 0.07	3.34 ± 0.26	38.28 ± 0.02	24.82 ± 1.65	**78.73 ± 2.59**
FOt	0.06 ± 0.02	6.30 ± 0.12	0.15 ± 0.01	3.16 ± 0.01	2.32 ± 0.06	18.99 ± 0.67	84.12 ± 5.64	**115.04 ± 6.29**
AOt	0.12 ± 0.02	6.56 ± 0.19	1.16 ± 0.09	6.42 ± 0.03	6.46 ± 0.35	31.78 ± 0.69	32.76 ± 7.76	**85.13 ± 1.23**
FCOt	0.03 ± 0.01	6.32 ± 0.10	0.15 ± 0.00	3.19 ± 0.11	2.09 ± 0.04	17.64 ± 0.34	68.71 ± 0.68	**98.10 ± 0.96**
ACOt	0.09 ± 0.02	5.97 ± 0.10	0.77 ± 0.20	5.97 ± 0.14	3.94 ± 0.46	39.66 ± 6.63	20.07 ± 5.93	**76.38 ± 2.50**

2.3.3.2 Long-term experiment

In the first part of the long-term experiment cumulative production of $^{14}CO_2$-C was significantly higher in the forest soil than in arable soil ($F_{1,16}$=7.44, P=0.02), whereas in the second part it was significantly higher in the arable soil than in the forest soil ($F_{1,16}$=23.9, P=0.0002; Table 2.2). Clay ($F_{1,16}$=1.22, P=0.29; $F_{1,16}$=0.02, P=0.9) and *O. tyrtaeum* ($F_{1,16}$=0.76, P=0.40; $F_{1,16}$=0.8, P=0.38) did not affect cumulative $^{14}CO_2$-C production. The rate of $^{14}CO_2$-C production ($^{14}CO_2$-C microcosm^{-1} h^{-1}) was high initially, then rapidly decreased until day four and slightly decreased to the end of the experiment (Fig. 2.2.b). Soil significantly affected the rate of $^{14}CO_2$-C production, but the effect varied with Time (Time x Soil; $F_{7,112}$=441.1, P<0.0001). Initially the rate of $^{14}CO_2$-C production was significantly higher in the forest than in the arable soil, whereas it was the opposite later.

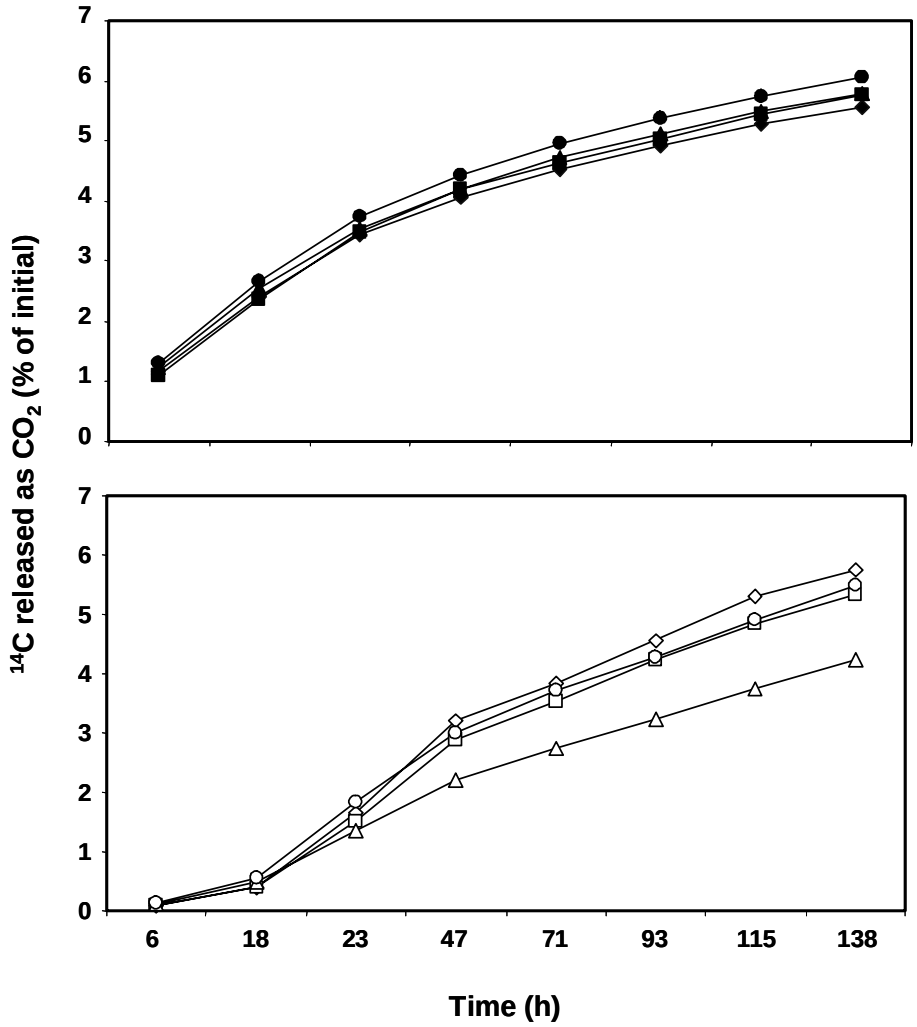

Figure 2.2.b: Production of $^{14}CO_2$ in pure Soil (rhombi), with addition of Clay (squares), in presence of *Octolasion tyrtaeum* (triangles) and with addition of Clay in presence of *Octolasion tyrtaeum* (circles) in Forest (closed symbols) and Arable soil (open symbols) during s the long-term experiment in presence of *Octolasion tyrtaeum*. Means of three replicates.

The effect of soil persisted during the second part of the experiment with significant higher rate of $^{14}CO_2$-C production in the arable soil than in the forest soil ($F_{1,16}$=13.1, P=0.0023). Clay and *O. tyrtaeum* did not significantly affect the rate of $^{14}CO_2$-C production during the long-term experiment (Fig. 2.2.c).

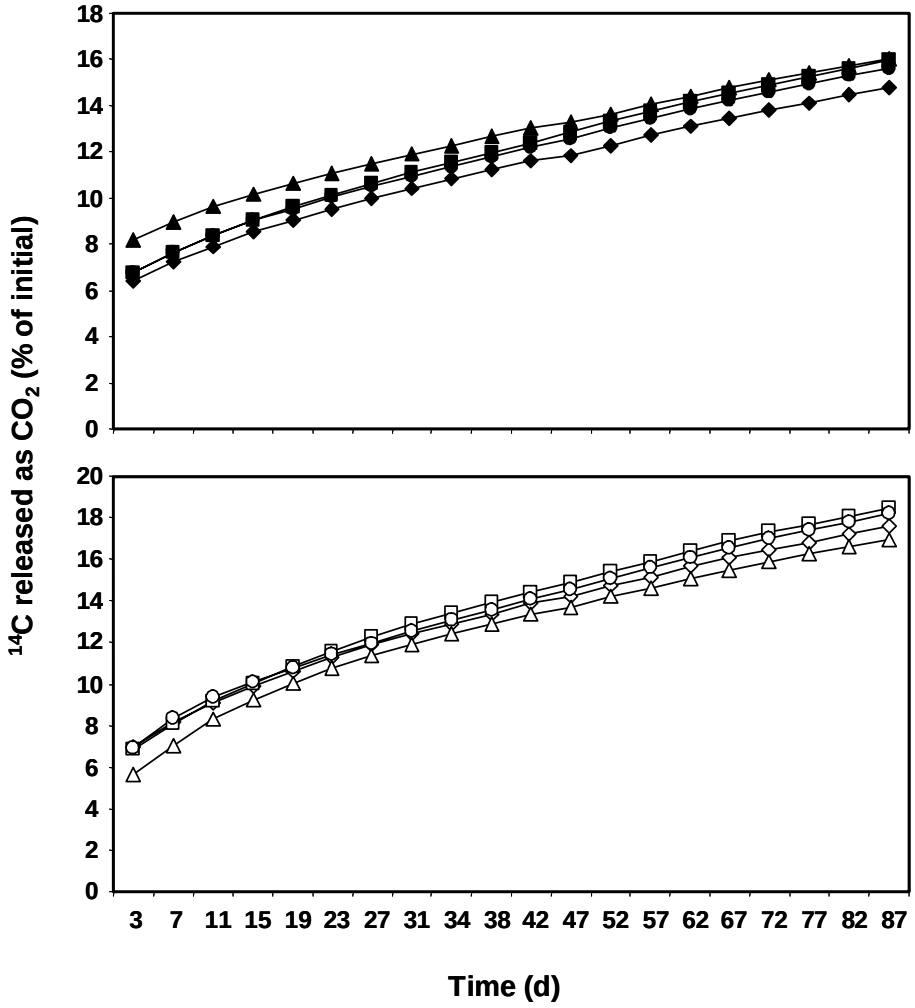

Figure 2.2.c: Production of $^{14}CO_2$ in pure Soil (rhombi), with addition of Clay (squares), in presence of *Octolasion tyrtaeum* (triangles) and with addition of Clay in presence of *Octolasion tyrtaeum* (circles) in Forest (closed symbols) and Arable soil (open symbols) during the long-term experiment after the extraction of *Octolasion tyrtaeum*. Means of three replicates.

2.3.4 Accumulation of ^{14}C in humic fractions

2.3.4.1 Short-term experiment

At the end of the short-term experiment most of the ^{14}C was recovered in the soil (Table 2.1). Total recovery of ^{14}C was significantly affected by Soil ($F_{1,16}$=242.4, P<0.0001) with on average 99.1 ± 8.4% and 75.1 ± 5.9% recovered from forest and arable soil, respectively. The addition

of clay significantly decreased total recovery of ^{14}C from 93.4 ± 14.0% without and 81.5 ± 11.2% with addition of Clay ($F_{1,16}$=63.7, P<0.0001). *Octolasion tyrtaeum* did not affect recovery of ^{14}C in soil ($F_{1,16}$=0.01, P=0.91).

Relative distribution of ^{14}C in humic fractions was strongly affected by soil with significant lower amount of ^{14}C in the DOM fractions ($F_{1,16}$=104.9, P<0.0001), the FA fractions ($F_{1,16}$=3941, P<0.0001) and the HA fractions ($F_{1,16}$=742.9, P<0.0001) and significant higher amount of ^{14}C in the humin fraction ($F_{1,16}$=1867.7 P<0.0001) in the forest than in the arable soil (Fig. 2.3.a).

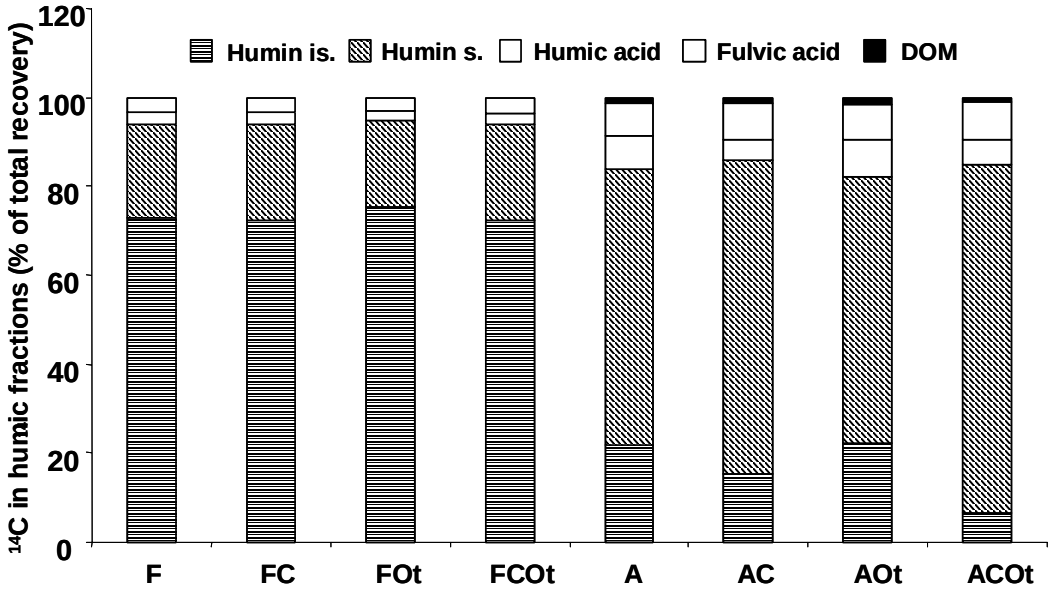

Figure 2.3.a: Relative distribution of radioactivity from ^{14}C-labelled catechol in the humic fractions of the Forest (F) and Arable soil (A) with and without Clay (C) and in presence and absence of *Octolasion tyrtaeum* (Ot) after the short-term incubation. Means of three replicates.

Overall, *O. tyrtaeum* did not affect the amount of ^{14}C in forest soil, but significantly increased it in the FA fraction (Soil x *O. tyrtaeum*; $F_{1,16}$=18.1, P=0.0006) and HA fraction (Soil x *O. tyrtaeum*; $F_{1,16}$=13.9, P=0.002) and decreased it in the humin fraction (Soil x *O. tyrtaeum*; $F_{1,16}$=16.6, P=0.0009) in the arable soil. The addition of clay significantly increased the relative amount of ^{14}C in the FA fraction (Soil x Clay; $F_{1,16}$=6.8, P=0.002) and humin fraction (Soil x Clay; $F_{1,16}$=36.4, P<0.0001), but strongly decreased it in the HA fraction of the arable soil (Soil x Clay, $F_{1,16}$=84.3, P<0.0001). However, the effect of clay on the amount of ^{14}C in the FA fraction depended on soil and *O. tyrtaeum*; the addition of clay increased the relative amount of

^{14}C in the FA fraction of the arable soil in absence of *O. tyrtaeum*, but increased it in the forest soil in presence of *O. tyrtaeum* (Soil x Clay x *O. tyrtaeum*; $F_{1,16}$=18.2, P=0.0005).
The relative distribution of ^{14}C within the humin fraction was strongly affected by soil with 56.7 ± 8.8% of the ^{14}C incorporated into the soluble fraction of the arable soil (supernatant following silylation) and 19.8 ± 0.9% into that of the forest soil ($F_{1,15}$=514.6, P<0.0001; Fig. 2.3.a). Clay significantly increased the incorporation of ^{14}C into the soluble fraction of the arable soil (Soil x Clay, $F_{1,15}$=12.2, P=0.003). *Octolasion tyrtaeum* did not affect ^{14}C distribution in the humin fraction ($F_{1,15}$=0.05, P=0.83).

2.3.4.2 Long-term experiment

In the long-term experiment total recovery of ^{14}C in soils was not affected by soil ($F_{1,16}$=2.9, P=0.11), clay ($F_{1,16}$=1.1, P=0.31) and *O. tyrtaeum* ($F_{1,16}$=1.3, P=0.28), with on average 48.2 ± 5.5% and 51.8 ± 5.4% recovered in the arable and forest soil, respectively (Table 2.2).
The relative distribution of ^{14}C in humic fractions was strongly affected by soil with significant lower amount of ^{14}C in the DOM fractions ($F_{1,16}$=299.4, P<0.0001), the FA fractions ($F_{1,16}$=181.5, P<0.0001) and the HA fractions ($F_{1,16}$=189.4, P<0.0001) and significant higher amount of ^{14}C in the humin fraction ($F_{1,16}$=926.8, P<0.0001) in the forest than in the arable soil (Fig. 2.3.b). Overall, clay did not affect the amount of ^{14}C in forest soil, but significantly decreased it in the DOM fraction (Soil x Clay; $F_{1,16}$=33.9, P<0.0001) and HA fraction (Soil x Clay; $F_{1,16}$=30.6, P<0.0001) and increased it in the humin fraction (Soil x Clay; $F_{1,16}$=91.9, P<0.0001) in the arable soil. *Octolasion tyrtaeum* significantly increased the relative amount of ^{14}C in the DOM fraction (Soil x *O. tyrtaeum*; $F_{1,16}$=11.4, P=0.004) and HA fraction (Soil x *O. tyrtaeum*; $F_{1,16}$=20.3, P=0.0004) but strongly decreased it in the humin fraction of the arable soil (Soil x *O. tyrtaeum*; $F_{1,16}$=50.2, P<0.0001). However, *O. tyrtaeum* increased the amount of ^{14}C in the DOM fraction of the arable soil only in treatments without clay (Soil x Clay x *O. tyrtaeum*; $F_{1,16}$=8.4, P=0.01).
The relative distribution of ^{14}C within the humin fraction was strongly affected by Soil ($F_{1,16}$=1143, P<0.0001) with 55.1 ± 3.2% of ^{14}C incorporated into the soluble humin fraction of the arable soil and 23.6 ± 2.1% into that of the forest soil (Fig. 2.3.b). *Octolasion tyrtaeum* significantly decreased the incorporation of ^{14}C into the soluble humin fraction ($F_{1,16}$=8.9, P=0.009), whereas clay tended to increase it ($F_{1,16}$=4.5, P=0.051).

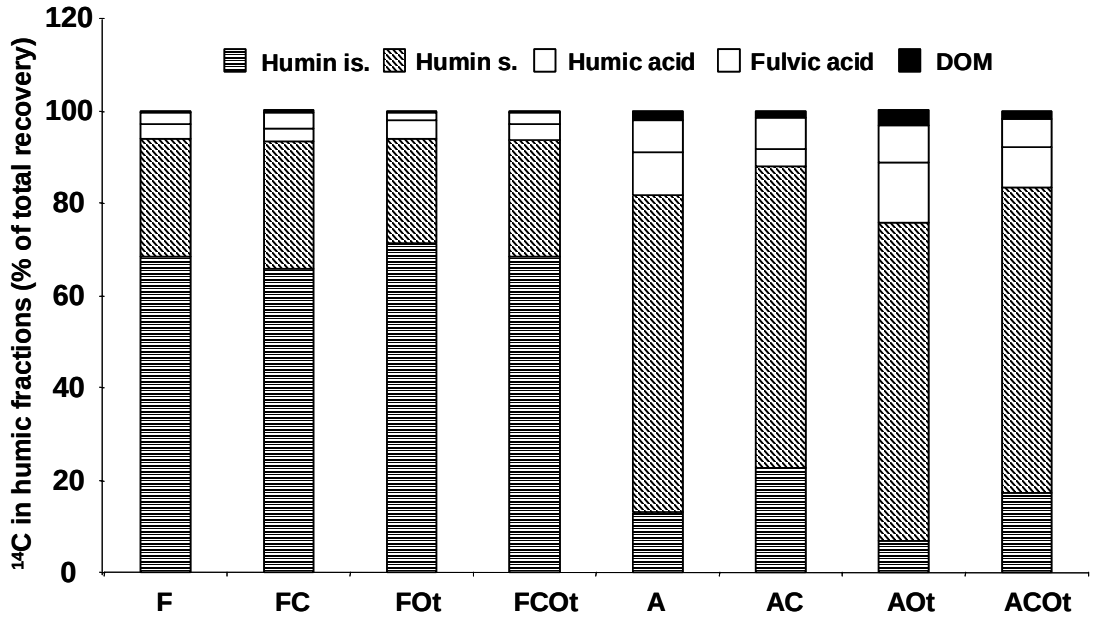

Figure 2.3.b: Relative distribution of radioactivity from ^{14}C-labelled catechol in the humic fractions of the Forest (F) and Arable soil (A) with and without Clay (C) and in presence and absence of *Octolasion tyrtaeum* (Ot) after the long-term incubation. Means of three replicates.

2.3.5 Leachate

On average 105 ± 6.1 ml of the added 120 ml of water added were recovered as leachate; the amount of leachate was not affected by Soil ($F_{1,16}$=1.6, P=0.22), Clay ($F_{1,16}$=0.3, P=0.61) and *O. tyrtaeum* ($F_{1,16}$=2.7, P=0.12). Cumulative leaching of ^{14}C was low but significantly affected by Soil ($F_{1,16}$=264.5, P<0.0001) with on average 1.15 ± 0.2% and 0.32 ± 0.1% of the added ^{14}C leached from the arable and forest soil, respectively. Clay ($F_{1,16}$=0.89, P=0.36) and *O. tyrtaeum* ($F_{1,16}$=3.17, P=0.07) did not affect cumulative leaching of ^{14}C.

Table 2.2: Distribution and recovery (%) of radioactivity from ^{14}C-labelled catechol in Arable (A) and Forest soil (F) with and without Clay (C) and in presence and absence of *Octolasion tyrtaeum* (Ot) in the long-term experiment. Means of three replicates.

	Ot	leachate	CO_2 1	CO_2 2	DOM	FA	HA	Humin s.	Humin is.	total recovery
F		0.36 ± 0.03	5.58 ± 0.05	9.05 ± 0.04	0.22 ± 0.03	1.43 ± 0.27	1.66 ± 0.29	11.87 ± 1.03	38.26 ± 5.36	**68.43 ± 6.12**
A		1.18 ± 0.10	5.60 ± 0.68	11.52 ± 0.24	0.96 ± 0.02	3.35 ± 0.64	4.43 ± 0.94	22.06 ± 4.08	17.05 ± 1.76	**66.16 ± 7.50**
FC		0.37 ± 0.03	5.98 ± 0.20	9.71± 0.84	0.27 ± 0.07	1.66 ± 0.25	1.48 ± 0.32	12.30 ± 1.94	35.39 ± 4.02	**67.16 ± 6.29**
AC		1.24 ± 0.30	5.37 ± 0.04	12.72 ± 0.44	0.68 ± 0.06	2.84 ± 0.29	1.67 ± 0.43	21.54 ± 0.53	16.24 ± 2.49	**62.31 ± 2.95**
FOt	0.05 ± 0.02	0.23 ± 0.14	5.84 ± 0.17	10.53 ± 3.79	0.19 ± 0.02	0.97 ± 0.35	2.25 ± 0.70	10.86 ± 1.13	40.36 ± 3.17	**71.27 ± 6.03**
AOt	0.46 ± 0.13	1.28 ± 0.04	5.46 ± 0.25	13.05 ± 0.05	1.53 ± 0.01	3.96 ± 0.97	6.22 ± 0.38	19.14 ± 0.31	17.52 ± 0.94	**68.62 ± 1.69**
FCOt	0.06 ± 0.06	0.32 ± 0.02	5.97 ± 0.20	9.16 ± 0.10	0.27 ± 0.02	1.16 ± 0.13	1.68 ±0.11	10.51 ± 1.10	34.26 ± 3.79	**63.38 ± 4.84**
ACOt	0.42 ± 0.42	0.93 ± 0.06	5.67 ± 0.08	12.29 ± 0.43	0.91 ± 0.22	3.27 ± 0.16	4.79 ± 1.33	24.66 ± 3.40	20.10 ± 0.30	**73.03 ± 4.27**

2.4. Discussion

2.4.1 Total recovery of applied ^{14}C

Total recovery of ^{14}C applied as catechol ranged from 76.4 ± 2.5 to 115.0 ± 6.3% in the short-term experiment and from 62.3 ± 2.7 to 73.0± 4.3 % in the long-term experiment. Although low recoveries particularly in the long-term experiment are somehow undesirable, they are in line with other studies (Kretzschmar and Ladd, 1993, Rüttimann-Johnson and Lamar, 1997, Waldrop et al., 2004). For example, Bailey and McGill (2002) incubated ^{14}C-pyrene in creosote-contaminated soil for twelve months and recovered only 62 to 81% in the first six month, suggesting that, due to the lack of volatilization, low recovery may be ascribed to a heterogeneous distribution of ^{14}C-pyrene in the soil in the beginning of the experiment. Presumably, insufficient homogeneity of ^{14}C catechol in the long-term experiment resulted in an underestimation of ^{14}C stabilized in the soil when analysing only an aliquot of the soil.
Total recovery of ^{14}C in the short-term experiment was higher, in part higher than 100% and significantly affected by the soil and clay. Recoveries of ^{14}C in excess of 100% also have been reported previously (Ji et al., 2000; Li and Brune, 2005), suggesting that some humic fractions may not be separated completely. However, only in two of 16 treatments we recovered on average more than 100% of the added ^{14}C catechol, suggesting that the extraction method is appropriate for the separation of humic fractions.

2.4.2 Accumulation of radiolabel in earthworm tissue

Overall, incorporation of ^{14}C catechol into the earthworm tissue was low, with 0.1% in the short-term and 0.44% of the added carbon in the long-term experiment. Information about the incorporation of phenolic compounds into the tissue of soil feeding invertebrates is scarce. Ji et al. (2000) recovered 8.4% of ^{14}C-labelled humic acids in the tissue of the soil-feeding termite *Cubitermes orthognathus* after incubation in tropical forest soil for ten days. In contrast, only 0.7% of aromatic ^{14}C-labelled humic acids were recovered in the tissue of cetoniid beetle larvae (*Pachnoda ephippiata*) incubated in soil for eight days (Li and Brune, 2005). Obviously, the incorporation of phenolic compounds into the tissue of soil feeding invertebrates varies between taxa, due to different digestion systems and symbiotic gut microflora.
In the present study the low incorporation of radiolabel into the tissue of *O. tyrtaeum* suggests that endogeic earthworms preferentially assimilate soil carbon compounds rather than phenolic compounds. Generally, the concentration of phenolic compounds strongly affects the

palatability of plant residues and the digestion by earthworms (Neuhauser et al., 1978). However, *O. tyrtaeum* quickly transformed the soils into casts, suggesting that the palatability of the mineral soil was not affected by the addition of ^{14}C-catechol, and that the concentration of phenolic compounds in soil is only of minor importance. Earthworms possess no or only a weak indigenous gut microflora (Schönholzer et al., 1999, Egert et al., 2004) and are little effective in the degradation of aromatic compounds (Neuhauser et al., 1978). Therefore, we suggest that the inability of digesting phenolic compounds, rather than their palatability was responsible for the low incorporation of ^{14}C into the earthworm tissue, and further that mainly microorganisms and the soil mineral matrix affected the fate of catechol in the soil.

2.4.3 Mineralization of soil organic matter

Earthworms significantly alter soil physical properties, such as water holding capacity, aeration and pore size (Devligher and Verstraete, 1997; Subler and Kirsch, 1998) and improve the availability of inorganic and organic compounds in soils, thus modify the biomass and activity of the decomposer community (Lee, 1985; Scheu, 1987a; Robinson et al., 1992; Salmon and Ponge, 2001). Generally, earthworms protect carbon from microbial mineralization by enclosing organic residues into cast aggregates (Guggenberger et al., 1995; Edwards and Bohlen, 1996). The stabilization of carbon, however, mainly occurs in ageing casts, whereas in fresh casts microbial activity is increased due to increased availability of nutrients (Tiunov and Scheu, 2000, Marhan and Scheu, 2006).

Particularly, in the arable soil in the short-term experiment and in the first part of the long-term experiment *O. tyrtaeum* significantly increased CO_2 production suggesting that *O. tyrtaeum* enhanced microbial activity in casts by improving the availability of nutrients from soil as well as by the excretion of mucoproteins, NH_4^+ and urea (Lee, 1985, Bohlen et al., 2004). Considering the different microbial biomass and nutrient contents of the soils, we suggest that the activity of *O. tyrtaeum* was only of minor importance for microorganisms in the nutrient rich forest soil, but strongly affected microorganisms in the nutrient poor arable soil by increasing carbon and nutrient availability in casts.

Early during the second part of the long-term experiment the production of CO_2 from the arable soil transformed into casts was higher than in non-processed soil, suggesting alleviation of the earthworm effect due to increased nutrient limitation later. In contrast, the production of CO_2 from the forest soil transformed into casts was significantly lower than in non-processed soil, suggesting protection of carbon inside of cast aggregates.

2.4.4 Mineralization of ^{14}C catechol

Phenolic compounds in the soil are subject to transformations resulting either in their stabilization or mobilization. Beside abiotic polymerization, leading to the formation of humic substances (Wang and Huang, 2000) and oxidation by Fe(III) and Mn(IV) (Dec et al., 2001; Pracht et al., 2001), they are polymerized and degraded by microorganisms. Several studies investigated the fate of phenolic compounds in pure cultures of bacteria, fungi and yeasts (Varga and Neujahr, 1970; Healy and Young, 1978; Leatham et al., 1983) as well as in soils (Cheng et al., 1983; Lehmann et al., 1987; Lehmann and Cheng, 1988; Cecchi et al., 2004). However, to our knowledge this is the first study investigating the effect of microorganisms on the fate of phenolic compounds in soils as mediated by earthworms.

Overall, the mineralization of catechol was high initially, but decreased later, suggesting that catechol was exploited quickly but then became unavailable and was protected from further mineralization. However, the soils studied significantly differed in their pattern of $^{14}CO_2$-C production; whereas initially the production of $^{14}CO_2$-C was higher in the forest than in the arable soil, it was contrary later. Presumably, due to higher microbial biomass in the forest soil, catechol was quickly mineralized but became unavailable quickly, due to nutrient limitation and stabilized in the soil matrix. In the arable soil catechol was mineralized more slowly due to the low microbial biomass but remained accessible for microbial decay until the end of the experiment.

Octolasion tyrtaeum did not affect the mineralization of ^{14}C-catechol in the long-term experiment, but increased it in the arable soil in the short-term experiment, suggesting that in soils with low microbial biomass and activity earthworms increase the activity of microorganisms. However, the increase in the mineralization of catechol in presence of *O. tyrtaeum* in the arable soil was counteracted by clay, suggesting that clay stabilize phenolic compounds during the gut passage through earthworms. This is in accordance to previous studies in which clay homogeneously mixed into the soil stabilized organic matter and protected it from microbial degradation (Shaw and Pawluk, 1986). Hence, we suggest that the intimate mixing of clay and phenolic compounds during the gut passage through earthworms promotes their stabilization in cast aggregates by binding with clay particles.

2.4.5 Stabilization of ^{14}C in humic fractions

The major part of the radioactivity added as ^{14}C catechol remained in the soil until the end of the experiment. Overall, the humin fraction contained the largest part, indicating that the labelled

catechol was stabilized by binding with the soil matrix. However, integration of catechol into the humic fractions differed between the two soils studied, with higher recovery of ^{14}C in the humin fraction, particular in the insoluble humin fraction, in the forest than in the arable soil. In addition, neither *O. tyrtaeum* nor clay affected the distribution of catechol in the humic fractions of the forest soil, suggesting that both are of little importance for the stabilization of phenolic compounds in soils with high clay content. The initially high mineralization on catechol in the forest soil and the relative low content of radiolabel in the labile humic fractions at the end of the experiments, suggests that catechol was rapidly attacked by microorganisms but quickly became stabilized by binding with the soil matrix.

Although in the arable soil most of the catechol was recovered from the humin fraction, mainly the soluble fraction, a considerable part was recovered from the labile DOM, FA and HA fraction, which are more easily accessible for microbial decay. *Octolasion tyrtaeum* increased the amount of ^{14}C in these labile fractions, suggesting that earthworms mobilized rather than stabilized phenolic compounds in the arable soil, which is supported by the increased mineralization of catechol in the arable soil with *O. tyrtaeum*.

In contrast, the addition of clay increased the amount of ^{14}C in the humin fractions in the arable soil, but the effect of clay on the distribution of ^{14}C within the humin fraction varied with time; it was decreased in the short-term experiment but increased it in the long-term experiment. The incorporation of carbon into particle size fractions depend on time. Carbon in organic residues quickly enters the coarse soil fractions, but these carbon pools are labile and easily mineralizable. In contrast, clay associated carbon pools are build up slowly and are more stable (Hassink et al., 1997), suggesting that the different time scale of accumulation and mineralization accounted for the increased amount of ^{14}C in the insoluble humin fraction in the long-term experiment.

2.5 Conclusions

Results of the present study support the general assumption that endogeic earthworms are little effective in the degradation of aromatic rings and affect phenolic compounds in soils mainly by controlling microbial activity and changing their association with the mineral soil matrix. In soils with high organic matter content, high clay content and high microbial biomass, endogeic earthworms are only of little importance, whereas in poor soils endogeic earthworms strongly increase microbial activity and thus mineralization of phenolic compounds. In these soils the additional availability of clay in combination with earthworms results in a decrease of the mineralization of phenolic compounds, suggesting that the intimately mixing during the gut

passage through earthworms promotes the stabilization of phenolic compounds in casts by binding with clay particles and protects carbon from microbial mineralization.

Chapter

3

Response of soil microorganisms and endogeic earthworms to cutting of grassland plants – a laboratory experiment

Abstract

It is hypothesized that defoliation and herbivory alter the availability of soluble carbon in the rhizosphere thereby the biomass and activity of soil microorganisms, and presumably higher trophic levels. We established a laboratory experiment investigating effects of cutting of shoots of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) on soil microorganisms and juveniles of the endogeic earthworm species *Octolasion tyrtaeum* (Savigny) and *Aporrectodea caliginosa* (Savigny). Plants were grown in microcosms for 4 weeks, cut 5 cm above the ground and left for regrow for another 4 weeks. Then, microcosms were destructively sampled.

Defoliation reduced total root biomass of *L. perenne* and *T. repens* by 48.6 and 67.3%, respectively but generally root biomass of *L. perenne* significantly exceeded that of *T. repens*. Both plant species increased microbial biomass in the soil with the effect of *L. perenne* being more pronounced than that of *T. repens*. In contrast, microbial activity remained unaffected. Although defoliation increased the availability of carbon in the rhizosphere as indicated by the increase in microbial biomass, the biomass of both earthworm species decreased during the experiment.

This suggests that earthworms were unable to exploit the microbial biomass pool in soil, rather, rhizosphere microorganisms appeared to have effectively competed with endogeic earthworms and exploited root exudates.

3.1. Introduction

Plants allocate considerable amounts of carbon belowground for the growth of roots to acquire nutrients and water. In addition, large amounts of carbon are either actively secreted into the rhizosphere, or passively diffuse in response to the root-soil concentration gradient. The carbon input into the rizosphere soil is estimated to account for 5-30% of the net assimilation of plants (Liljeroth et al., 1990; Johansson, 1992; Swinnen et al., 1995). Most of the carbon enters the soil at the sub-apical and the root-hair zone (Hale et al., 1978; Curl and Truelove, 1986). This deposition of carbon affects microorganisms in the rhizosphere, as the compounds are readily assimilated (Campbell et al., 1997; Grayston et al., 1998). Thus, the carbon released into the rhizosphere soil affects microbial biomass, activity and community composition, and therefore several soil ecosystem processes such as soil organic matter decomposition and nutrient mineralization.

Defoliation through aboveground herbivory alters the allocation and release of carbon into the rhizosphere and may affect microbial community structure and organic matter dynamics in the rhizosphere; however, the effects of defoliation appear to be variable. Defoliation increased the exudation of carbon into rhizosphere soil in some studies (Holland et al., 1996; Paterson and Sim, 1999, 2000), whereas in others effects were neutral (Todorovic et al., 1999, Bazot et al., 2005) or negative (Miller and Rose, 1992). In addition, the response of the microbial community in the rhizosphere soil on defoliation is variable. For instance, Holland (1995) and Mawdsley and Bardgett (1997) found microbial biomass in the rhizosphere to be increased after defoliation, whereas others did not find any changes (Kuzjakov et al., 2002; Mikola et al., 2001). Microorganisms are the major primary decomposers and the nutritional basis for a large number of soil invertebrates including amoebae, flagellates, nematodes and earthworms (Hunt et al., 1987; De Ruiter et al., 1995; Berg et al., 2001). Hence, defoliation and subsequent alteration in carbon rhizodeposition, microbial biomass and community composition is likely to propagate at higher trophic levels. However, information on the effect of defoliation on the biomass of invertebrates in the rhizosphere is sparse and limited to certain groups such as nematodes (Bazot et al., 2005; Ilmarinen et al., 2005; Mikola et al., 2005). The response of major groups of decomposer invertebrates such as earthworms have not been investigated in a rigorous way. Although endogeic earthworms are known to primarily consume soil and associated humified organic residues it is still debated if microorganisms significantly contribute to earthworm nutrition. Endogeic earthworms primarily feed in the rhizosphere (Spain et al, 1990; Spain and Le Feuvre, 1997) thereby they may also ingest roots (Cortez and Bouche, 1992). Predominantly, however, they are consuming rhizodeposits, rhizobiota such as mycorrhizae, saprophytic fungi, nematodes and protozoa (Bonkowski and Schaefer, 1997;

Doube and Brown, 1998). Hence, if defoliation alters the amount of rhizodeposites and thereby the microbial biomass and community composition in the rhizosphere, it likely also affects endogeic earthworms.

Endogeic earthworms are among the most important decomposers in temperate ecosystems and significantly modify soil physical properties through burrow construction and casting thereby affecting microbial biomass, activity and community composition (Edwards and Bohlen, 1996). They primarily feed on organic matter in the soil which they intimately mix during the gut passage. Fresh cast aggregates of endogeic earthworms are hotspots of microbial activity due to increased nutrient and carbon availability (Devliegher and Verstraete, 1997). During aging of casts, however, organic matter enclosed in casts becomes protect against microbial attack (Guggenberger et al., 1995; Tiunov and Scheu, 1999; Marhan and Scheu 2006).

Commonly, earthworm nutrition is considered to depend on the input of resources from above the ground, i.e. plant residues in particular leaf litter. However, a large amount of carbon enters the soil via plant roots suggesting that endogeic earthworms may also benefit from belowground resource input. A large fraction of resources entering the soil via roots constitute of sugars forming a major component of root exudates. In fact, it has been documented that earthworms are limited by the availability of carbon resources and benefit from e.g. the addition of glucose to the soil (Tiunov and Scheu 2004). Root exudates, on the other hand are readily consumed by microorganisms. Therefore, endogeic earthworms and rhizosphere microorganisms likely interact and compete for carbon resources in the rhizosphere and these interactions may vary with the amount of exudates produced.

The aims of the study were to determine (1) if plant defoliation affects microbial biomass and activity in the rhizosphere, (2) if endogeic earthworms benefit from the defoliation of the plants, and (3) if the mineralization of soil carbon is affected by the defoliation of the plants and the presence of endogeic earthworms.

3.2 Material and methods

3.2.1 Soil, earthworms and plants

In September 2004 arable soil was collected from the upper 10 cm of the long-term fertilization experiment in Bad Lauchstädt (Saxony-Anhalt, Germany) and sieved (4 mm mesh size) to remove stones and coarse plant residues. For defaunation the soil was stored at -20°C for one week and kept at 4°C one week before the experiment was set-up. Prior to adding to microcosms chemical and physical characteristics and microbial biomass of the soil were

determined (n = 4). The particle size distribution as determined according to Stemmer et al. (1998) was 11.9% sand, 70.5% silt and 16.1% clay. The water holding capacity and soil pH determined as described in Schinner et al. (1993) was 52% and 7.0, respectively. Total carbon and nitrogen concentration as measured by an elemental analyser (NA 1500, Carlo Erba, Milan, Italy) were 1.70% and 0.16%, respectively. Microbial biomass as determined by the substrate-induced respiration method (SIR) (Anderson and Domsch, 1978; Scheu, 1992) was 210 µg C_{mic} g dry weight $soil^{-1}$.

In September 2004 juvenile earthworms were sampled by hand in a 130-year old beech wood on limestone near Göttingen (Southern Lower Saxony, Germany). Details on the site are given in Schaefer (1991). Earthworms were transferred into the laboratory and identified. The endogeic earthworm species *Aporrectodea caliginosa* (Savigny) and *Octolasion tyrtaeum* (Savigny) dominant in the beech wood were chosen for the experiment. Earthworms were kept in containers filled with experimental soil at 4°C until the experiment was set-up.

In total 240 seeds of *Trifolium repens* L. (white clover) and *Lolium perenne* L. (perennial ryegrass) were sown individually in plastic tubes with a height of 90 mm and a diameter of 16 mm filled with experimental soil. Tubes were incubated in a climate controlled greenhouse at 18°C, a relative humidity of 80% and watered every day with distilled water. Three weeks later, when the plants had reached 10 cm in height the experiment was started by adding of the earthworms.

3.2.2 Experimental set-up

A total of 105 microcosms were filled with 1200 g fresh weight soil to a height of 150 mm, resulting in a bulk density of 0.98 g cm^{-3}. Microcosms consisted of plastic tubes with a height of 190 mm and a diameter of 100 mm. Microcosms were closed at the bottom by plastic lids to avoid leaching of water and nutrients. To assure a continuous water supply, three glass fibre wicks (Ortmann, Hilden, Germany) approximately 400 mm in length were water saturated and positioned in the depth of 20, 60 and 100 mm below the soil surface. The three wicks were connected via a flexible plastic tube to a 500 ml plastic bottle filled with distilled water. Microcosms were closed on the top by plastic lids. An opening in the lid allowed the placement of the plants into the microcosms, seeded in separate plastic tubes. A vessel was attached to the lid which could be filled with alkali (1N KOH) to trap CO_2 in the headspace.

One plastic tube either containing one *T. repens* or *L. perenne*, and one juvenile earthworm, either *A. caliginosa* (0.22 ± 0.05 g) or *O. tyrtaeum* (0.20 ± 0.06 g), were added to the microcosms to establish the following treatments: soil only **(S)**, soil + *O. tyrtaeum* **(O),** soil + *A.*

caliginosa **(A)**, soil + *T. repens* **(T)**, soil + *L. perenne* **(L)**, soil + *T. repens* + *O. tyrtaeum* **(TO)**, soil + *T. repens* + *A. caliginosa* **(TA),** soil + *L. perenne* + *O. tyrtaeum* **(LO),** soil + *L. perenne* + *A. caliginosa* **(LA).** In half of the microcosms of each treatment plants were cut after four weeks (denoted by **d**). Treatments were replicated seven times; four empty microcosms served as control for CO_2 measurements. Microcosms were incubated eight weeks in a climate controlled greenhouse at 18°C, with a relative humidity of 80% and a 16 to 8 h day to night cycle.

3.2.3 Sampling and analytical procedure

After four weeks half of the plants were cut 5 cm above the ground (defoliation treatments). Shoots were dried at 65°C for three days and weighed. During incubation CO_2 production was determined three times before (day 5, 9 and 28) and four times after defoliation (day 33, 35, 47 and 57) as described in Macfadyen (1970). Briefly, CO_2 evolved in the microcosms in a period of two days was trapped in alkali (1 N KOH), and an aliquot (0.5 ml) was measured titrimetrically with 0.1 N HCl after precipitation of carbonate with saturated $BaCl_2$ solution.
At the end of the experiment plants were cut at ground level, dried at 65°C for three days and weighed. Soil cores were removed from the microcosms and divided into three layers (H1, 0-3 cm; H2, 4-8 cm; H3, 9-15 cm). Water content of the soil was determined in an aliquot as described in Schinner et al. (1993). Approximately 20 g fresh weight soil of each layer was stored for microbial analyses. Roots enclosed in each layer were washed, dried at 65°C for three days and weighed. Earthworms were collected, weighed, killed by freezing at -20°C for one day and dried at 65°C for three days.
Microbial biomass was determined using the chloroform fumigation extraction (CFE) method as described in Joergensen (1996). Briefly, 5 g fresh weight root-free rhizosphere soil was weighed into glass vessels. The vessels were placed in a desiccator lined with wet filter paper to maintain humidity, and containing a vessel filled with about 50 ml ethanol-free chloroform ($CHCl_3$) and anti-bumping granules. The desiccator was evacuated using a vacuum pump until the $CHCl_3$ boiled; then, the desiccator was incubated at room temperature. After 24 h the filter paper and the vessel with $CHCl_3$ were removed, and the desiccator was flushed with air. Fumigated and non-fumigated control soil samples were transferred into 50 ml Falcon tubes containing 20 ml 0.025 M K_2SO_4, shaken for 30 min and centrifuged at 4560 g for 30 min. Supernatants were transferred into 20 ml plastic vessels using a pipette equipped with 2 µm gaze filters. Organic C in the supernatants was determined as CO_2 by infrared absorption after combustion at 850°C using a Dimatoc 100 automatic analyzer (Dimatec, Essen, Germany). Microbial biomass C was

calculated as $C_{mic} = E_C / k_{EC}$, where E_C = (organic C extracted from fumigated soils) – (organic C extracted from non-fumigated soils) and $k_{EC} = 0.45$ (Wu et al., 1990; Joergensen, 1996).

3.2.4 Data analysis

Data were analysed by two or three factor analysis of variance (ANOVA) with the factors Defoliation (with and without), Plant either with two levels (*L. perenne*, *T. repens*), three levels (without plants, *L. perenne*, *T. repens*) for the analysis of carbon mineralization before defoliation, and Earthworm either with three levels (without earthworms, *O. tyrtaeum*, *A. caliginosa*) or two levels (*O. tyrtaeum*, *A. caliginosa*) for the analysis of earthworm biomass. Repeated measures analyses of variance (RM-ANOVA) was used to analyse variations between the three soil layers. Significant layer and treatment interactions were further investigated using ANOVA for each layer; differences between means were evaluated using Tukey´s HSD test. Homogeneity of variances was analysed using Levine test; to increase homogeneity of variance data were log-transformed if necessary. A statistical probability $P<0.05$ was considered significant. STATISTICA 7.0 (Statsoft, Tulsa, USA) and SAS 8.0 (Statistical Analysis System, SAS Institute Inc., Cary, USA) software packages were used for statistical analyses.

3.3. Results

3.3.1 Earthworms

In total one of the 35 individuals of *A. caliginosa* and eight of the 35 individuals of *O. tyrtaeum* added to the microcosms died until the end of the experiment. Earthworm body mass generally decreased but the decline significantly differed between earthworms species ($F_{1,20}=26.7$, $P<0.0001$), with *A. caliginosa* loosing 23.5 ± 11.6% and *O. tyrtaeum* 38.7 ± 6.7% of their initial body mass. Analysis of the earthworm species separately showed that the biomass of *A. caliginosa*, but not that of *O. tyrtaeum*, was affected by both the presence of the plants and the defoliation of the plants (Plant x Defoliation: $F_{1,8}=6.31$; $P=0.04$); the defoliation of *T. repens* significantly increased biomass loss of *A. caliginosa* from -7.5% to -31.5%, whereas the defoliation of *L. perenne* slightly decreased it from -27.4% to -22.6%.

3.3.2 Plants

Biomass of shoots of *L. perenne* (0.72 g dry weight) cut off after four weeks significantly exceeded that of *T. repens* (0.10 g dry weight; $F_{1,12}$=153.4, P<0.0001); earthworms generally did not affect shoot biomass ($F_{2,12}$=0.98, P=0.04). After eight weeks shoot biomass did not differ between plant species ($F_{1,24}$=0.88, P=0.36), but was significantly affected by defoliation ($F_{1,24}$=58.27, P<0.0001) with 0.84 g dry weight leaves harvested from plants previously defoliated and 1.79 g dry weight from non-defoliated plants. Again, earthworms did not affect plant biomass at the end of the experiment ($F_{2,24}$=1.16, P=0.33). Total shoot biomass of both sampling dates depended on both plant species and defoliation (Plant x Defoliation; $F_{1,24}$=6.05, P=0.02). The biomass of non-defoliated *L. perenne* (1.87 g dry weight), non-defoliated *T. repens* (1.73 g dry weight) and defoliated *L. perenne* (1.62 g dry weight) did not significantly differ, whereas the biomass of defoliated *T. repens* was significantly reduced (0.88 g dry weight; Fig. 3.1).

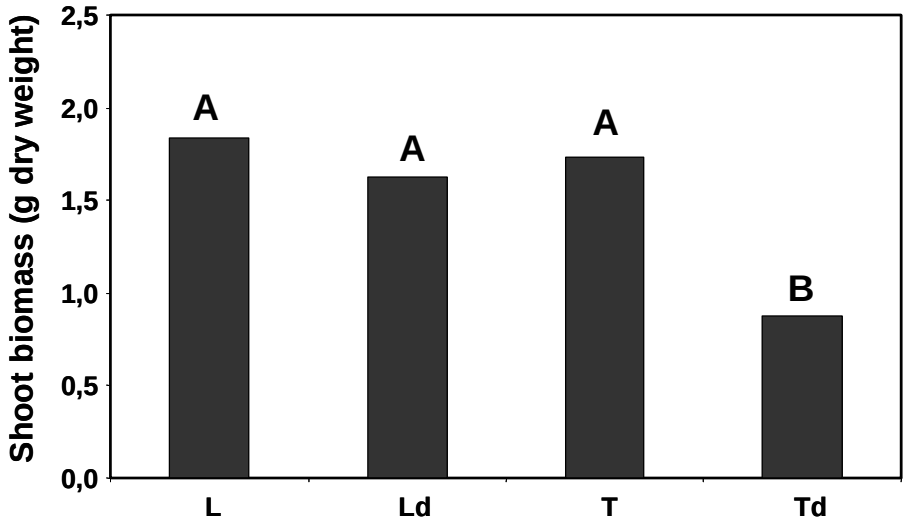

Figure 3.1: Effect of defoliation (d), on total shoot biomass (i.e. the sum of defoliated shoot mass and harvested shoot mass) of *Lolium perenne* (L) and *Trifolium repens* (T). Means of three replicates. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

Generally, root biomass of *L. perenne* significantly exceeded that of *T. repens* and this was more pronounced in deeper soil layers (Layer x Plant; $F_{2,48}$=101.04, P<0.0001). Root biomass of *L. perenne* increased with soil depth, whereas that of *T. repens* decreased from H1 to H2 and then remained in H3 (Fig. 3.2). Defoliation also affected root biomass but the effect varied between soil layers and plant species (Layer x Defoliation x Plant; $F_{2,48}$=21.39, P<0.0001). Defoliation decreased root biomass of both plant species in H1 from 0.26 to 0.14 g, did not significantly affect root biomass of *T. repens* in H2 (0.06 g) and H3 (0.07 g), but significantly decreased root biomass of *L. perenne* in H2 from 0.56 to 0.28 g and in H3 from 0.96 to 0.45 g (H2; $F_{1,24}$=13.61, P=0.001; H3; $F_{1,24}$=24.10, P<0.0001). Earthworm generally did not affect root biomass (Layer x Earthworm; $F_{4,48}$=2.12, P=0.09).

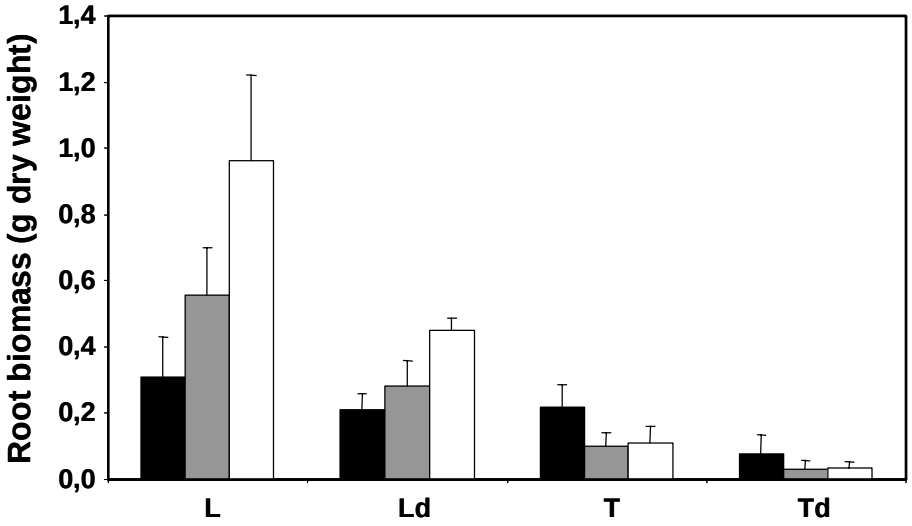

Figure 3.2: Root biomass of *Lolium perenne* (L) and *Trifolium repens* (T) in H1 (0-3 cm, black bars), H2 (4-8 cm, grey bars) and H3 (9-15 cm, white bars) as affected by defoliation (d). Means of three replicates ± 1 S.D.

3.3.3 Microbial biomass

Earthworms did not significantly affect microbial biomass in the soil ($F_{1,4}$=1.38, P=0.30). Microbial biomass significantly differed between H1 (580 µg C_{mic} g^{-1} dry weight soil) and both H2 (707 µg C_{mic} g^{-1} dry weight soil) and H3 (701 µg C_{mic} g^{-1} dry weight soil; $F_{2,16}$=5.25, P=0.02). Plant species and defoliation did not affect microbial biomass in H1 and H2, but significantly affected it in H3 with 607 µg C_{mic} g^{-1} dry weight soil in the rizosphere of *T. repens* and 795 µg C_{mic} g^{-1} dry weight soil in that of *L. perenne* ($F_{1,8}$=14.09, P=0.006), and 632 and 771

µg C_{mic} g^{-1} dry weight soil in the rizosphere of non-defoliated and defoliated plants, respectively ($F_{1,8}$=7.66, P=0.02; Fig. 3.3).

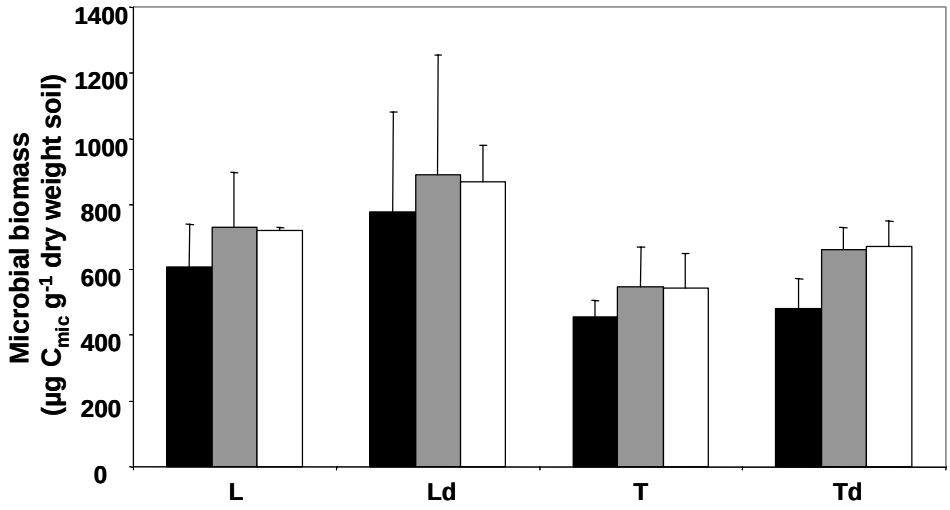

Figure 3.3: Microbial biomass in H1 (0-3 cm, black bars), H2 (4-8 cm, grey bars) and H3 (9-15 cm, white bars) as affected by defoliation (d) of *Lolium perenne* (L) and *Trifolium repens* (T). Means of three replicates ± 1 S.D.

3.3.4 CO_2 production

CO_2 production in treatments without plants remained almost constant throughout the experiment with on average 2.29 ± 0.41 mg C microcosm^{-1} d^{-1} (Fig. 3.4). Until day five CO_2 production was not affected by the presence of *T. repens* and *L. perenne* ($F_{2,36}$=0.06, P=0.95), but significantly increased in presence of *O. tyrtaeum*, particularly in treatments without plants ($F_{2,36}$=4.78, P=0.01). However, later in the experiment earthworms generally did not affect CO_2 production. At day 19 CO_2 production was significantly increased in presence of *L. perenne* ($F_{2,36}$=122.47, P<0.0001), whereas at day 28 both plant species significantly increased CO_2 production ($F_{2,36}$=147.58, P<0.001) with the effect being more pronounced with *L. perenne* than with *T. repens* (11.04 ± 0.45 and 4.68 ± 1.51 mg C microcosm^{-1} d^{-1}, respectively).

After defoliation, CO_2 production significantly differed between plant species (Fig. 3.4). It rapidly declined in treatments with *L. perenne* to 6.32 ± 0.73 mg C microcosm^{-1} d^{-1} and then remained almost constant until the end of the experiment. In contrast, CO_2 production in treatments with *T. repens* slightly decreased to 3.44 ± 0.76 mg C microcosm^{-1} d^{-1}, but then rapidly increased to 9.30 ± 1.20 mg C microcosm^{-1} d^{-1} at the end of the experiment. CO_2 production in microcosms with non-defoliated *L. perenne* further increased to 13.30 ± 1.48 mg

C microcosm^{-1} d^{-1} until day 33 and then decreased steeply to 9.93 ± 1.10 mg C microcosm^{-1} d^{-1} at the end of the experiment. In contrast, CO_2 production in microcosms with non-defoliated *T. repens* continuously increased to 15.14 ± 1.04 mg C microcosm^{-1} d^{-1} until the end of the experiment.

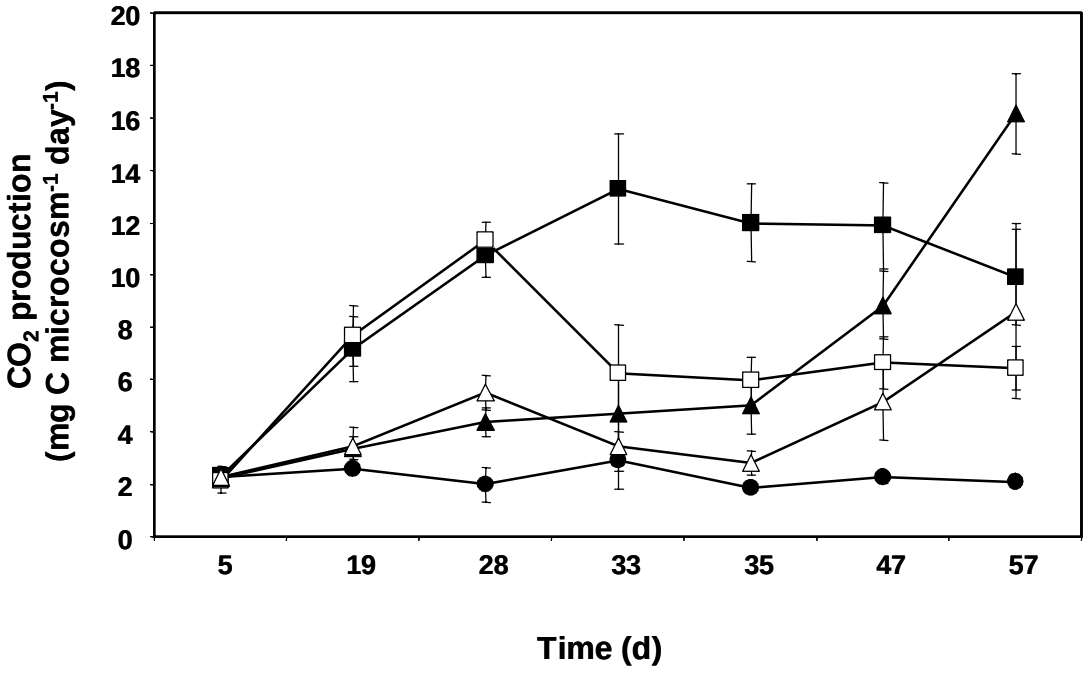

Figure 3.4: CO_2 production in treatments without plants (circle), and with *Lolium perenne* (square) or with *Trifolium repens* (triangle) either with non-defoliated (closed symbols) or defoliated plants (open symbols). Means of three replicates ± 1 S.D

3.3.5 Soil water content

The water content in the three soil layers ranged from 17.86 to 26.31% of dry weight; it was affected by the presence of plants and earthworms, but the effect varied between soil layers (Layer x Plant; $F_{2,48}$=4.52, P=0.016; Layer x Earthworm; $F_{4,48}$=3.50, P=0.014). In H1 the soil water content (23.04 ± 2.18%) was neither affected by plants ($F_{1,24}$=2.30, P=0.14), nor by earthworms ($F_{2,24}$=1.93, P=0.17); in H2 it was significantly higher in presence of *T. repens* (24.50 ± 1.89%) compared to *L. perenne* (22.88 ± 2.81%; $F_{1,24}$=7.06, P=0.014); in H3 it was also not affected by earthworms ($F_{2,24}$=1.09, P=0.35), but similar to H2 it was significantly higher in treatments with *T. repens* (25.68 ± 2.52%) than in those with *L. perenne* (23.62 ± 2.09%; $F_{1,24}$=16.16; P=0.0005). Further, both earthworms affected the soil water content in H2

($F_{2,24}$=3.79, P=0.037) with increasing water content in the order with *A. caliginosa* (22.78 ± 2.44%), with *O. tyrtaeum* (23.48 ± 2.91%) and without earthworms (24.81 ± 1.78%).

3.4. Discussion

3.4.1 Response of microorganisms

Results of the present study suggest that the presence of plants and plant defoliation beneficially affect microorganisms in the rhizosphere. Both plant species significantly increased microbial biomass and activity in soil, confirming that microorganisms benefit from root exudates and rhizodeposites (Holland, 1995; Mawdsley and Bardgett, 1997). However, the response of microorganisms differed between plant species. At the end of the experiment microbial biomass in the rhizosphere of *L. perenne* significantly exceeded that of *T. repens*. This contrasts results of the study of de Neergaard and Gorissen (2004) who reported higher microbial biomass in clover than in grass rhizosphere soil. The contrasting results presumably are due to different performance of legumes and grasses in the two experiments; the biomass of roots of legumes exceeded that of grasses in the study of de Neergaard and Gorissen (2004) whereas the opposite was the case in our experiment. Compared to bulk soil, microbial biomass and activity is known to be increased in nutrient rich microsites such as the rhizosphere (Poll et al. 2006; Brown et al., 2000; Furlong et al., 2002). Hence, the higher microbial biomass under grass compared to clover in our experiment likely was caused by higher root derived resources rather than by the different plant species.

In addition to the presence of plants, defoliation significantly increased microbial biomass in soil. This is in accordance to previous studies in which the defoliation of *L. perenne, T. repens* and other plant species resulted in an increased microbial biomass (Holland, 1995; Mawdsley and Bardgett, 1997). Defoliation increases the amount of dead roots and therefore the input of resources into the soil (Guitian and Bardgett, 2000; Ilmarinen et al., 2005). Also, root exudation may increase after defoliation (Paterson and Sim, 2000).

Although microbial biomass in the rhizosphere under grass exceeded that under clover at the end of the experiment, CO_2 production in clover treatments exceeded that of grass treatments. Since CO_2 production in the present study comprised of root respiration and microbial respiration, we suggest that differences in CO_2 production between plant species likely were due to differences in root biomass and rhizodeposition.

3.4.2 Response of earthworms

Interactions between plants and earthworms are extensively investigated. However, studies primarily focused on the effect of earthworms on the growth of plants (Scheu, 2003) or on the consumption of plant residues and subsequent growth of earthworms (Curry, 2004); little is known on the effects of living plants on earthworms. Endogeic earthworms colonize the upper layer of the mineral soil and primarily consume soil and associated organic residues (Bouche, 1972). They do not feed indiscriminately but select and exploit nutrient rich microsites (Wolter and Scheu, 1999; Marhan and Scheu, 2006). The rhizosphere forms such a nutrient rich microenvironment, with increased availability of dead roots and root exudates as well as microorganisms. Rovira et al. (1987) observed that endogeic earthworms primarily burrow close to the roots of wheat. Similarly, Hirth et al. (1998) found *Aporrectodea rosea* to preferentially burrow close to the roots of perennial ryegrass, suggesting that roots, rhizosphere soil and associated microorganisms form part of the diet of endogeic earthworms.

Contrary to our expectations the growth of *A. caliginosa* and *O. tyrtaeum* was neither affected by the defoliation of the plants nor by the presence of plants. Although, the presence and defoliation of both plant species strongly affected microbial biomass in the rhizosphere, indicating an increased availability of carbon resources, both earthworm species lost weight. Generally, the growth of soil animals such as endogeic earthworms is suggested to be limited by the availability of nitrogen (White, 1993). However, studies investigating soil nitrogen dynamics in presence of endogeic earthworms documented increased leaching of nitrogen due to mineralization and excretion, indicating that nitrogen may not be the limiting factor for endogeic earthworms (Marhan and Scheu, 2005, 2006). Rather, the availability of carbon proved to limit the growth of endogeic earthworms (Seastedt et al., 1988; Scheu and Schaefer, 1998; Tiunov and Scheu, 2004). In addition to earthworms, microorganisms are known to be carbon limited, and it has been shown that both compete for labile carbon resources in soil, with endogeic earthworms effectively competing with microorganisms (Tiunov and Scheu, 2004). However, results of the present study suggest that in the rhizosphere microorganisms are the superior competitor for root exudates and other root derived resources exploiting these pools more rapidly than earthworms thereby reducing carbon availability to earthworms.

In addition to soil carbon, soil microorganisms themselves may contribute to the nutrition of earthworms. However, in the present study earthworms did not affect microbial biomass in soil. Although microbial biomass in the defoliated treatments was significantly increased, earthworms lost weight, suggesting that they were unable to exploit the increased microbial pool of carbon. This is consistent with earlier studies suggesting that microorganisms, in

particular bacteria, do not form a major part of the diet of earthworms (Fischer et al. 1995, Wolter and Scheu 1999, Egert et al. 2004). Even microbial-feeders such as bacterivorous nematodes do not necessarily benefit from defoliation of plants (Merill et al., 1994; Todd, 1996; Bazot et al., 2005), suggesting that rhizosphere resources are not easily accessible to decomposer invertebrates, such as earthworms.

3.4.3 CO_2 production

Contrary to our hypothesis, earthworms did not affect CO_2 production suggesting that they did not affect mineralization of soil carbon in the present experiment. Generally, soil macrofauna is only responsible for a small part of the total CO_2 production of terrestrial ecosystems, it is microorganisms and plant roots that produce the major part of CO_2 emitted from soils (Swift et al., 1979). However, microbial biomass and activity, and thereby soil CO_2 production is strongly affected by soil invertebrates, in particular earthworms which function as ecosystem engineers controlling the availability of resources to microorganisms by mixing organic material and mineral soil (Jones et al., 1997; Lavelle, 1997). Due to the lack of litter materials in our experiment the major function of earthworms carbon cycling was prevented. This likely was responsible for the lack of earthworms in affecting soil CO_2 production. Further, the biomass of earthworms may have been too low to significantly affect CO_2 production. Despite endogeic earthworms are very active and ingest two to five times their own weight of soil and organic matter per day only 10% of the soil added likely have been transformed into earthworm casts by the end of the experiment

3.5. Conclusions

Our data indicate that the defoliation of perennial ryegrass and white clover increases the release of root exudates into the rhizosphere, which are utilized by rhizosphere microorganisms for biomass production. On the contrary, juvenile endogeic earthworms did neither benefit from the release of root exudates, nor from the increased microbial biomass in the soil, and lost weight. Hence, we suggest that in the rhizosphere microorganisms outcompete juvenile endogeic earthworms and more effectively exploit root derived resources presumably due to their close association with plant roots.

Chapter

4

Fungi and endogeic earthworms - antagonists in stabilization of litter carbon in soils

Abstract

The interaction of litter decomposing fungi and endogeic earthworms (*Octolasion tyrtaeum* (Savigny)) on the translocation and stabilization of litter derived carbon into the upper layer of a mineral soil was investigated in a microcosm experiment. Arable soil with and without *O. tyrtaeum* was incubated with $^{13}C/^{15}N$ labelled rye leaves positioned either on or above the soil surface, to distinguish between biotic and chemical/physical translocation of nutrients by fungi and leaching.

Contact of leaves to the soil increased ^{13}C translocation whereas presence of *O. tyrtaeum* reduced the incorporation of ^{13}C into the mineral soil in all treatments. Although biomass of *O. tyrtaeum* decreased during the experiment, more ^{13}C and ^{15}N was incorporated into earthworm tissue in treatments with contact of leaves to the soil. Contact of leaves to the soil and the presence of *O. tyrtaeum* increased cumulative $^{13}CO_2$-C production by 18.2% and 14.1%, respectively.

The concentration of the fungal bioindicator ergosterol in the soil tended to be increased and that of the fungal specific phospholipid fatty acid 18:2ω6 was significantly increased in treatments with contact of leaves to the soil. Earthworms reduced the concentration of ergosterol and18:2ω6 in the soil by 14.0% and 43.2%, respectively. Total bacterial PLFAs in soil were reduced in presence of *O. tyrtaeum* but did not respond to the addition and position of the rye leaves. In addition, the bacterial community in treatments with *O. tyrtaeum* differed from that without earthworms and shifted towards an increased dominance of Gram-negative bacteria.

The results indicate that litter decomposing fungi translocate and stabilize litter derived carbon in their mycelial network in the upper mineral soil. Endogeic earthworms reduce fungal biomass by grazing and thereby counteract the fungal stabilization of carbon. The results suggest that litter decomposing fungi and endogeic earthworms function as antagonists in the stabilization of litter derived carbon in the upper layer of mineral soils.

4.1 Introduction

In most terrestrial ecosystems 80-90% of the aboveground net primary production enters the soil food web as dead plant litter with estimates of a worldwide litter input in the range of 50 x 10^9 tons per year (Isidorov and Jdanova, 2002). Bacteria and fungi forming up to 90% of the soil microbial biomass and are mainly responsible for the decomposition of litter. Generally, fungi are the most abundant primary decomposers in the soil-litter interface in terrestrial ecosystems, playing an important role in the global carbon cycle (Dix and Webster, 1995; Schimel et al, 1999). They are well adapted to the heterogeneous distribution of nutrients in soils (Cain et al., 1999) due to formation of widespread mycelial networks, enabling the translocation of nutrients from high to low nutrient patches, thereby improving nutrient supply and exploitation of litter carbon resources.

Particularly the effect of wood decaying fungi on decomposition processes and nutrient cycling has been investigated. They are known to translocate significant amounts of phosphorous and nitrogen in their mycelial network (Lindahl et al., 1999) thereby increasing decomposition. However, bidirectional translocation of nutrients in the mycelial network of wood decaying fungi has also been documented (Lindahl et al., 2001).

The effect of litter decomposing fungi on nutrient translocation is less investigated. Some studies document a translocation of nitrogen from the mineral soil into the litter layer, presumably speeding up litter decomposition (Hart et al., 1993; Frey et al., 2000). In contrast, information on carbon translocation by litter decomposing fungi is sparse. Frey et al. (2003). reported that decomposing fungi translocate litter derived carbon into soil macroaggregates and concluded that this might be an important mechanism for the stabilization of carbon in the upper mineral soil.

Earthworms form the majority of the soil macrofauna and play an important role in organic matter processing and nutrient cycling in temperate ecosystems. By fragmenting plant residues and mixing organic matter with mineral soil earthworms generally provide favourable habitats for microorganisms, thereby strongly affecting microbial activity and microbial community structure (Tiunov and Scheu, 1999; Tiunov et al., 2001). However, earthworms may also decrease microbial biomass in the soil by grazing (Brown, 1995).

Endogeic earthworm species often dominate the earthworm community in soils. They live in non permanent horizontal burrows in the upper soil layer where they consume large quantities of mineral soil and associated organic residues (Edwards and Bohlen, 1996). They do not feed randomly, but selectively exploit patchy distributed soil aggregates enriched in organic matter (Wolter and Scheu, 1999; Marhan and Scheu, 2006). These nutrient rich microsites are heavily

colonized by microorganisms. Although investigated in detail, the fate of microorganisms during the gut passage of earthworms and the role of microorganisms as food source for earthworms is still debated, and appears to differ between ecological groups of earthworms and microorganisms investigated (Bonkowski et al., 2000; Horn et al., 2003; Egert et al., 2004). Both endogeic earthworms and litter decomposing fungi predominantly colonize the upper mineral soil and therefore likely interact in decomposition processes; however, the effect of endogeic earthworms on fungal biomass and fungal stabilization of litter derived carbon has not been investigated.

The objective of this study was to investigate, (1) the fungal translocation and stabilization of litter derived carbon in the upper layer of the mineral soil, and (2) the modification of this processes by endogeic earthworms. Using ^{13}C and ^{15}N labelled rye leaves, carbon mineralization as well as incorporation of carbon and nitrogen into the upper layer of the mineral soil and into the tissue of earthworms was quantified. Using phospholipid fatty acids and ergosterol as marker molecules enabled us to determine the effects of earthworms on bacterial and fungal abundance and microbial community composition. We hypothesised that endogeic earthworms decrease the fungal translocation of litter derived carbon. As a consequence, earthworms might contribute to the mobilization of litter derived carbon in the upper mineral soil by ingesting and disrupting mycelial networks of litter decomposing fungi.

4.2 Materials and methods

4.2.1 Experimental set-up

Arable soil was taken in May 2004 from the upper 10 cm of the long-term fertilization experiment in Bad Lauchstädt (Germany, Saxony-Anhalt). The soil consisted of 11.9% sand, 70.5% silt and 16.1% clay with a carbon and nitrogen content of 1.70% (^{13}C 1.084 atom%) and 0.16%, respectively. Further details on the study site are given in Körschens (1994). The soil was sieved (4 mm) to remove stones and larger plant residues and stored for two months at 5°C. Before the soil was used in the experiment it was frozen for defaunation at -20°C for one week.

In June 2004, adult endogeic earthworms of the species *Octolasion tyrtaeum* (Savigny) were sampled by hand in a 120 year old beech forest on limestone about 10 km east of Göttingen (South Lower Saxony). To avoid contamination with soil from the beech forest, earthworms were kept in containers filled with experimental soil for 4 weeks before they were used in the experiment.

Labelled rye leaves were obtained as described in Schmidt and Scrimgeour (2001). Briefly, a total of 480 rye seeds ("Avanti", Saaten-Union GmbH, Isernhagen, Germany) were sawn in six boxes filled with experimental soil. When plants reached the second leaf stage they were labelled by watering the plants once a week with 0.4 g $^{15}NH_4^+$ (99atom% ^{15}N; Campro Scientific, Berlin, Germany) in 1500 ml distilled water. Twice a week the plants were labelled with ^{13}C by spraying a solution of 250 ml distilled water containing increasing amounts of urea (99atom% ^{13}C; Campro Scientific, Berlin, Germany) starting with 0.03 g ^{13}C and increasing to 0.4 g at the end. After five weeks plants were cut at ground level dried at 65°C for three days and cut into pieces (< 2 cm). An aliquot was ground to powder and approximately 2.5 mg was weighed into tin capsules for isotope analysis.

In July 2004 the experiment was set up; 174 g fresh weight soil (150 g dry weight compacted to a bulk density of 1.23 g cm^{-3}), one specimen of *O. tyrtaeum* (average biomass 0.39 ± 0.04 g) and 0.3 g dry weight labelled rye leaves (38.8% C, 1.49 atom% ^{13}C, 0.96% N, 33.30 atom% ^{15}N) were added to 56 microcosms to establish the following treatments:

1. soil only
2. soil + *O. tyrtaeum*
3. leaves only
4. soil + leaves with contact to the soil
5. soil + leaves without contact to the soil
6. soil + leaves with contact to the soil + *O. tyrtaeum*
7. soil + leaves without contact to the soil + *O. tyrtaeum*

Treatments were replicated eight times. Four empty microcosms served as control for CO_2 measurements. Microcosms consisted of transparent plastic tubes (height 150 mm, inner diameter 60 mm) fixed air tight on ceramic plates. Microcosms could be closed on the top with air tight lids. Lowering the atmospheric pressure in the box under the microcosms allowed sampling of leaching water in vessels which were placed underneath each microcosm. Labelled rye leaves were placed on plastic rings with gaze (64 µm mesh size) which enabled fungi to grow from added rye leaves into the soil and vice versa, but avoided incorporation of leaves into the mineral soil by *O. tyrtaeum*. Half of the rings were placed on the soil surface (treatment with contact of leaves to the soil), the other half were adjusted 3 cm above the soil surface (treatment without contact of leaves to the soil) to distinguish between biotic translocation of carbon and nitrogen by fungi and chemical/physical translocation by leaching. Microcosms were incubated in a climate chamber in darkness at 20°C and watered weekly with 10 ml distilled water.

4.2.2 Sampling and analytical procedure

To investigate the temporal dynamics of decomposition processes half of the microcosms were sampled after six weeks, the other half after 12 weeks. During the experiment leaching water, a pooled sample of 1 week, was weighed and analysed for dissolved organic carbon and total dissolved nitrogen on a Dimatoc 100 TOC/DOC TN_b-analyser (Dimatec Analysentechnik, Essen, Germany).

CO_2 evolved in the microcosms was trapped in 2 ml alkali (1 M NaOH) in vessels attached to the lids. Trapped CO_2 was measured in an aliquot by titration with 0.1 M HCl after precipitation of carbonate with saturated $BaCl_2$ (Macfadyen, 1970). The amount of ^{13}C in CO_2 ($^{13}CO_2$-C) was determined by precipitation of alkali in saturated $BaCl_2$ in 50 ml centrifuge vessels. 2 ml alkali were transferred to vessels containing 40 ml distilled water and centrifuged for 2 min at 2500 rpm. After centrifugation supernatants were discarded, vessels were filled with 40 ml water and centrifuged again. The washing and centrifugation procedure was repeated five times until the supernatants were of neutral pH. Pellets were dried at 65°C for three days, and approximately 0.3 mg of the $BaCO_3$ was analysed for ^{13}C. Determination of total CO_2 and $^{13}CO_2$-C was carried out alternating each day early in the experiment and every third day later.

After six and twelve weeks the remaining rye leaves were removed from the microcosms dried at 65°C for three days and weighed. Samples were ground to powder and approximately 2.5 mg were analysed for ^{13}C and ^{15}N. To extract the earthworms from microcosms without disturbing the soil cores, microcosms were waterlogged with 50 ml distilled water. When earthworms appeared on the soil surface, they were collected, weighed and killed by freezing. The anterior part of the earthworm was cut off and approximately 0.8 mg were analysed for ^{13}C and ^{15}N. To remove the added water from soil cores, microcosms were drained for a further day as described above. Then, soil cores were carefully removed and divided into three layers (H1: 0-1 cm, H2: 1-3 cm, H3: 3-5 cm). Due to rapidly decreasing microbial biomass and activity with increasing distance from nutrient resources the two lower soil layers were excluded from further analysis (Poll et al. 2006). An aliquot of H1 was dried at 65°C for two days and the water content was calculated. Samples were ground to powder and approximately 30 mg were analysed for ^{13}C. Isotope ratios ($^{13}C/^{12}C$; $^{15}N/^{14}N$) were measured by a system of an elemental analyser (NA 1500, Carlo Erba, Milan, Italy) coupled with a trapping box (type CN, Finnigan, Bremen, Germany) and an isotope ratio mass spectrometer (MAT 251, Finnigan, Bremen, Germany) (Reineking et al., 1993). Vienna Pee Dee Belemnite served as standard for ^{13}C; atmospheric N_2 for ^{15}N. Acetanilide (C_8H_9NO, Merck, Darmstadt, Germany) was used for internal calibration. The amount of ^{13}C and ^{15}N excess in earthworm tissue and the amount of ^{13}C excess in bulk soil

were calculated by subtracting natural signatures from the measured ^{15}N and ^{13}C atom%. Mineralization of labelled rye leaves was calculated from data of CO_2 evolved (mg CO_2) and amount of ^{13}C (atom%) and expressed as cumulative ^{13}C production (µg $^{13}CO_2$-C microcosm^{-1}).
Microbial parameters were determined by measurement of ergosterol concentration in H1 and rye leaves, and PLFA contents in the soil. Ergosterol was measured in 1 g fresh weight soil and 0.5 g fresh weight rye leaves by a modified method described in Djajakirana et al. (1996). Briefly, ergosterol was extracted with 100 ml ethanol for 30 min by horizontal shaking at 250 rev min^{-1}. After centrifugation at 3700 rpm for 40 min, 40 ml of the supernatant was vaporized in a rotation vacuum evaporator (RVC 2-25, Martin Christ GmbH, Osterode, Germany) at 60°C for 12 h. The pellets were dissolved in 1 ml methanol, transferred in 1.5 ml HPLC vials using 0.45 µm cellulose-acetate filters and stored at -20°C until measurement. Ergosterol was measured by HPLC analysis (Beckmann Coulter, System Gold 125, Fullerton, USA) at 22°C using a 250 mm x 4.6 mm Spherisorb ODS II 5 µm column with a mobile phase of pure methanol, a flow rate of 1 ml min^{-1} and detected at 282 nm (Beckmann Coulter, System Gold 166 UV-detector, Fullerton, USA).
Lipids were extracted from soil, fractionated and quantified as described in Bardgett et al. (1996). Separated phospholipid fatty acid methyl-esters were identified by chromatographic retention time and mass spectral comparison with a mixture of standard qualitative bacterial acid methyl-ester and fatty acid methyl-ester that ranged from C11 to C20 (Fa. Supelco, Bellefonte, USA). For each sample the abundance of individual phospholipid fatty acid methyl-esters was expressed per unit dry weight. The nomenclature for PLFAs followed that of Frostegård et al. (1993). The sum of eleven PLFAs (i15:0, a15:0, 15:0, i16:0, 16:1ω7, 17:0, i17:0, cy17:0, 18:1ω7 and cy19:0) was used to represent bacterial biomass in the soil (Frostegård and Bååth, 1996). In addition to ergosterol the concentration of the fungal specific fatty acid 18:2ω6 was used as an indicator of fungal biomass (Federle, 1986).

4.2.3 Data analysis

Data were analysed by two or three factor analysis of variance (ANOVA) with the factors *O. tyrtaeum* (without and with), Time (week 6 and week 12) and Leaves as Presence of leaves with three levels (without leaves, leaves without contact to soil and leaves with contact to soil) and further if this was significant as Position of leaves with two levels (without contact to soil and with contact to soil) to differentiate between treatments with and without contact to the soil. For analyses of CO_2, and the cumulative leaching of carbon and nitrogen only the twelve week sampling was included in the analysis. Prior to analyses the data were inspected for

homogeneity of variance (Levene test) and log-transformed if required to match the prerequisites of ANOVA. Tukey´s honestly significant difference (HSD) test was used for comparison of means. A statistical probability $P<0.05$ was considered significant. To compare the overall structure of the PLFA profiles between the treatments, Discriminant function analysis (DFA) was used. Squared Mahalanobis distances between group centroids and reliability of sample classification were determined and results were graphically presented in two dimensional space. For the interpretations of discreminant roots linear correlations were calculated between the discriminant function scores for each sample and the distribution of single PLFAs. Statistical analyses were carried out using STATISTICA 7.0 (Statsoft, Tulsa, USA) and SAS 8.0 (Statistical Analysis System, SAS Institute Inc., Cary, USA) software packages.

4.3 Results

4.3.1 Earthworms

All except one *O. tyrtaeum* survived until the end of the experiment. The biomass of all earthworms decreased and was affected by the time of incubation with significant stronger decrease in the twelve week treatment (-45.6%) than in the six week treatment (-32.4%, $F_{1,12}=22.4$, $P<0.0001$). The presence of rye leaves only tended to influence earthworm biomass; in microcosms without rye leaves it declined to 64.5% and 50.4% of their initial biomass, whereas in microcosms with leaves to 68.3% and 52.1% without contact, and 69.9% and 60.6% with contact, after six and twelve weeks of incubation, respectively.

Overall, the nitrogen concentration in earthworm tissue significantly increased during the experiment ($F_{3,12}=5.9$, $P=0.012$) from an initial of 11.8% to 12.9% at the end of the experiment, but was not affected by the treatments. Earthworm tissue carbon concentration remained at 47.4%. The amount of ^{13}C and ^{15}N in earthworm tissue significantly increased in microcosms with rye leaves ($F_{2,11}=35.6$, $P=0.0002$ and $F_{2,11}=56.9$, $P<0.0001$, respectively). The time of incubation had no effect ($F_{1,7}=0.01$, $P=0.91$), whereas the position of rye leaves significantly affected the amount of ^{13}C in the earthworm tissue ($F_{1,7}=13.7$, $P=0.007$; Fig. 4.1a). It increased from an initial of 1.085 atom% to an average of 1.089 and 1.093 atom% without and with contact of rye leaves to the soil, respectively. Furthermore, the position of the rye leaves affected the amount of ^{15}N in earthworm tissue with significant higher incorporation in treatments with contact of leaves to the soil than in those without contact ($F_{1,6}=41.7$, $P=0.0006$;

Fig. 4.1b). However, this varied with Time; differences were only significant after six weeks of incubation ($F_{1,6}$=12.19, P=0.013). In microcosms with contact of leaves to the soil the amount of ^{15}N in earthworm tissue increased from an initial of 0.37 to 0.83 atom% after six weeks and remained on the same level until the end of the experiment. In microcosms without contact of leaves to the soil the amount of ^{15}N in earthworm tissue only increased to 0.49 and 0.73 atom% after six and twelve weeks; i.e. in treatments without contact of leaves to the soil the increase was considerably slower.

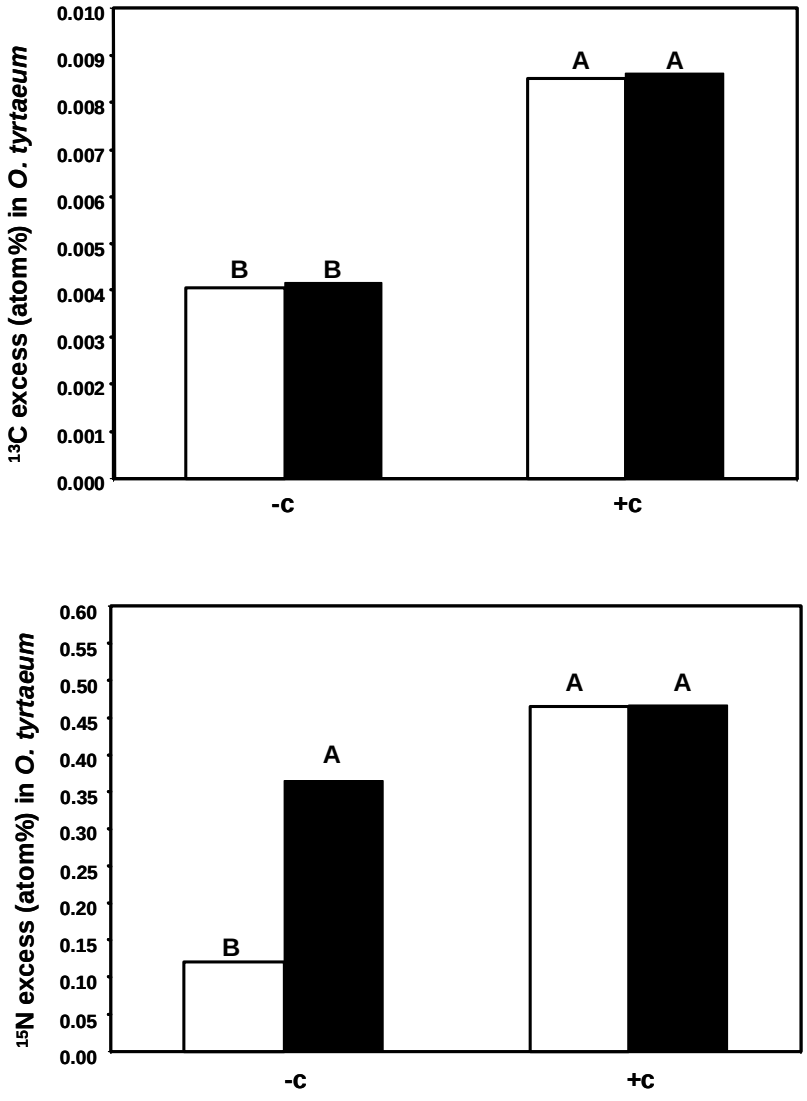

Figure 4.1: Effect of the position of rye leaves (without soil contact (-c) and with soil contact (+c)) and time of incubation (white bars: six weeks; black bars: twelve weeks) on **(a)** the amount of ^{13}C and **(b)** the amount of ^{15}N excess in *Octolasion tyrtaeum*. Means of three replicates. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

4.3.2 Rye leaves

The biomass of rye leaves strongly decreased during the experiment but mass loss significantly varied between treatments (Fig. 4.2). Generally, it was higher in treatments with than in those without contact of leaves to the soil (82.7% and 66.1%, respectively; $F_{1,14}$=61.4, P<0.0001). *Octolasion tyrtaeum* increased mass loss of rye leaves only in treatments with contact of leaves to the soil, but the effect depended on time of incubation. While *O. tyrtaeum* did not affect mass loss of rye leaves after six weeks, it was significantly increased by 86.5% after twelve weeks of incubation (*O. tyrtaeum* x Time x Position of leaves: $F_{1,14}$=6.4, P=0.02; Fig. 4.2).

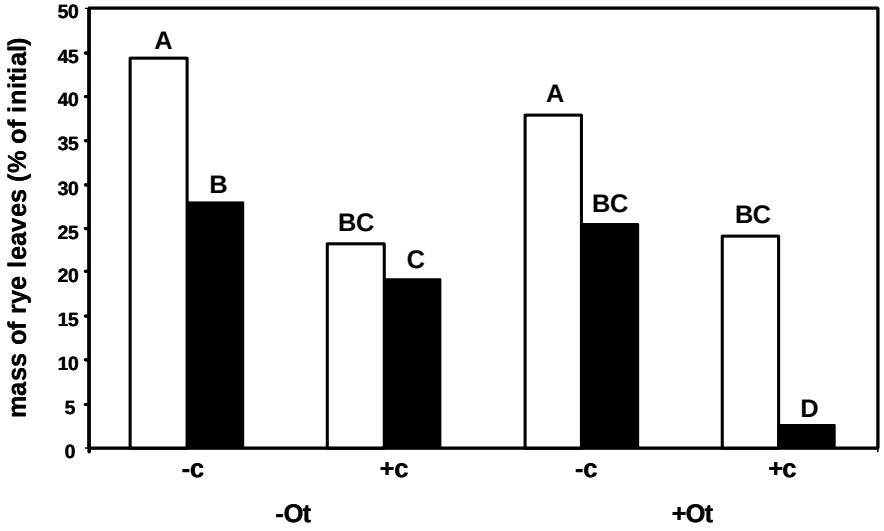

Figure 4.2: Effect of the position of rye leaves (without soil contact (-c) and with soil contact (+c)), time of incubation (white bars: six weeks; black bars: twelve weeks) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on mass loss of rye leaves. Means of three replicates. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

4.3.3 Soil

The water content of the soil significantly varied with the presence of *O. tyrtaeum* ($F_{1,24}$=50.2, P<0.0001) and Time ($F_{1,24}$=20.1, P=0.0001) with increased water content in treatments with *O. tyrtaeum* (water content 24.4%) compared to treatments without *O. tyrtaeum* (20.6%), and decreased water content in the twelve week samples (21.3%) compared to the six week samples (23.7%). Furthermore, the presence of rye leaves affected the water content ($F_{2,24}$=10.6, P=0.03)

with the lowest water content in treatments with leaves in contact to the soil (21.4%), the highest in treatments without contact (23.2%) and an intermediate content in treatments without addition of rye leaves (22.8%).

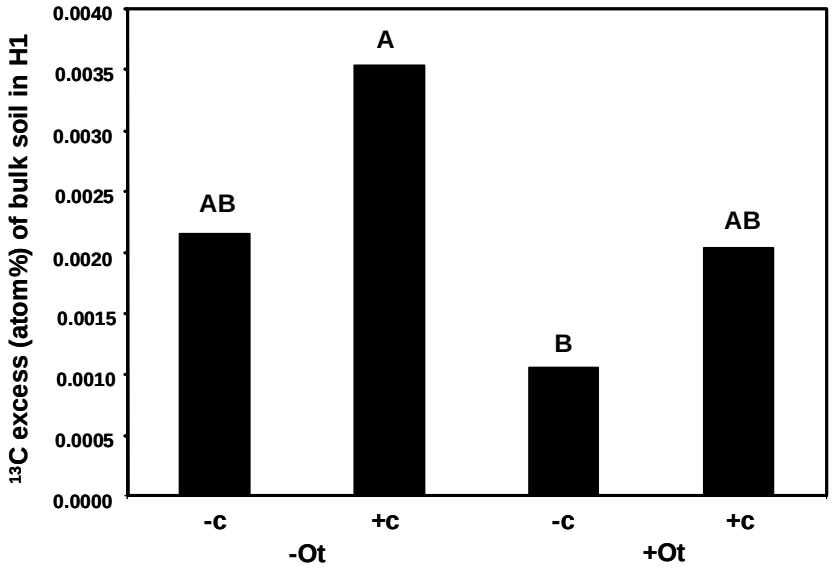

Figure 4.3: Effect of the position of rye leaves (without soil contact (-c) and with soil contact (+c)) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on the amount of ^{13}C excess in soil (0-1 cm). Pooled data of the six and twelve week treatments. Different letters indicate significant difference between means at $P<0.05$ (Tukey´s HSD test).

Overall, the carbon concentration of the soil slightly decreased during the experiment from an initial of 1.70% to 1.68% at the end of the experiment but was not significantly affected by the presence of *O. tyrtaeum* ($F_{1,24}=0.07$, $P=0.79$), the presence of rye leaves ($F_{2,24}=0.58$, $P=0.57$) or time of incubation ($F_{1,24}=2.54$, $P=0.12$). In treatments with rye leaves *O. tyrtaeum* decreased soil carbon content, whereas in treatments without rye leaves it was increased (*O. tyrtaeum* x Presence of leaves; $F_{2,24}=3.49$, $P=0.05$). Furthermore, the presence of rye leaves on soil carbon varied with time (Time x Presence of leaves; $F_{2,24}=4.5$, $P=0.02$). In treatments without rye leaves the carbon concentration increased at both samplings, whereas it increased at the first sampling and decreased at the second sampling in treatments without contact of leaves to the soil. In contrast, in treatments with contact of leaves the carbon concentration in the soil decreased at the first sampling and increased at the second sampling. However, the differences were low and ranged between 0.02 and 0.12%.

The amount of ^{13}C in the upper soil layer in treatments with rye leaves was slightly increased compared to those without, and also varied with the position of the rye leaves; it was significantly higher in treatments with than in those without contact of leaves to the soil ($F_{1,16}$=4.8, P=0.04; Fig. 4.3). Independent of the position of rye leaves, *O. tyrtaeum* reduced the ^{13}C excess in the soil ($F_{1,16}$=5.8, P=0.03; Fig. 4.3); the time of incubation did not affect the incorporation of ^{13}C into the soil ($F_{1,16}$=0.14, P=0.71).

4.3.4 Carbon mineralization

Generally, CO_2 production remained on a similar level throughout the experiment. It was lowest in the soil only treatment and in the treatment with soil only and *O. tyrtaeum* with an average of 1.66 ± 0.37 and 2.26 ± 0.44 ml CO_2 microcosm^{-1} day^{-1}, respectively. *Octolasion tyrtaeum* and the presence of rye leaves significantly increased CO_2 production, but the effect varied with time; both factors increased the CO_2 production primarily early in the experiment (Fig. 4.4a).

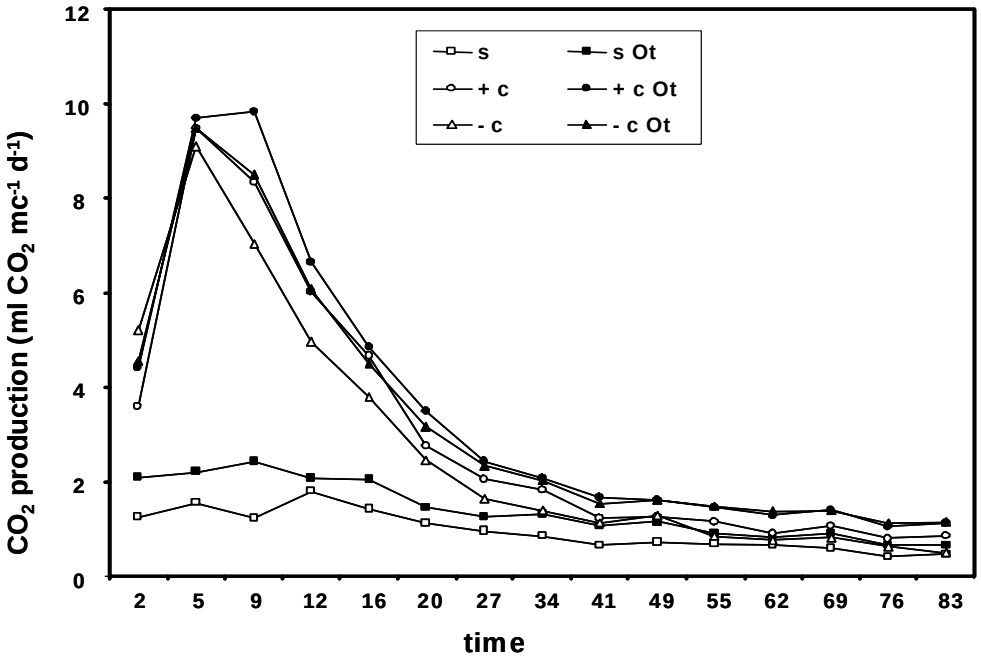

Figure 4.4a: Effect of the presence of rye leaves (without leaves (s), leaves without soil contact (-c), leaves with soil contact (+c)) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on CO_2 production during 83 days of incubation. Means of three replicates.

Cumulative CO_2 production was significantly enhanced by the presence of rye leaves ($F_{2,16}$=135.3, P<0.0001) with higher mineralization in treatments with than without contact of leaves to the soil. *Octolasion tyrtaeum* significantly increased cumulative CO_2 production in microcosms with rye leaves ($F_{1,16}$=28.6, P<0.0001; Fig. 4.4b).

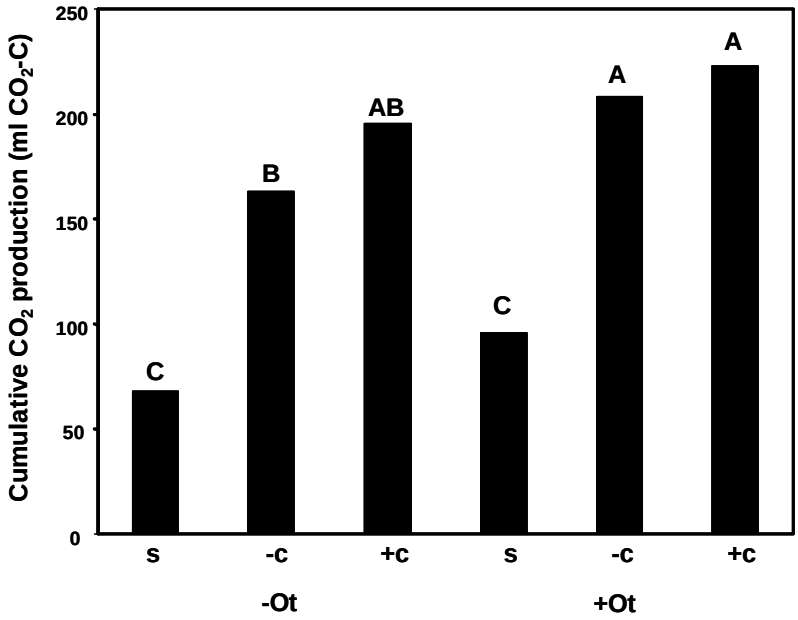

Figure 4.4b: Effect of the presence of rye leaves (without leaves (s), leaves without soil contact (-c), leaves with soil contact (+c)) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) cumulative CO_2 production after twelve weeks. Means of three replicates. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

Also, the contact of leaves to the soil ($F_{1,8}$=26.5, P=0.0009) and the presence of *O. tyrtaeum* ($F_{1,8}$=15.1, P=0.005) significantly increased the cumulative $^{13}CO_2$ production on average by 18.2% and 14.1%, respectively (Fig. 4.5). Generally, the contact of leaves to the soil increased the production of $^{13}CO_2$ during the whole experiment, whereas the effect *O. tyrtaeum* occurred primarily later in the experiment (data not shown).

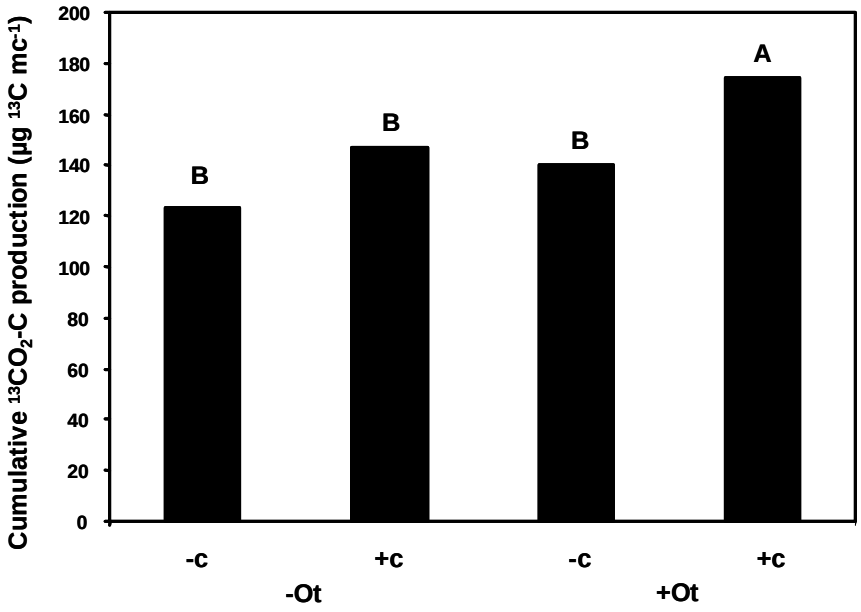

Figure 4.5: Effect of the position of rye leaves (without soil contact (-c) and with soil contact (+c)) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on cumulative $^{13}CO_2$-C production after twelve weeks. Means of three replicates. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

4.3.5 Leachate

Octolasion tyrtaeum strongly increased the cumulative leaching of nitrogen; 4.58 and 1.86 mg N were leached in treatments with and without *O. tyrtaeum*, respectively ($F_{1,12}$=526.2, P<0.0001). The presence but not the position of the rye leaves also significantly affected leaching of nitrogen with cumulative leaching of 3.39 and 3.40 mg N per microcosms in treatments with and without contact of leaves to the soil, respectively, and of 2.87 mg N in treatments without rye leaves ($F_{2,12}$=8.92, P=0.004). In contrast, cumulative leaching of carbon was reduced in presence of *O. tyrtaeum*; 2.78 and 3.38 mg C were leached per microcosm in treatments with and without *O. tyrtaeum*, respectively ($F_{1,12}$=16.9, P=0.001). The position but not the presence of the rye leaves also affected leaching of carbon with cumulative leaching in treatments without contact of leaves to the soil (on average 3.55 mg C) exceeding that in treatments with contact (2.95 mg C) and treatments without addition of rye leaves (2.73 mg C; $F_{2,12}$=11.3, P=0.002)

4.3.6 Microorganisms

The total amount of bacterial PLFAs in the soil ranged from 2.68 to 12.87 nmol g^{-1} dry mass with 18:1ω7 being most abundant (1.67 nmol g^{-1} dry mass) and 15:0 were being least abundant (0.11 nmol g^{-1} dry mass). It was significantly reduced in presence of *O. tyrtaeum* ($F_{1,23}$=47.9, P<0.0001; Fig. 4.6), but the effect decreased from the first sampling ($F_{1,12}$=53.7, P<0.0001) to the second sampling ($F_{1,12}$=9.7, P=0.01). Neither the time of incubation ($F_{1,23}$=3.7, P=0.06), nor the presence of rye leaves ($F_{2,23}$=0.97, P=0.39) significantly affected the total amount of bacterial PLFAs in the soil.

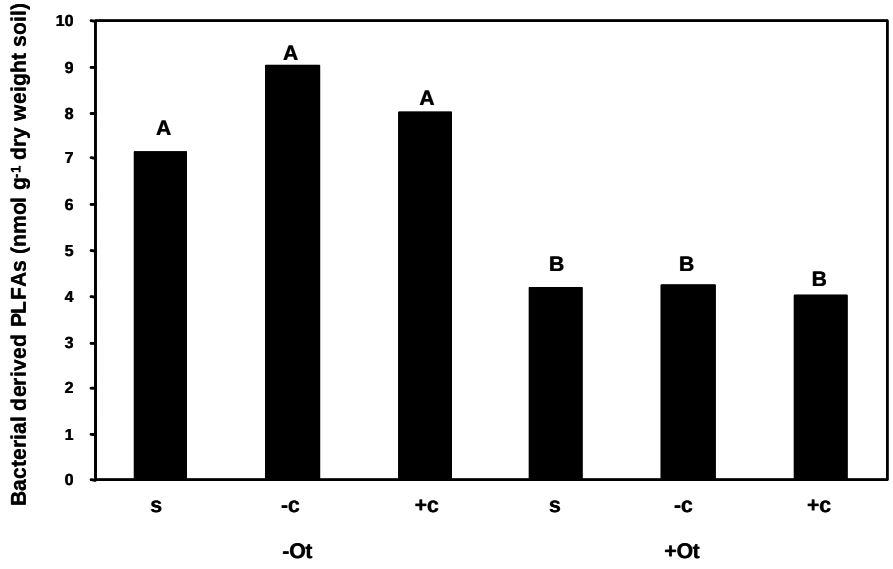

Figure 4.6: Effect of the presence of rye leaves (without leaves (s), leaves without soil contact (-c), leaves with soil contact (+c)) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on amount of bacterial derived PLFAs in soil (0-1 cm). Pooled data of the six and twelve week treatments. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

In addition, individual bacterial PLFAs significantly responded to the presence of *O. tyrtaeum*. Overall, *O. tyrtaeum* reduced the amount of bacterial PLFAs in the soil, except that of cy19:0 which was not affected ($F_{10,20}$=12.48, P<0.0001). *Octolasion tyrtaeum* reduced the relative concentration of i15:0, a15:0, 15:0, 16:1ω7, cy17:0 and increased that of i16:0, 18:1ω7 and cy19:0, whereas the relative concentration of 17:0 and i17:0 was not affected ($F_{10,20}$=9.44, P<0.0001). Accordingly, DFA separated the PLFA profiles of treatments with *O. tyrtaeum* from those without with root 1 accounting for 74% of the discriminatory power (Fig. 4.7). Bacterial PLFA profiles in treatments with *O. tyrtaeum* were not further separated by root 2, accounting

for 19% of the discriminatory power. However, they differed strongly between treatments without and with leaves in contact to the soil; the PLFA profile of treatments with leaves without contact to the soil was intermediate and did not significantly differ from both (Table 4.1). The concentration of PLFAs except cy19:0 correlated positively with root 1, and 18:1ω7 correlated negatively with root 2 (Table 4.2).

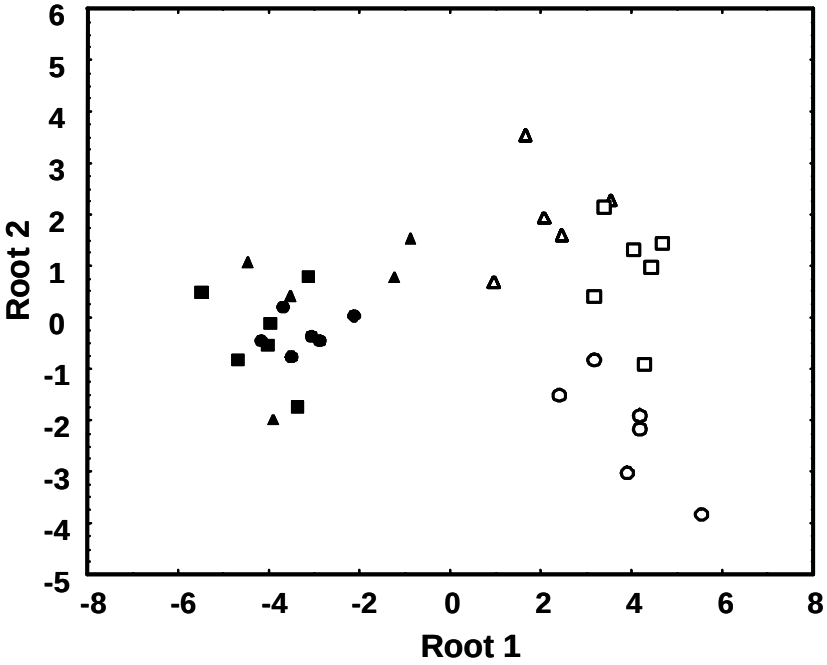

Figure 4.7: Discriminant function analysis (DFA) of phospholipid fatty acid (PLFAs) profiles in soil without leaves (triangle), with leaves with soil contact (circle), with leaves without soil contact (square) with (closed symbols) or without (open symbols) *Octolasion tyrtaeum*.

Table 4.1. Squared Mahalanobis distances between group centroids and reliability of discrimination on data on phospholipid fatty acids in the soil without litter and Octolasion tyrtaeum (S), with *O.n tyrtaeum* (Ot) and litter (L) with and without litter in contact to the soil (-c).

	SL	SL-c	SOt	SLOt	SL-cOt
S	20.8**	5.7^{ns}	28.7**	35.6***	45.5***
SL		10.2^{ns}	52.1***	55.3***	68.8***
SL-c			47.8***	54.5***	68.5***
SOt				1.9^{ns}	6.2^{ns}
SLOt					3.9^{ns}

*P<0.05, ** P<0.01, *** P<0.001, ns, not significant

Table 4.2. Linear correlation (r-values) of the total amount of PLFA (nmol g dry weight soil^{-1}) to root 1 and root 2 of the discriminant function analysis.

PLFA	Root 1	Root 2
i15:0	0.6631***	0.2735^{ns}
a15:0	0.6042***	0.2964^{ns}
15:0	0.5612**	-0.0716^{ns}
i16:0	0.8196***	-0.0517^{ns}
16:1ω7	0.7992***	0.1292^{ns}
i17:0	0.9389***	-0.0476^{ns}
cy17:0	0.8709***	-0.0511^{ns}
17:0	0.6428***	-0.2118^{ns}
18:1ω7	0.8455***	-0.3963**
cy19:0	-0.2065^{ns}	-0.1576^{ns}

** P<0.01, *** P<0.001, ns, not significant

The concentration of ergosterol in rye leaves ranged from 58 to 955 µg g^{-1} dry mass and was strongly affected by the treatments. Generally, it decreased from 624 µg g^{-1} dry mass after six weeks to 318 µg g^{-1} dry mass after twelve weeks of incubation ($F_{1,16}$=22.3, P=0.0002). The concentration of ergosterol was significantly higher ($F_{1,16}$=9.4, P=0.008) in treatments without (582 µg g^{-1} dry mass) compared to those with contact of leaves to the soil (360 µg g^{-1} dry mass; Fig. 4.8). However, this was mainly due to the presence of *O. tyrtaeum* which did not affect ergosterol concentration in rye leaves in treatments without contact, but significantly decreased it by 33.0% after six weeks and by 69.1% after twelve weeks in treatments with contact of leaves to the soil (*O. tyrtaeum* × Position of leaves; $F_{1,16}$=4.7, P=0.04; Fig. 4.8).

The concentration of ergosterol in the soil ranged from 0.31 to 0.64 µg g^{-1} dry mass soil. *Octolasion tyrtaeum* significantly reduced the concentration of ergosterol by 14.01% ($F_{1,24}$=8.23, P=0.0085; Fig. 4.9). Neither presence of the leaves ($F_{2,24}$=0.63, P=0.54), nor the time of incubation ($F_{1,24}$=1.84, P=0.19) significantly affected the ergosterol concentration in soil. However, without *O. tyrtaeum* the concentration of ergosterol tended to be higher in treatments with (0.55 ± 0.13 µg g^{-1} dry mass) than in those without contact of leaves to the soil (0.49 ± 0.06 µg g^{-1} dry mass), whereas in presence of *O. tyrtaeum* it tended to be lower in treatments with (0.44 ± 0.08 µg g^{-1} dry mass) than in those without contact of leaves to the soil (0.47 ± 0.08 µg g^{-1} dry mass).

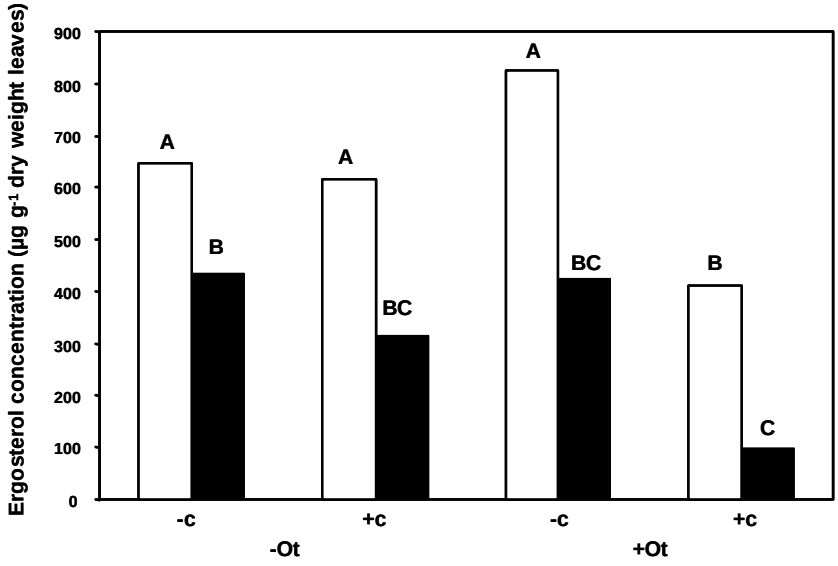

Figure 4.8: Effect of the position of rye leaves (without soil contact (-c) and with soil contact (+c)), time of incubation (white bars: six weeks; black bars: twelve weeks) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on concentration of ergosterol in remaining rye leaves. Means of three replicates. Different letters indicate significant difference between means at $P<0.05$ (Tukey´s HSD test).

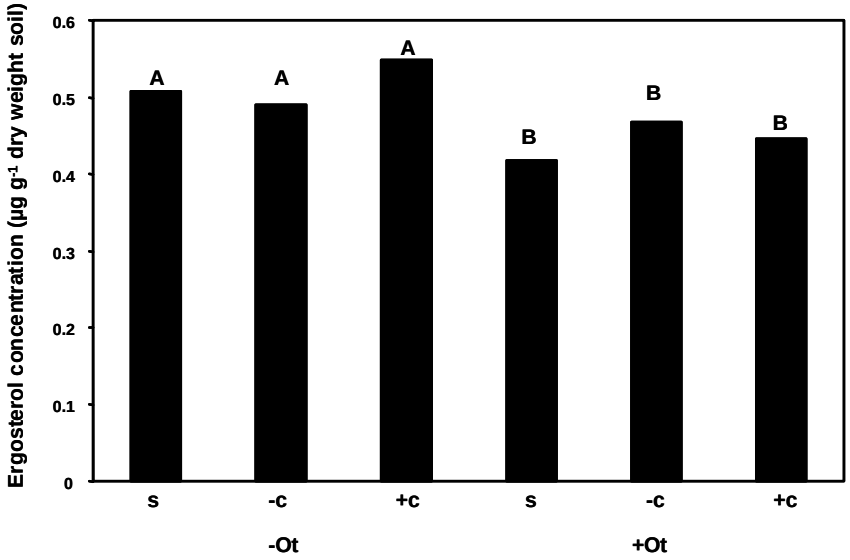

Figure 4.9: Effect of the presence of rye leaves (without leaves (s), leaves without soil contact (-c), leaves with soil contact (+c)) and presence of *Octolasion tyrtaeum* (without *O. tyrtaeum* (-Ot) and with *O. tyrtaeum* (+Ot)) on concentration of ergosterol in soil (0-1 cm). Pooled data of the six and twelve week treatments. Different letters indicate significant difference between means at $P<0.05$ (Tukey´s HSD test).

The fungal PLFA 18:2ω6 responded similar to that of ergosterol but the response was generally more pronounced. The position of rye leaves significantly affected the concentration of 18:2ω6 in the soil ($F_{1,23}$=4.2, P=0.03) with the highest concentration in treatments with contact (0.40 nmol g^{-1} dry weight) the lowest in those without contact to the soil (0.21 nmol g^{-1} dry weight); treatments without rye leaves being intermediate (0.27 nmol g^{-1} dry weight). Furthermore, *O. tyrtaeum* significantly reduced the concentration of 18:2ω6 with 0.21 and 0.37 nmol g^{-1} soil dry weight in treatments with and without *O. tyrtaeum*, respectively ($F_{1,23}$=7.1, P=0.01).

4.4. Discussion

4.4.1 Translocation processes between rye leaves and soil

We hypothesized that litter decomposing fungi translocate litter derived carbon into the upper mineral soil, and thereby contribute to the stabilization of carbon in soils. Contact of leaves to the soil significantly increased the decomposition of the rye leaves but fungal biomass did not differ between treatments with and without contact of leaves to the soil suggesting that increased decomposition of the rye leaves did not result in the formation of fungal biomass. Most decomposing fungi live in the upper soil layer but some colonize the phyllosphere of plants (Osono, 2003). The activity of litter decomposing fungi often is limited by the availability of nitrogen (Gallardo and Schlesinger, 1994; Ekblad and Nordgren, 2002). To compensate nitrogen deficiency fungi translocate nitrogen from the mineral soil through their mycelial network into the litter, thereby increasing litter decay (Frey et al., 2000). The decomposition of the rye leaves and the growth of fungi in the present study occurred on two different time scales which presumably were due to changes in the availability of nitrogen. In the treatment without contact of leaves to the soil fungi exclusively depended on the nutrients in the leaves, resulting in slow decomposition. In treatments with contact of leaves to the soil, additional nutrients from the soil enabled fungi to decompose the rye leaves much faster. The respiration of total CO_2 and $^{13}CO_2$ during the first few weeks of the experiment in the treatment with contact of leaves to the soil was significantly increased, suggesting that translocation of nutrients into the rye leaves by fungi was quick.

The concentration of ^{13}C in the soil supports our hypothesis that fungi translocate and stabilize litter carbon in the mineral soil. The incorporation of ^{13}C in treatments with contact of leaves to the soil exceeded that in treatments without contact by 39%, suggesting that the additional incorporation of carbon into the mineral soil in treatments with contact of leaves to the soil resulted from translocation by fungi. Increased fungal biomass in the soil in treatments with

compared to those without contact of leaves to the soil provided further evidence for translocation of carbon by fungi. The concentration of ergosterol tended to be increased and the concentration of the PLFA 18:2ω6 was significantly increased. Concentrations of ergosterol and 18:2ω6 are commonly used for the quantification of the fungal biomass in soils (Frostegård and Bååth, 1996) but both methods have limitations (Ruess et al., 2002; Drijber et al., 2000); hence calculations of fungal biomass are more reliable if based on multiple indicators. As suggested previously (Mille-Lindblom et al., 2004) the PLFA 18:2ω6 is a more sensitive indicator than ergosterol, presumably due to faster decay after fungal death.

4.4.2 Modification of translocation and stabilization processes by earthworms

Generally, earthworms favour microbial abundance and activity by comminution of litter, and mixing it with mineral soil as well as by excretion of mucus and urine, thereby increasing microbial access to litter carbon resources and nutrient availability (Brown, 1995). However, earthworms may also decrease microbial biomass in the soil by grazing (Brown, 1995), but the importance of bacteria and fungi for earthworm nutrition still is not fully understood (Bonkowski et al., 2000; Horn et al., 2003; Egert et al., 2004). Certainly, saprophagous animals as endogeic earthworms rely on plant residues entering the soil.

Incorporation of leaves and direct feeding of *O. tyrtaeum* on the added rye leaves was prevented in our experiment. Therefore, the nutrition of earthworms relied on leave derived nutrients incorporated into the soil by fungi and/or leached out of the rye leaves. Overall, the biomass of *O. tyrtaeum* decreased during the experiment, but this was less pronounced in treatments with rye leaves having contact to the soil. Further, *O. tyrtaeum* incorporated significantly more ^{13}C and ^{15}N in treatments with than in those without contact of leaves to the soil, suggesting that rye leave materials indeed served as additional food. *Octolasion tyrtaeum* decreased concentrations of ergosterol and of the fungal specific PLFA 18:2ω6 in the soil, indicating that *O. tyrtaeum* fed on fungi; since fungi translocated litter resources into the soil this likely contributed to the increased incorporation of ^{13}C (and ^{15}N) into the earthworm tissue in treatments with contact of leaves to the soil.

Decreased accumulation of ^{13}C in the upper mineral soil (-42.4%) strongly support our hypothesis that endogeic earthworms reduce the stabilization of litter derived carbon by grazing on fungi. Fungal dominated systems are slow-cycling systems with low rates of nutrient turnover (Coleman et al., 1983). Previous studies indicated that earthworms disturb these fungi dominated systems by comminution of litter and incorporating it into deeper soil layers

(bioturbation), thereby reducing the availability of carbon resources to fungi (Scheu and Parkinson, 1994). The fast-cycling bacterial community becomes favoured, resulting in increased mineralization of organic matter. In the present study, however, *O. tyrtaeum* significantly decreased fungal as well as bacterial biomass. In addition, the experimental set-up prevented the incorporation of the rye leaves by earthworms; indicating that the decline of fungal biomass was caused by grazing and disruption of fungal hyphae and not by bioturbation.
As indicated by the incorporation of ^{13}C into the soil in treatments without contact of leaves to the soil, ^{13}C leached out of the rye leaves and entered the soil. Labile and easily available carbon entering the soil is rapidly incorporated and mineralized by microorganisms which are limited by the availability of labile carbon (Mikola and Setälä, 1998; Joergensen and Scheu, 1999). However, in our experiment bacterial biomass in soil in treatments without *O. tyrtaeum* did not respond to the presence of rye leaves, but PLFA profiles indicated that bacterial community composition changed and this was most pronounced in treatments with contact of leaves to the soil. This shift was predominantly due to an increased relative amount of the fatty acid 18:1ω7, an indicator for Gram-negative bacteria. This indicates that litter resources were in fact used by bacteria and resulted in selective growth of certain groups.
Octolasion tyrtaeum generally reduced the bacterial biomass in the soil and altered PLFA profiles. In contrast to treatments without *O. tyrtaeum*, the presence of rye leaves did not affect bacterial community composition in presence of *O. tyrtaeum*. As in treatments without *O. tyrtaeum* the increased relative amount of the fatty acid 18:1ω7 was mainly responsible for the difference between treatments with and without *O. tyrtaeum*, suggesting that earthworms reduced the availability of leave nutrients in particular for Gram-negative bacteria. This is in accordance to other studies indicating that earthworms may effectively compete with bacteria for carbon resources (Scheu and Schaefer, 1998; Scheu et al., 2002; Tiunov and Scheu, 2004).

4.5. Conclusions

The present study indicates that endogeic earthworms reduce bacterial biomass in the soil while effectively competing with bacteria for labile carbon resources that enter the soil. In addition, data on bacterial PLFA profiles indicate that endogeic earthworms significantly alter the composition of the bacterial community in soil primarily by favouring Gram-negative bacteria. Most importantly, however, endogeic earthworms decrease fungal biomass by grazing and disruption of fungal hyphae thereby reducing the translocation of litter carbon in their mycelial network into the upper layer of the mineral soil and counteracting the fungal-mediated

stabilization of carbon in soils. This indicates that fungi and endogeic earthworms function as antagonists in the stabilization of litter derived carbon in the upper layer of mineral soils.

Chapter

5

Earthworms and the soil microbial food web - Insight into a `black box´ using RNA stable isotope probing

Abstract

The flux of bacterial carbon through the soil microbial community as affected by the passage through the gut of endogeic earthworms (*Aporrectodea caliginosa* (Savigny)) was investigated in a microcosm experiment. Arable soil with and without *A. caliginosa* was inoculated with ^{13}C labelled *Serratia marcescens* cells and incubated for one day. Casts and un-processed soil were incubated for further five days and the microbial community was analysed by means of terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments and rRNA stable isotope probing (SIP).

The T-RFLP profiles of the bacterial communities of bulk soil and casts differed markedly with two dominant OTUs in that of the casts which were either absent or hardly abundant in the soil. Furthermore, cumulative $^{13}CO_2$-C production was strongly increased in presence of *A. caliginosa* and in casts compared to the soil.

After one day of incubation T-RFLP and cloning analyses of the "heavy" ^{13}C labeled bacterial RNA of casts and soil revealed among *S. marcescens* eleven different bacterial lineages, with *Proteobacteria* and *Actinobacteria* being the most abundant. Both clone libraries, however, differed in the abundance of *Acidobacteria*, *Firmicutes* and *Planctomycetes*. After six days *S. marcescens* clones were absent and microbial community in the soil was almost similar to that of casts at day one. The clone library of the casts, in contrast, consisted of only five different lineages and was dominated by members of the phylum *Firmicutes*, predominantly *Staphylococcus* spp.

The results indicate that the passage through the gut of endogeic earthworms considerably changes the activity and composition of the microbial community, and therefore the mineralization and incorporation of bacterial carbon through the soil microbial community. In addition, our results provide evidence for the abundance of an indigenous microbial community in the gut of the endogeic earthworm species *A. caliginosa*.

5.1 Introduction

Earthworms are among the most important members of the soil invertebrate fauna in temperate ecosystems (Edwards and Bohlen, 1996). Through burrow construction, mixing of organic matter and mineral soil, comminution of organic matter and casting they beneficially affect soil structure and chemical properties, and thus the biomass, activity and community composition of microorganisms (Brown, 1995; Tiunov and Scheu, 1999; Tiunov et al., 2001). Since microorganisms, particularly bacteria and fungi, are the primarily decomposers of organic matter in soils, earthworms are key species in controlling carbon and nitrogen cycling in soils (Jones et al., 1994; Lavelle et al., 1997; Scheu and Setälä, 2002). The most important effect of earthworms on microorganisms presumably is the passage through the earthworm intestinal tract which results in changes in microbial biomass, activity and community composition in cast aggregates.

Studies of the biomass, activity and community composition of microorganisms in cast aggregates and in the gut of several earthworm species have been traditionally performed using cultivation-based techniques (Tiwari et al., 1989; Kristufek et al., 1992), direct counting by microscopic techniques (Parle, 1963; Jolly et al., 1993; Schönholzer et al., 1999) and by the measurement of enzyme activities and respiration (Scheu, 1987; Zhang et al., 2000; Scheu et al., 2002); each of these techniques has limitations. For instance, it has been estimated that only 1-3% of bacterial species in soils can be cultivated (Alexander, 1961; Rappe and Giovannoni, 2003). The measurement of enzyme activities and respiration may provide insight into the activity and biomass of microorganisms but only poorly reveal microbial community composition.

Over the last twenty years identification of the phylogenetic diversity of the microbial community using molecular markers, such as the small subunit ribosomal RNA (16S rRNA) has become an established and robust method (Amann et al., 1995) and has recently been used in the identification of microorganisms in casts and gut of earthworms (Furlong et al., 2002; Singleton et al., 2003; Egert et al., 2004). However, the previous studies were limit to the taxonomic identity of microorganisms and failed to attribute specific metabolic functions to particular microorganisms within the community. Without this link a mechanistic understanding of effects of earthworms on carbon cycling and mineralization processes may not be possible.

In recent years stable-isotope probing (SIP), a coupling of molecular biological methods with stable-isotope abundance in biomarkers, has provided a powerful tool for linking microbial identity with metabolic functions in soils (Radajewski et al., 2003; Friedrich, 2006; Madsen, 2006). SIP relies on the application of isotopically enriched substrates (^{13}C, ^{15}N) into natural

environments, and the identification of metabolically active microorganisms assimilating this substrate by selective recovery and analysis of isotope-enriched biomarkers such as phospholipid fatty acids (PLFAs) (Boschker et al., 1998; Tillman et al., 2005), DNA (Radajewski et al., 2000) and RNA (Manefield et al., 2002a; Lueders et al., 2004a, 2004b, 2006). Each of the biomarkers has limitations and strengths. Briefly, compared to nucleic acids, PLFA patterns provide weak informations on the taxonomic level of microorganisms and uncultivated microorganisms cannot be identified since their PLFA patterns are unknown (Dumont and Murrell, 2005, Friedrich, 2006). DNA-based SIP requires relatively long incubation times since useful ^{13}C-labelling of DNA requires the replication of cells (Manefield et al., 2002b; Mahmood et al., 2005). Hence, RNA-based SIP is regarded to be the favoured technique to identify metabolic active microorganisms in short term incubations, since RNA synthesis occurs at a high rate and labelling can also occur without the need for DNA synthesis or replication of the cells.

Commonly, SIP experiments have been carried out adding commercially manufactured stable-isotope-enriched substrates, such as ^{13}C phenol or ^{13}C methanol into soils and the incorporation into primary degraders was followed (for recent reviews see Friedrich, 2006; Madsen, 2006). However, microorganisms themselves provide a large pool of carbon resources in soils; the amount of prokaryotic carbon is estimated to be in the range of 22-215 PgC, representing up to 15% of the total carbon in soils (Whitman et al., 1998). Despite this large carbon pool studies about the fate of microbial biomass carbon in soil food webs using SIP are limited (Lueders et al., 2006). In addition, the response of the soil microbial community to the addition of stable-isotope-enriched substrates as affected by biotic or abiotic factors has never been investigated using SIP.

In the present study, we followed the fate of ^{13}C labelled *Serratia marcescens* cells added to agricultural soil in presence and absence of the endogeic earthworm species *Aporrectodea caliginosa*. The gram-negative soil bacterium *S. marcescens* was chosen as it has been commonly isolated from soil, water, animals and the surface of plants, indicating a strong adaptive and survival potential and the ability to utilize a wide range of nutrients. The incorporation of ^{13}C into microorganisms which had assimilated this carbon resource was analysed by RNA-based SIP. We hypothesized that the passage through the intestinal tract of *A. caliginosa* considerably changes the activity and composition of the microbial community, and therefore the incorporation and mineralization of carbon of *S. marcescens* to soil bacteria..

5.2 Material and Methods

5.2.1 Soil and earthworms

In September 2005 soil was sampled from the upper 10 cm of a meadow located on the northeast corner of the Jena Biodiversity Experiment field site ("The Jena Experiment"). The experimental field is located on the flood plain of the Saale river (altitude 130 m NN) at the northern edge of Jena (Jena-Löbstedt, Thuringia, Germany). The soil is a Fluvisol (FAO, Unesco 1997) with pH 8.1 and a carbon and nitrogen content of 4.6% and 0.3%, respectively and a ^{13}C content of 1.091 atom%. The mean annual temperature on the experimental field is 9.3°C and the annual precipitation is 587 mm. For further details see Roscher et al. (2004). The soil was sieved (2 mm) to remove stones and coarse plant residues and stored at 4°C until the experiment was set up.

Earthworms were extracted in April 2006 on the experimental field by the oktett method (DEKA 4000, Deka Gerätebau, Marsberg, Germany) as described in Tieleman (1989), transferred to the laboratory in containers filled with soil from the study site and identified (Sims and Gerard, 1985). The endogeic earthworm species *Apporectodea caliginosa* (Savigny), dominant within the earthworm community on the experimental field was chosen for the experiment. Earthworms were kept in containers filled with soil from the study site at 4°C. Five days before the experiment was set up earthworms were transferred into containers with moist filter paper and kept at 15°C for defecation.

5.2.2 Serratia marcenscens

Serratia marcescens was kept on solid NB medium. The starter culture was grown from a single colony in 50 ml Erlenmeyer flasks containing 10 ml M9 medium, 10 µl SL 10 and 200 µl ^{13}C-labelled Glucose (99 atom%) on a rotary shaker (30°C, 150 rpm) for 12 h. For full labelling 20 µl of the culture were transferred into new 50 ml Erlenmeyer flasks containing ^{13}C medium as described above and incubated for further 12 h. Early-stationary cells were harvested by centrifugation at 4000 rpm for 2 min. Cells were washed twice in M9 medium and optical density (OD) was measured in an aliquot using a photometer (BioPhotometer, Eppendorf, Hamburg, Germany). To determine number of cells the measured OD was compared to a given calibration curve, resulting in 1.75×10^5 cells μl^{-1}.

5.2.3 Experimental set up and sampling

In July 2006 the experiment was set up. Glass flasks (150 ml) closed air tight by rubber septa were used for incubating the soil; 0.7 g dry weight soil (0.8 g fresh weight) were filled into glass vials (height 45 mm, diameter 20 mm) and placed inside the flasks. The soil was inoculated with 28.6 ml of ^{13}C labeled *S. marcescens* culture in 75.7 ml M9 medium resulting in a final concentration of 5 x 10^6 cells g^{-1} dry weight soil with a gravimetric water content of 28.8%, corresponding to 60% of the water holding capacity. For cast production 500 ml glass flasks were used and 0.8 g fresh weight soil labeled as described above were added into 1000 µl pipette tips. The tips were inserted into the base of plastic vials (height 25 mm, diameter 35 mm) accessible to earthworms placed in the vials to the labeled soil. One individual of *A. caliginosa* was added per vial. The experimental set up allowed sampling of cast aggregates but avoided burrowing of the earthworms. After one day cast aggregates were removed, weighed and transferred into glass vials placed into flasks as described for the incubation of the soil; earthworms and uningested soil were stored at –20°C until further analysis. Ten replicates per treatment were established; three empty flasks, three flasks with unlabelled soil and three flasks with unlabelled soil and one individual of *A. caliginosa* served as control for quantification of mineralization. Flasks were incubated in darkness in a climate chamber at 20°C for 15 days.

During the experiment CO_2 and $^{13}CO_2$ evolved were determined every 1 or 2 days. CO_2 in the headspace was determined in an aliquot (500 µl) by gas chromatography using a gas chromatograph (SRI 9300 A, SRI Instruments, Las Vegas, USA) equipped with a PoraPak Q column (80/100 mesh, 1.8 m length, Alltech, Deerfield, USA). $^{13}CO_2$ in the headspace was determined in an aliquot (500 µl) using a gas chromatography-isotope ratio mass spectrometry (GC-IRMS) system as described in Conrad et al. (2000).

Soil and casts were sampled after one and six days and stored at –20°C until further analysis. Aliquots of soil and casts were dried at 65°C for two days and water content was calculated. Dried samples were ground to powder and approximately 30 mg were analysed for ^{13}C. To avoid contamination with gut materials, the anterior part of the earthworm was cut off and approximately 0.8 mg were analysed for ^{13}C. The isotope ratios ($^{13}C/^{12}C$) were measured by a system of an elemental analyser (NA 1500, Carlo Erba, Milan, Italy) coupled with a trapping box (type CN, Finnigan, Bremen, Germany) and an isotope ratio mass spectrometer (MAT 251, Finnigan, Bremen, Germany) (Reineking et al., 1993). Vienna Pee Dee Belemnite served as standard for ^{13}C; acetanilide (C_8H_9NO, Merck, Darmstadt, Germany) was used for internal calibration. The amount of ^{13}C excess in earthworm tissue and the amount of ^{13}C excess in bulk soil were calculated by subtracting natural signatures from the measured ^{13}C atom%.

Mineralization of *S. marcescens* biomass was calculated from data of CO_2 evolved (mg CO_2) and amount of ^{13}C (atom%) and expressed as ^{13}C production (µg $^{13}CO_2$-C g^{-1} dry weight soil).

5.2.4 RNA extraction

Nucleic acids were extracted from soil and casts after one and six days according to Lueders et al. (2004). To inhibit loss of nucleic acids all steps were performed at 4°C. Briefly, 0.8 g fresh weight soil and casts were homogenised in 750 µl 120 mM $NaPO_4$ buffer and 250 µl TNS for 45 s at 6.5 m s^{-1} in a bead beater cell disruptor (Savant FastPrep Bio 101, Qbiogene, Inc., Carlsbad, USA) with zirconium beads in 2 ml Eppendorf tubes. Cell debris, zirconium beads and soil were removed by centrifugation at 14.000 rpm for 10 min. Beating and centrifugation were repeated to ensure maximum recovery of nucleic acids. Supernatants were transferred into 2 ml Eppendorf "Phase lock gel heavy" tubes containing 1 vol of Phenol/Chloroform/Isoamylalkohol (PCI) (25/24/1 vol/vol/vol, pH 8) and centrifuged at 14.000 rpm for 5 min. After centrifugation supernatants were transferred into 2 ml "Phase lock gel heavy" tubes containing 1 vol Chloroform-Isoamylalkohol (CI) (24:1, vol/vol) and centrifuged for 10 min at 14.000 rpm. Supernatants were recovered and nucleic acids were precipitated adding 2 vol PEG. After centrifugation (1 h, 14.000 rpm) nucleic acids were washed with 500 µl 70% ethanol and eluted in 50 µl elution buffer (EB, Qiagen, Hilden, Germany).
RNA was separated from co-extracted DNA by a modified protocol according to Lueders et al. (2004). Briefly, 35 µl of the primary extracts were transferred to 2 ml Eppendorf tubes containing 40 µl 10 x DNAse buffer (RQ1-buffer, Promega, Madison, USA), 10 µl RNAse-free DNAse (RQ1, 1u $µl^{-1}$, Promega) and 315 µl RNAse-free water and incubated for 30 min at 37°C. After incubation extracts were transferred into 2 ml "Phase lock gel heavy" tubes and purified with PCI and CI as described above. Supernatants were transferred to 2 ml Eppendorf tubes containing 0.1 vol 3 M NaAc and 2.5 vol 100% ethanol and precipitated at -20°C overnight. After centrifugation (1 h, 14.000 rpm) pellets were washed with 500 ml 70% ethanol and eluted in 30 µl EB.

5.2.5 Gradient centrifugation

RNA gradient centrifugation were performed according to Manefield et al. (2002a). Briefly, 500 ng RiboGreen-quantified rRNA were transferred into 6 ml centrifugation tubes (Sorvall, Asheville, USA) containing 4.6 ml of 2 g ml^{-1} cesium trifluoroacetate (CsTFA, Amersham Bioscience, Buckinghamshire, England), 175 µl formamide (Amresco, Ohio, USA) and 730 µl

Gradient buffer (GB; 0.1 M Tris-HCl, pH 8; 0.1 M KCl; 1 mM EDTA). The centrifugation media was mixed gently, average density was checked using a digital refractometer (AR200, Reichert, USA) and adjusted to 1.795 g ml^{-1} by adding gradient buffer if necessary. Gradient centrifugation was performed at 39.000 rpm at 20°C for >65 h. After centrifugation gradients were fractionated from bottom to top into 14 equal fractions (~400 ml) by displacing the gradient medium with water at the top of the tube using a syringe pump. The density of an aliquot (75 ml) of each CsTFA gradient fractions was determined and nucleic acids were precipitated with 1 vol of isopropanol, washed with 500 ml 70% ethanol and eluted in 30 µl EB.

5.2.6 Reverse Transcription PCR and T-RFLP

Bacterial 16s RNA genes were amplified using the Access RT-PCR System Kit (Promega) with the universal eubacterial primer combination of 27f-FAM (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3', 6-carboxyfluorescein-labelled) and 907r (5'-CCG-TCA-ATT-CCT-TTR-AGT-TT-3') resulting in 16S rDNA products of 900 bp. Reactions were carried out in a final volume of 50 µl containing 0.5 µl of each primer, 0.5 µl 10 x BSA, 0.5 µl 10 mM dNTP´s, 2 µl 25 mM $MgSO_4$, 8 µl AMV/Tfl 5 x RT buffer, 0.8 µl Tfl DNA Polymerase, 0.8 µl AMV Reverse Transcriptase and 2 µl RNA extract of each gradient fraction.
Samples were amplified in a preheated (94°C) thermocycler (GeneAmp PCR System 9700, Applied Biosystems, USA) by using the following protocol: an initial denaturation step of 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 68°C for 1 min. Cycling was completed by a final extension at 68°C for 10 min. To verify the purity of PCR products, visualization by standard agarose gel electrophoresis were performed for PCR products of each gradient fraction. PCR products were purified using the MinElute PCR Purification Kit according to manufactures protocol (Qiagen). One hundred ng of purified PCR products were digested with 0.25 µl Msp1 restriction enzyme (1u $µl^{-1}$, Promega), 1 µl 10 x MSP1 buffer (Promega) and 1 µl BSA in final volume of 10 µl for 3 h at 37°C. Digested PCR products were purified with Autoseq G-50 columns (Amersham Biosciences) according to manufactures protocol. After purification 1 µl PCR product was mixed with 11 µl Hi-Di formamide (Applied Biosystems) and 0.2 µl of 6-carboxy-X-rhodamine-labelled Size Standard MM1000 (Bio-Ventures, Murfreesboro, Tennessee, USA), denaturated (3 min at 95°C) and analysed on an ABI 310 Genetic analyser (Applied Biosystems) with a GeneScan-1000 ROX standard. T-RFLP electropherograms were analysed with GeneScan 2.1 Software (Applied Biosystems).

5.2.7 Cloning and sequence analysis

Based on the T-RFLP fingerprints representative `heavy´ gradient fractions of soils and casts after one and six days (CsTFA buoyant densities [g $ml^{-1}$]: soil day 1: 1.8206, day 6: 1.8206, cast day 1: 1.8206, day 6: 1.8155) were chosen for cloning. Bacterial 16s RNA genes were amplified from the `heavy´ fractions using the Access RT-PCR System Kit (Promega) with the primer combination 27f / 907r according to the protocol described above. PCR products were purified using the MinElute PCR Purification Kit according to manufactures protocol (Qiagen). Cloning was performed using the pGEM-T Vector System II (Promega). Ligation of PCR products was carried out according to manufactures protocol and vectors were transformed into *E. coli* competent cells by heat shock. Amplification of clones was performed using a M13-forward / M13-reverse primer combination. Reactions were carried out in a final volume of 50 µl containing 0.5 µl of each primer, 5 µl 10 PCR buffer, 5 µl 500 µM DNTPs, 3 µl 25 mM $MgCl_2$, 0.25 µl Taq polymerase (5 u μl^{-1}) and 1 µl purified clone extract. Samples were amplified in a preheated (94°C) thermocycler (GeneAmp PCR System 9700, Applied Biosystems, USA) by using the following protocol: an initial denaturation step of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min. Cycling was completed by a final extension at 72°C for 5 min. Purity of PCR products was visualized by standard agarose gel electrophoresis.
Clones were selected randomly and sequenced by the ADIS DNA core facility (Max-Planck-Institute for Plant Breeding Research, Cologne, Germany) on an ABI Prism 3700 sequencer (Applied Biosystems) using Big-Dye-terminator cycle sequencing chemistry according to the manufactures instructions.

5.2.8 Data analysis

Data on incorporation of ^{13}C into earthworm tissue and concentration of C in the earthworm tissue were analysed by one-factor analysis of variance (ANOVA) with the factor *Serratia marcescens* (with and without). Cumulative production of $^{13}CO_2$ was analysed by one-factor ANOVA with the factor *Aporrectodea caliginosa* (with and without), and rates of $^{13}CO_2$ production were analysed by repeated measures analyses of variance (RM-ANOVA). Significant time and treatment interactions were further investigated using ANOVA for each sampling event; differences between means were evaluated using Tukey´s HSD test. Prior to analyses data were inspected for homogeneity of variance (Levene test) and log-transformed if

required; a statistical probability P<0.05 was considered significant. To compare the bacterial community in soil and cast one and six days after the amendment of ^{13}C labelled *S. marcescens* cells, data on relative frequencies of phylogenetic lineages in the clone libraries were arcsinus-transformed and analyzed using the principal component analysis (PCA).
CANOCO (Ter Braak and Smilauer, 2002), STATISTICA 7.0 (Statsoft, Tulsa, USA) and SAS 8.0 (Statistical Analysis System, SAS Institute Inc., Cary, USA) software packages were used for statistical analyses.

5.3. Results

5.3.1 Mineralization of bacterial ^{13}C

Initially, 5 x 10^6 ^{13}C labeled cells of *S. marcescens* were added per g^{-1} dry weight soil, resulting in a concentration of 1.264 atom% ^{13}C in the soil. During incubation the concentration of ^{13}C in the soil significantly decreased but the decline depended on time and *A. caliginosa* with a decline of -0.105 and -0.039 atom% one day and -0.112 and -0.097 atom% six days after the amendment of ^{13}C biomass in casts and soils, respectively (Time x *A. caliginosa*; $F_{1,4}$=102.17, P=0.0005; Fig. 5.1).

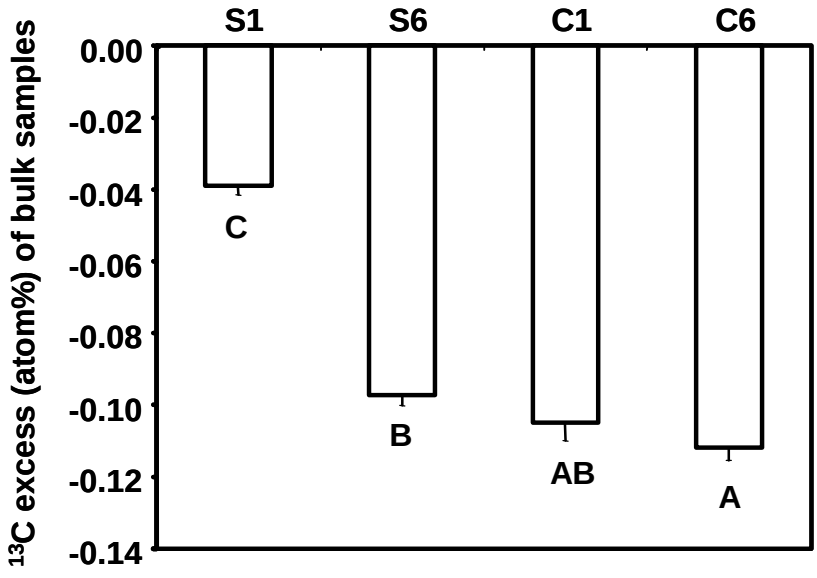

Figure 5.1: ^{13}C excess in soils (S) and casts (C) one day (1d) and six days (6d) after the amendment of ^{13}C-labeled *Serratia marcescens* cells. Means of three replicates ± 1 S.D. Different letters indicate significant difference between means at P<0.05 (Tukey´s HSD test).

The concentration of ^{13}C in the tissue of *A. caliginosa* slightly increased from an initial of 1.0841 to 1.0854 atom% after one day ($F_{1,6}$=42.0, P=0.0006). The carbon content was not affected and remained at 47.81 ± 2.72% ($F_{1,6}$=5.13, P=0.064).

Cumulative production of $^{13}CO_2$-C was significantly increased in presence of *A. caliginosa* with 12.26 ± 1.12 and 5.12 ± 0.38 µg ^{13}C g^{-1} dry weight soil emitted as CO_2 with and without *A. caliginosa*, respectively ($F_{1,6}$=146.06, P<0.0001), but the effect varied with time (Time x *A. caliginosa*; $F_{3,18}$=4.85, P=0.01); it was more pronounced in presence of *A. caliginosa* during the first day than during days 2 to 6, when earthworms had been removed (Fig. 5.2).

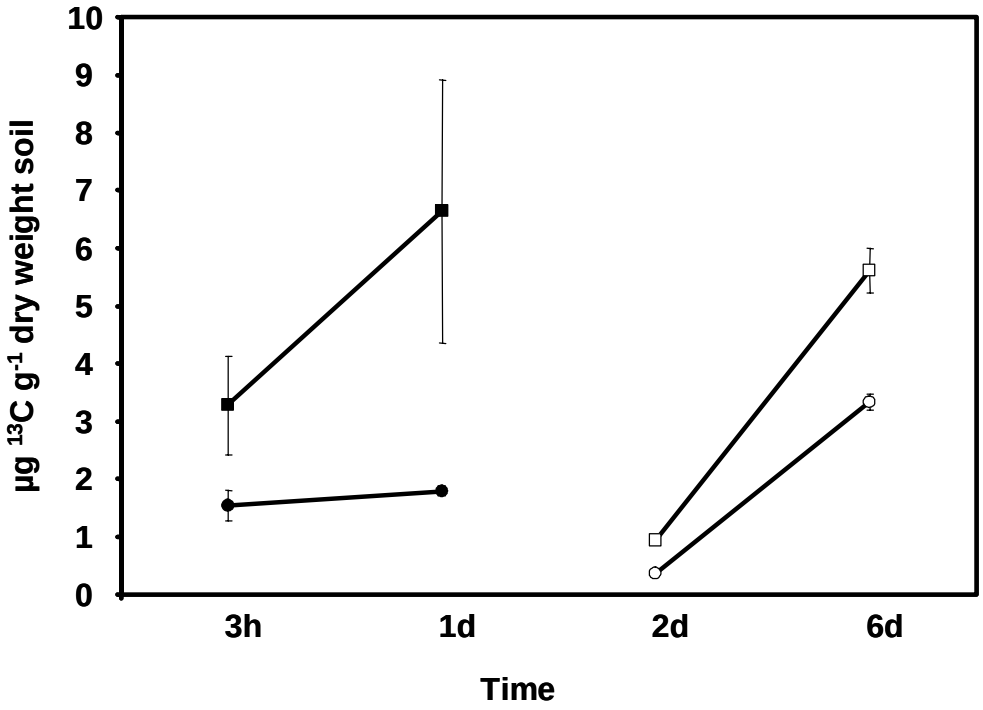

Figure 5.2: $^{13}CO_2$-C production (µg ^{13}C g^{-1} dry weight soil) in soils (circle), and casts (square) during 6 days of incubation. Means of three replicates ± 1 S.D

5.3.2 Bacterial communities in bulk samples

The bacterial community composition was analyzed by T-RFLP fingerprinting of 16S rRNA gene fragments of the control soil, the initial soil with *S. marcescens* (0h), and of soils and casts after one day (1d) and six days (6d). All T-RFs with heights smaller than 50 relative fluorescence units were excluded from the analyses. T-RFs similar in size of 1-2 bp were treated as operational taxonomic units (OTUs). Major OTUs in the profiles were defined as those with a relative electropherogram peak height of > 1.5% of the total electropherogram peak height, resulting in a total of 47 different OTUs. The OTUs dominating these patterns were defined as those with a relative peak height of > 5% of the electropherogram peak height of the major OTUs, resulting in ten dominant OTUs accounting for 57 to 75% of the total peak height of major OTUs.

Table 5.1: Relative percentile frequencies of dominant bacterial operational taxonomic units (OTUs) in the Control soil, the Initial soil, and Soils and Casts one day (1d) and six days (6d) after the amendment of ^{13}C-labeled *Serratia marcescens* cells.

OTU	Control	Initial	Soil 1d	Soil 6d	Cast 1d	Cast 6d
57	5.41	-	-	5.69	-	-
80	-	0.48	n.d.	n.d.	16.52	10.75
129	11.60	7.86	8.68	8.78	6.92	9.88
140	7.90	9.82	7.52	7.33	7.77	8.98
144	6.53	5.83	6.31	6.25	8.25	-
150	19.63	17.51	18.09	17.71	17.54	17.91
160	10.65	9.47	10.24	11.19	11.86	11.56
199	-	-	-	6.22	-	-
489	n.d.	6.64	9.72	n.d.	-	-
532	n.d.	n.d.	n.d.	n.d.	6.26	5.25

n.d., not detected; -, not dominant

The addition of *S. marcescens* little affected the microbial community composition in the soil (Fig. 5.3). The T-RFLP profiles of the control soil and the initial soil were dominated by the 129-, 140-, 144-, 150- and 160-bp OTUs. The *S. marcescens* characteristic OTU (489-bp) corresponded to 5.94% of the total electropherogram peak height and to 6.64% of that of the major OTUs of the initial soil, but was not detected in the control soil. The 57-bp OTU accounted for 5.41% of the electropherogram peak height of the major OTUs of the control soil, but did not occur in the initial soil in a major part. The T-RFLP profiles did not change until day one, but the 489-bp OTU disappeared after six days, and a new dominant OTU (199-bp) was detected (Table 5.1, Fig. 5.3).

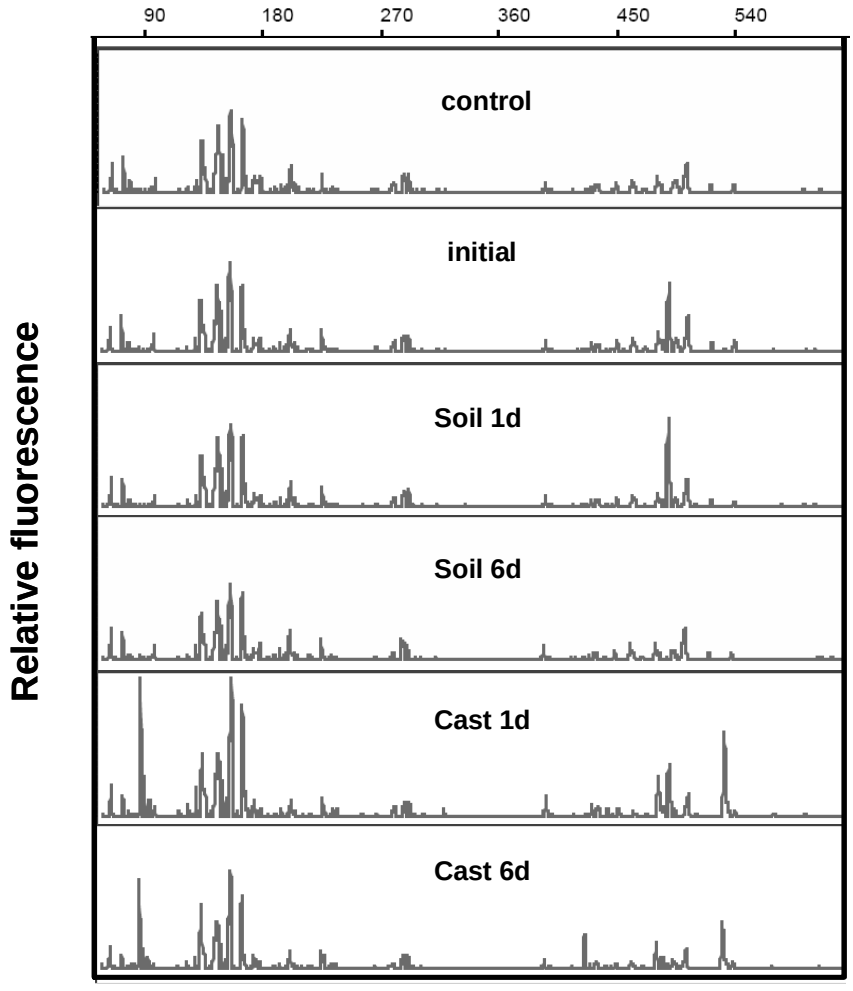

Figure 5.3: T-RFLP fingerprints of 16S rRNA gene fragments of the control soil, the initial soil with *Serratia marcescens* (0h), and of soils and casts one day (1d) and six days (6d) after the amendment of ^{13}C-labeled *Serratia marcescens* cells.

The T-RFLP profile of the cast one day after the amendment of ^{13}C labeled *S. marcescens* cells considerably differed from that of the initial soil (Table 5.1, Fig. 5.3). Two additional dominant OTUs (80- and 532-bp) were detected, and the amount of the 489-bp OTU decreased to 4.13%. After six days the 489-bp OTU accounted for 0.6% of the total profile and the 144-bp OUT did not occur in a major part (Table 5.1).

5.3.3 RNA-based stable isotope probing

To identify members of the microbial community which incorporated the ^{13}C label added as ^{13}C labeled *S. marcescens* cells, "light" unlabeled and "heavy" ^{13}C labeled rRNA molecules from the control soil, the initial soil, and from soils and casts after one day and six days were fractionated by density gradient centrifugation, quantified by reverse transcription PCR and analyzed by T-RFLP fingerprinting.

With a buoyant density (BD) ranging from 1.7168 to 1.8080 g ml^{-1} the control soil exhibited no bacterial rRNA matching the prerequisites for stable isotope labeling. In contrast, the initial soil contained bacterial rRNA displayed a BD ranging from 1.6297 to 1.8359 g ml^{-1} indicating an ample amendment of ^{13}C labeled *S. marcescens* biomass into the soil. T-RFLP fingerprinting of the bacterial community in the control soil confirmed that the "heavy" fractions were dominated by the 489-bp OTU (Table 5.2), whereas the "light" fractions were highly diverse (data not shown).

One day after the amendment of ^{13}C-biomass the soil contained large amounts of "heavy" bacterial rRNA and the T-RFLP profiles revealed significant changes within the bacterial community with four new dominant OTUs (89-, 130-, 155- and 167-bp) occurring in the heavy fraction (Table 5.2). After six days "heavy" bacterial rRNA was still present in the soil and highly diverse, with 56-, 142-, 150- and 162-bp OTUs being the most dominant. The 489-bp OTU was absent in the "heavy" fractions and hardly detected in the "light" fractions.

The T-RFLP profile of the "heavy" fraction of the cast one day after the amendment of ^{13}C-biomass differed from those of the initial soil and soil at day one (Table 5.2). It was dominated by the OTU with 54-bp which was hardly detected in the soil. In addition, OTUs with 62-, 89-, 150-, 167- and 489-bp occurred in substantial amounts. Six days after the amendment of ^{13}C-biomass the 489-bp OTU was absent in the "heavy" fractions and T-RFLP profile was dominated by 43-, 53-, 155- and 167-bp OTUs.

Table 5.2: Relative percentile frequencies of dominant bacterial operational taxonomic units (OTUs) in "heavy" fractions of the Initial soil, and Soils and Casts one day (1d) and six days (6d) after the amendment of ^{13}C-labeled *Serratia marcescens* cells.

OTU	Initial	Soil 1d	Soil 6d	Cast 1d	Cast 6d
43	7.23	11.47	2.46	n.d.	42.37
53	n.d.	-	n.d.	29.23	6.66
56	-	n.d.	6.23	n.d.	n.d.
62	n.d.	n.d.	n.d.	6.16	-
89	n.d.	22.09	n.d.	14.40	4.86
130	-	7.10	4.73	-	-
142	-	3.45	13.05	4.75	n.d.
150	-	-	12.08	9.35	-
155	1.96	8.65	n.d.	-	25.83
162	3.95	15.12	11.87	3.73	-
167	-	6.91	2.09	8.77	11.93
489	86.85	7.32	n.d.	8.41	n.d.

n.d., not detected; -, not dominant

To phylogenetically characterize the bacteria which incorporated ^{13}C, we constructed two 16S rRNA clone libraries of the "heavy" fractions of soils after one day (BD, 1.8206 g ml^{-1}, 42 clones) and six days (BD, 1.8206 g ml^{-1}, 54 clones) and two of the "heavy" fractions of casts after one day (BD, 1.8206 g ml^{-1}; 44 clones) and six days (BD, 1.8155 g ml^{-1}, 43 clones).

The clone library of the soil one day after the amendment of ^{13}C-biomass was dominated by *S. marcescens* sequences, accounting for 38% of total sequences analyzed. About 21% of the clones were related to different genera of the *Actinobacteria* lineage and remaining clones were related to various genera of α-, β- and γ-*Proteobacteria*, *Flavobacteria*, *Chloroflexi*, *Planctomycetes* and two uncultured *Acidobacteria* (Table 5.3, Fig 5.4a).

Six days after the amendment of ^{13}C-biomass sequences of *S. marcescens* were absent within the clone library of the "heavy" fraction and composition of phylogenetic lineages in the clone library was highly diverse with eleven different phylogenetic lineages in total. The clone library was dominated by different genera of the α-*Proteobacteria* and *Actinobacteria*, accounting for 29 and 17% of the total sequences, respectively. Furthermore, among β- and γ-*Proteobacteria*, *Acidobacteria*, *Chloroflexi* and *Planctomycetes*, previously detected, δ-*Proteobacteria*,

Cyanobacteria, *Nitrospina* and *Firmicutes* were present, whereas *Flavobacteria* were not detected (Table 5.3, Fig. 5.4b).

Table 5.3: Relative percentile frequencies of phylogenetic lineages in the bacterial community in the "heavy" fractions of Soils and Casts one day (1d) and six days (6d) after the amendment of ^{13}C-labeled *Serratia marcescens* cells.

Phylogenetic lineage	Soil 1d	Soil 6d	Cast 1d	Cast 6d
Serratia marcescens	38.10	n.d.	26.67	n.d.
α-Proteobacteria	9.52	31.48	13.33	2.27
β-Proteobacteria	9.52	9.26	8.89	4.55
γ-Proteobacteria	2.38	5.56	4.44	4.55
δ-Proteobacteria	n.d.	7.41	2.22	n.d.
Actinobacteria	21.43	16.67	15.56	4.55
Acidobacteria	7.14	7.41	n.d.	n.d.
Flavobacteria	7.14	n.d.	2.22	n.d.
Planctomycetes	2.38	11.11	11.11	n.d.
Chloroflexi	2.38	3.70	2.22	n.d.
Firmicutes	n.d.	3.70	11.11	84.09
Cyanobacteria	n.d.	1.85	n.d.	n.d.
Sphingobacteria	n.d.	n.d.	2.22	n.d.
Nitrospina	n.d.	1.85	n.d.	n.d.

n.d., not detected

The clone library of the casts one day after the amendment of ^{13}C-biomass consisted of eleven different phylogenetic lineages in total. Sequences of *S. marcescens* accounted for 28% of the clones. In addition, various genera of α-, β-, γ-and δ-*Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, and *Firmicutes* were identified. *Cyanobacteria*, *Nitrospina* and *Acidobacteria* sequences present in the clone library from soil were not detected in the cast, but sequences of *Flavobacteria*, and *Sphingobacteria* were detected (Table 5.3, Fig. 5.4c). The clone library of the "heavy" fraction of the cast six days after the amendment of ^{13}C-biomass considerably differed from clone libraries of the soils and from that of cast after one day. Five

different phylogenetic lineages were detected, with the lineage *Firmicutes* dominated by the genus *Staphylococcus* accounting for 84% of the total sequences. The remaining clones were related to α-, β-, γ-*Proteobacteria* and *Actinobacteria* (Table 5.3, Fig. 5.4d).

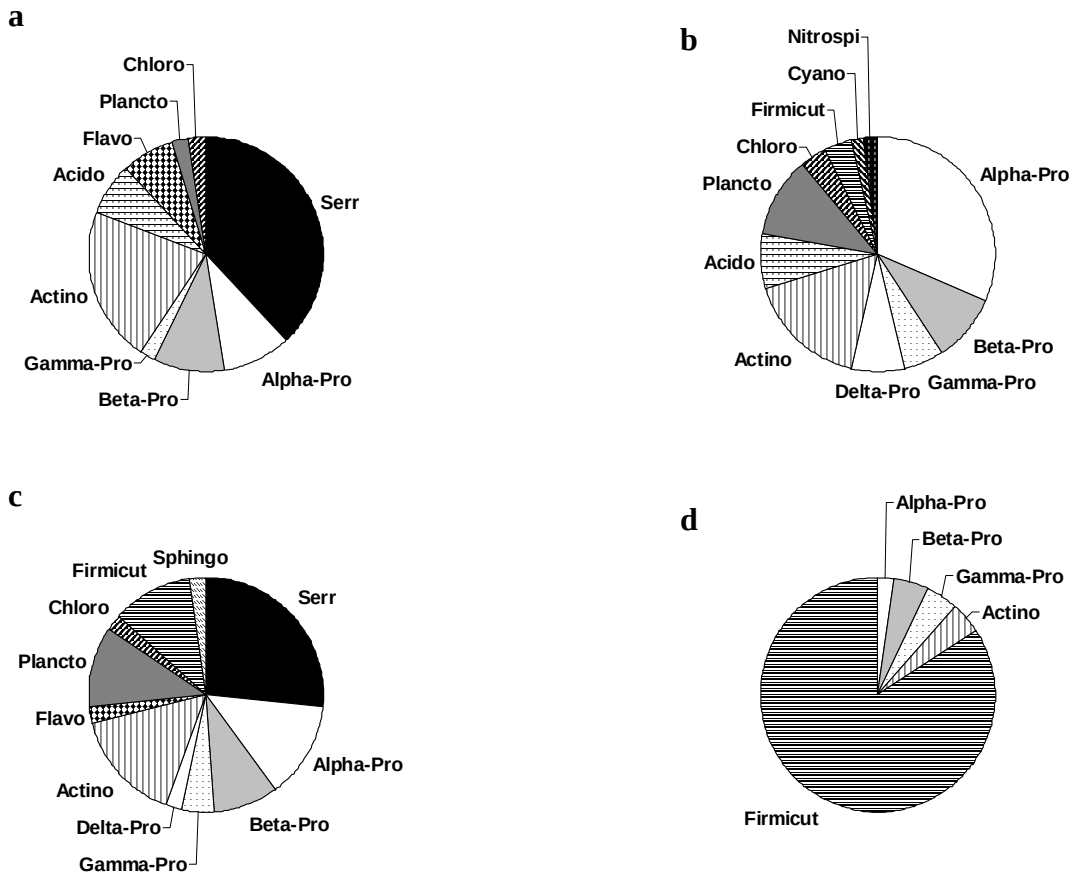

Figure 5.4: Phylogenetic affiliation and relative abundance of bacterial 16S ribosomal RNA clones in the clone library of the "heavy" fraction of **(a)** soil after one day, **(b)** soil after six days, **(c)** cast after one day and **(d)** cast after six days. Serratia marcescens (Serr), Alpha-Proteobacteria (Alpha-Pro), Beta-Proteobacteria (Beta-Pro), Gamma-Proteobacteria (Gamma-Pro), Delta-Proteobacteria (Delta-Pro), Actinobacteria (Actino), Acidobacteria (Acido), Flavobacteria (Flavo), Planctomycetes (Plancto), Chloroflexi (Chloro), Cyanobacteria (Cyano), Nitrospina (Nitrospi), Sphingobacteria (Sphingo), Firmicutes (Firmicut)

The principal component analysis (PCA) for clone libraries indicated that *Serratia marcescens* and the *Flavobacteria* preferentially occurred in cast and soil after one day, whereas the *Cyanobacteria* and the *Nitrospina* preferentially occurred in the soil after six days and the *Sphingobacteria* in cast after one day (Fig. 5.5). The *Firmicutes* were closely related to casts, while the remaining lineages responded little to the treatments. Overall, the first two PCA axes accounted for 80.2% of the variance in the clone libraries.

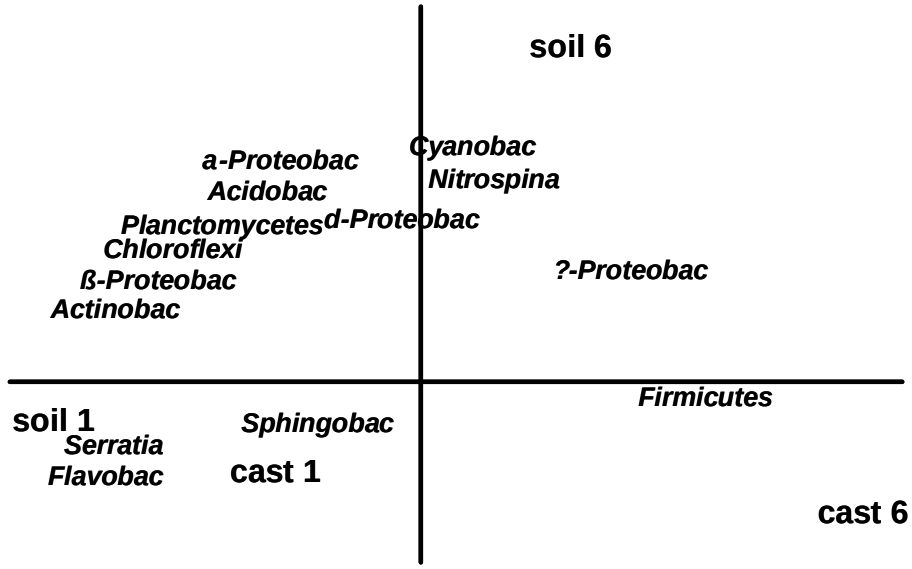

Figure 5.5: Principal components analysis (PCA) plot of bacterial lineages in Soils and Casts one day (1d) and six days (6d) after the amendment of ^{13}C-labeled *Serratia marcescens* cells. Serratia marcescens (Serr), Alpha-Proteobacteria (Alpha-Pro), Beta-Proteobacteria (Beta-Pro), Gamma-Proteobacteria (Gamma-Pro), Delta-Proteobacteria (Delta-Pro), Actinobacteria (Actino), Acidobacteria (Acido), Flavobacteria (Flavo), Planctomycetes (Plancto), Chloroflexi (Chloro), Cyanobacteria (Cyano), Nitrospina (Nitrospi), Sphingobacteria (Sphingo), Firmicutes (Firmicut)

5.4. Discussion

5.4.1 Effect of gut passage on bacterial community structure

The T-RFLP profiles of the bacterial communities of the soil differed markedly from those of the casts. Two dominant OTUs with 80- and 532-bp were detected in the T-RFLP profiles of the casts which were either absent (532-bp) or hardly abundant (80-bp) in the soil, suggesting that *A. caliginosa* harbors an indigenous microbial community in the gut

Symbiotic prokaryotes are common in the gut of soil invertebrates. They facilitate the digestion of resources, such as cellulose, hemicelluloses and lignin, which are inaccessible for soil invertebrates. Symbiotic prokaryotes have been detected in the gut of termites (Breznak, 1982, Paul et al., 1993), springtails (Hoffmann et al, 1998, Thimm et al., 1998), isopods (Kostanjsek et al., 2005) and millipedes (Byzov, 2005). The existence of an indigenous gut microflora in earthworms, however, is debated. Most studies have shown that bacterial communities in the gut and casts of earthworms primarily originate from the substrates earthworms were feeding on (Parle, 1963; Furlong et al., 2002; Singleton et al., 2003; Horn et al., 2003; Egert et al., 2004). Some studies, however, provided evidences for the abundance of an indigenous microflora community in the gut of earthworms (Jolly et al., 1993; Toyota and Kimura, 2000; Mendez et al., 2003; Sampedro et al., 2006).

Differences between bacterial communities in earthworm casts and non-processed soils are generally related to selective consumption of material with high concentrations of bacteria (Hendriksen, 1990). In addition, individual bacteria differently respond to the passage through the earthworm gut; some bacteria decrease in numbers, whereas other increase or pass through unaffected (Pedersen and Hendriksen, 1993; Fisher et al., 1997; Horn et al., 2003).

The abundance of the 80-bp OTU increased during the passage through the earthworm gut, suggesting that corresponding bacteria were promoted by favorable physicochemical conditions in the gut, but originated from the soil. Since the availability of oxygen is low in the gut of earthworms (Horn et al., 2003; Drake and Horn, 2006), we suggest that these bacteria were representatives of the anaerobic bacteria community.

The 532-bp OTU, in contrast, did not appear in the T-RFLP profiles of the soils, suggesting that corresponding bacteria originated from the earthworm gut. Although, we are aware of the low expressiveness of the experiment lacking statistical replications, we suppose that the presence of the 532-bp OTU indicates the existence of an indigenous microflora in the gut of *A. caliginosa*.

5.4.2 Flux of ^{13}C through the bacterial community

The primary objective of the present study was to investigate the incorporation of ^{13}C from isotopically labelled *S. marcescens* cells into the biomass of members of the soil bacterial community which had assimilated this carbon resource in presence and absence of *A. caliginosa* by means of RNA-SIP.

The T-RFLP profile of the "heavy" RNA fraction of the initial soil was dominated by the OTU characteristic for *S. marcescens* 16S rRNA genes, indicating ample availability of ^{13}C labeled *S. marcescens* biomass in the soil. After one day, however, the dominance of this peak decreased and the profiles of the "heavy" fractions of the soil and cast were highly diverse. Since the carbon isotope ratio of bacteria usually match the isotopic ratio of their food source (Abraham et al., 1998; Boschker and Middelburg, 2002), the T-RFLP profiles indicated that several members of the bacterial community became labeled by the utilization of ^{13}C labeled *S. marcescens* cells.

Cloning and sequencing of the "heavy" fractions of the soil and casts after one day identified a relatively diverse group of bacteria containing in addition to *S. marcescens* (soil 39%, cast 28%), representatives from eleven different lineages. Clone libraries of both soil and casts were dominated by different genera of the phylum *Proteobacteria* (soil 21%, cast 29%) and *Actinobacteria* (soil 21%, cast 16%) commonly isolated from soil, casts and burrows of earthworms (Furlong et al., 2002; DeRito et al., 2005; Lueders et al., 2006), indicating little effect of the passage through the earthworm gut on the use of bacterial carbon by the soil bacterial community in the short time.

However, the clone libraries of soil and casts differed in the abundance of *Acidobacteria*, *Firmicutes* and *Planctomycetes*. In the clone library of the soil three *Acidobacteria* were detected, but were absent in that of casts. Besides *Proteobacteria*, *Acidobacteria* are the most abundant bacteria in soils, accounting for up to 46% of soil bacteria (Janssen, 2006). They have also been found in earthworm casts, but were more abundant in soils than in casts (Furlong et al., 2002), suggesting that they might be digested or discriminated during the passage through the earthworm gut.

Different members of the lineage *Firmicutes*, belonging to the genera *Staphylococcus*, *Streptococcus* and *Paenibacillus* occurred in casts but not in the soil one day after the amendment of *S. marcescens* cells. *Firmicutes* have long been considered to be common members of the soil microbial community, but contribute only about 2% of soil clone libraries (Janssen, 2006). It has been documented that they occur in greater numbers in casts and burrows of earthworms than in un-processed soil, suggesting that *Firmicutes* are promoted during the passage through the earthworm gut (Furlong et al., 2002). In the present experiment, however,

Firmicutes were absent in the clone library of the soil after one day suggesting that they originated from the intestine of the earthworm. However, they might also have originated from the soil and promoted by favorable physicochemical conditions in the gut of *A. caliginosa*, as shown for *Bacillus megaterium* endospores passing the gut of *Lumbricus terrestris* (Fisher et al., 1997).

Members of the phylum *Planctomycetes* appeared in greater number in the cast library (five clones) than in the soil library (one clone) after one day. *Planctomycetes* have been commonly isolated from environmental samples, but contribute only about 2% of soil clone libraries (Janssen, 2006). All clones were closely related to uncultured *Planctomycetes* previously found in the biofilm of a fluidized-bed anaerobic digestor (Godon et al., 1997), suggesting that they were promoted by anaerobic conditions during the passage through the earthworm gut.

Overall, the increased production of $^{13}CO_2$-C in presence of *A. caliginosa* suggests that, although the bacterial community differed little between un-processed soil and cast, increased production of $^{13}CO_2$-C in cast was, in part, related to changes of the microbial community structure during the passage through the earthworm gut. In recent years, it became a major challenge in soil microbiology to ascribe microbial processes in the soil to specific microorganisms, but knowledge about the function of microbial communities and in particular, the function of specific microorganisms are limited (Pace, 1997; Hugenholtz et al., 1998; Torsvik and Ovreas, 2002). However, it has been demonstrated that *Acidobacteria* primarily occur in low–nutrient soils and being less physiologically active than α-*Proteobacteria* which dominate high-nutrient soils (Smit et al., 2001). These results correspond well with the increased number of α-*Proteobacteria* and the absence of *Acidobacteria* in casts after one day, indicating that the passage through the earthworm gut increases the availability of nutrients, and therefore the degradation of *S. marcescens* cells, i.e. the production of $^{13}CO_2$-C.

Six days after the amendment**,** *S. marcescens* clones were absent in both clone libraries, indicating complete degradation of the added cells independent from the gut passage through the earthworms; nevertheless, the clone libraries of casts and soil markedly differed. The clone library of the soil consisted of eleven different phylogenetic lineages, and was similar to the clone library of casts at day one. Sequences of α-*Proteobacteria* dominated, suggesting that the passage through the earthworm gut primarily accelerated the degradation of *S. marcescens* cells and made them accessible more quickly. The clone library of casts, in contrast, consisted of only five different lineages and was dominated by members of the phylum *Firmicutes*. In particular, most clones exhibited >99% sequence similarity to *Staphylococcus* spp., commonly isolated from soil, plants and animals (Brooks, 1963; Chesneau et al., 1993). *Staphylococcus* clones, however, were not only present in the clone library of day six, but also in that of day

one. In contrast, they were not detected in the libraries of the soil, suggesting that they either originated from the earthworm gut, or from soil, and have been promoted due to favorable physicochemical conditions in the gut. However, their dominance in the clone library of the cast after six days document that *Staphylococcus spp.* successfully competed with most microorganisms in using *S. marcescens* carbon. Furthermore, the higher production of $^{13}CO_2$-C in casts than in soil during days 2 to 6 indicates, that *Staphylococcus spp.* significantly contribute to the transformation of bacterial tissue into CO_2 and incorporating it into the biomass of indigenous bacterial community in soil.

Chapter

6

General Discussion

6. General Discussion

Soils are the most important component of the global carbon cycle and contain the largest active carbon pool on Earth. It is supposed that soils serve as a major sink for carbon but also contribute significantly to the flux of carbon to the atmosphere through the mineralization of soil organic matter (Cramer et al., 2001; Pacala et al., 2001; Schimel et al., 2001). Microorganisms, primarily bacteria and fungi are responsible for the mineralization of soil organic matter. Since earthworms directly and indirectly control the biomass, activity and community composition of microorganisms, they control the fate of soil organic matter which may lead either to its stabilization or mobilization. In the present work I investigated the interaction of two endogeic earthworm species (*Octolasion tyrtaeum* (Savigny), *Aporrectodea caliginosa* (Savigny)) on microbial biomass, activity and community composition with regard to the stabilization and mobilization of soil organic matter. Factors, presumably controlling the interaction of earthworms and microorganisms, have been investigated in five microcosm experiments under controlled environmental conditions in climate chambers.

6.1 Microbial biomass and community composition

Earthworms form the dominant component of the soil animal biomass and are regarded to be the most important soil animals participating in nutrient cycling and soil structure modification (Edwards and Bohlen, 1996; Spehn et al., 2000). By burrowing, mixing of organic matter with mineral soil and comminution of organic matter they strongly affect the structure and chemically composition of the soil and thereby microbial biomass and activity (Lee, 1985; Edwards and Bohlen, 1996; Tiunov and Scheu, 1999; Tiunov et al., 2001). In addition to these indirect effects on microorganisms, earthworms directly affect the microbial biomass and community composition by grazing (Brown, 1995; Bonkowski and Schaefer, 1997; Doube and Brown, 1998). Although investigated in detail, effects of earthworms on microorganisms are variable; earthworms increased microbial biomass and activity in some studies (Blair et al., 1995; Parmelee et al., 1998; Subler and Kirsch, 1998; Tiunov and Scheu, 1999), whereas in others effects were neutral or negative (Brown, 1995; Görres et al., 1997).

Performed experiments showed variable effects of earthworms on the microorganisms in soils. Juveniles of the endogeic earthworm species *O. tyrtaeum* and *A. caliginosa* did not affect microbial biomass in the rhizosphere of defoliated and non-defoliated white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) after eight weeks **(Chapter 3)**. In

contrast, adults of the species *O. tyrtaeum* reduced the bacterial and fungal biomass in the upper layer of mineral soil after six and twelve weeks when incubated with rye leaves positioned on the soil surface **(Chapter 4)**.

Endogeic earthworm species are very active, living in non permanent burrows in the upper mineral soil ingesting two to five times their own weight of soil and organic matter per day. They do not feed randomly, but selectively exploit nutrient hot spots (Spain et al, 1990; Spain and Le Feuvre, 1997; Wolter and Scheu, 1999; Marhan and Scheu, 2006). These hotspots, such as the rhizosphere or the soil litter interface, are preferentially colonized by microorganisms and earthworms. Since microorganisms provide a large pool of carbon resources in soils, we expected the microbial biomass to be decreased in the rhizosphere of *T. repens* and *L. perenne*, but effects of juveniles of the endogeic earthworm species *O. tyrtaeum* and *A. caliginosa* were neutral **(Chapter 3)**. Presumably, the biomass of earthworms was too low to significantly affect microbial biomass. Although based on biomass juvenile earthworms ingest larger amounts of soil and organic matter than adults due to their higher energy requirements (Scheu, 1987b; Curry et al., 1995), only 10% of the soil added likely have been transformed into casts until the end of the experiment. Similar to the results of this thesis, results of experiments on trophic transfers in the rhizosphere food web are variable; even microbial-feeders, such as bacterivorous nematodes do not necessarily affect microbial biomass in the rhizosphere (Merill et al., 1994; Todd, 1996; Bazot et al., 2005). Hence, further work is necessary to elucidate the interaction of earthworms and microorganisms in the rhizosphere.

In contrast to the rhizosphere, earthworms strongly decreased fungal and bacterial biomass in the upper layer of a mineral soil **(Chapter 4)**. Saprophytic fungi translocated and stabilized carbon from surface litter in their mycelial network in the upper mineral soil, thereby providing an easily available carbon resource for earthworms. *Octolasion tyrtaeum* exploited this carbon resource and reduced fungal biomass by grazing. In addition, *O. tyrtaeum* reduced the bacterial biomass, presumably by effectively competing with bacteria for labile and easily available carbon entering the soil as leachate. Microorganisms as well as endogeic earthworms are carbon limited, and it has been shown that both compete for labile carbon resources in soil, with endogeic earthworms effectively competing with microorganisms (Tiunov and Scheu, 2004).

In addition to microbial biomass, earthworms affected the community composition of microorganisms, with the microbial community in casts significantly differing from that in unprocessed soil. In the upper mineral soil *O. tyrtaeum* significantly reduced the amount of Gram-negative bacteria **(Chapter 4)**, suggesting that *O. tyrtaeum* preferentially fed on Gram-negative bacteria, or that Gram-negative bacteria are less adapted to physicochemical conditions in casts of endogeic earthworms. These results are in line with previous experiments, showing

that individual bacteria differentially responding to the passage through the earthworm gut; some bacteria decrease in numbers, whereas others increase or pass through unaffected (Pedersen and Hendriksen, 1993; Fisher et al., 1997; Horn et al., 2003). In contrast to Gram-negative bacteria **(Chapter 4)** some bacteria were selectively promoted due to favorable physicochemical conditions in the gut of *A. caliginosa* **(Chapter 5)**. Culture independent analysis of the bacterial community revealed that *Firmicutes*, particularly *Staphylococcus* spp. dominated the bacterial community in casts, but were absent (or reached low biomass) in the soil library, suggesting that they successfully competed with other microorganisms in earthworm casts.

6.2 Soil organic matter

Microorganisms considerably differ in their mineralization rate and efficiency, hence the effects of earthworms on microbial biomass and community composition, such as the selective consumption of individual microorganisms and the different response of microorganisms to earthworm ingestion, altered the fate of organic matter in soils. Earthworms differentially affected microbial biomass and community composition in the experiments, hence the fate of organic matter was variable.

The performed experiments demonstrated that earthworms primarily affect the retention of organic matter in soils by controlling the microbial biomass and community composition. By forming the habitat of microorganisms the soil macrofauna, in particular earthworms function as ecosystem engineers, thereby altering microbial mineralization of organic matter, and the production of CO_2 emitted from soils (Swift et al., 1979). Juveniles of the endogeic earthworm species *O. tyrtaeum* and *A. caliginosa* did not affect microbial biomass in the rhizosphere of defoliated and non-defoliated white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) **(Chapter 3)**, and also did not affect the mineralization of carbon. In contrast, *O. tyrtaeum* significantly decreased fungal and bacterial biomass in the upper layer of mineral soil when incubated with rye leaves positioned on the soil surface, and as a result increased the mineralization of carbon **(Chapter 4)**. The results demonstrated that microorganisms, in particular fungi stabilize carbon in their mycelial network in the upper mineral soil. Endogeic earthworms counteract this stabilization of carbon by grazing on fungal mycelia, indicating that endogeic earthworms contribute to the mineralization of carbon in soils by ingesting microorganisms. In a further experiment *A. caliginosa* considerably changed the community composition of bacteria in casts compared to unprocessed soil **(Chapter 5)**. Although, knowledge about the relation of taxonomic identity of microorganisms and mineralization of

soil organic matter is sparse, increased mineralization of carbon in casts compared to control soil indicate that endogeic earthworms increase the mineralization of soil organic matter by increasing the biomass of specific bacterial phylogenetic lineages in the casts.

In addition, the effect of endogeic earthworms on the fate of organic matter in the performed experiments was strongly affected by soil texture. For instance, the mineralization of phenolic compounds was strongly altered by the availability of clay **(Chapter 2)**. *Octolasion tyrtaeum* did not significantly affect the mineralization of carbon in forest soil, with high microbial biomass and clay content, but strongly increased microbial activity and thus mineralization of carbon in the arable soil with low clay content and microbial biomass **(Chapter 2)**. In the arable soil, however, the additional availability of clay in presence of earthworms resulted in a decreased mineralization of phenolic compounds, suggesting that the intimately mixing of organic matter and clay during the passage through the gut of earthworms promoted the stabilization of phenolic compounds in casts. This is in accordance to previous studies in which clay homogeneously mixed into the soil stabilized organic matter and protected it from microbial degradation by binding with clay particles (Jenkinson and Rayner, 1977; Shaw and Pawluk, 1986; Scullion and Malik, 2000).

6.3 Conclusions

In conclusion, the effect of endogeic earthworms on the stabilization and mobilization of soil organic matter strongly depends on their effect on microbial biomass and community composition in soils. Endogeic earthworms increased the mineralization of carbon by promoting specific microorganisms during the passage through their gut **(Chapter 5)**, but also by feeding on microorganisms which stabilize carbon in their biomass **(Chapter 4)**. The availability of clay, in contrast, decreases the mineralization of carbon due to intimate mixing during the gut passage through endogeic earthworms **(Chapter 2).** However, the present study primarily dealt with short-term experiments and further research, in particular long-term experiments, are needed to gain further insight into the interaction of endogeic earthworms and microorganisms in the stabilization and mobilization of soil organic matter.

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Acknowledgements

I thank my thesis supervisor **Prof. Dr. Stefan Scheu** for providing such an exciting research topic and for giving me the chance to work with him. His extensive knowledge and experience on soil ecology, earthworm sciences and beyond has greatly supported this work. He was always a source of motivation and never lost patience when listening to my scientific ideas or reading the first, second, third, ……. draft of my manuscripts.

I also thank **Prof. Dr. Angelika Schwabe-Kratochwil** for being the co-supervisor of my thesis, as well as **Prof. Dr. Felicitas Pfeifer** and **Prof. Dr. Stephan Kempe** for accepting to take part as examiners for my PhD dissertation.

Financial support by the **German Research Foundation (DFG)** is gratefully acknowledged (SPP 1090; Böden als Quelle und Senke für CO_2 - Mechanismen und Regulation der Stabilisierung organischer Substanz in Böden).

Many people provided excellent encouragement in supporting my studies on several ways. I hope I did not forget anybody.
Special thanks to my former "Chef" and "Hiwi" **Sven Marhan** for his friendship and for the suggestion of the interesting topic, introduction to earthworm sciences, thoughtful discussions, helpful comments and continuous support. I hope I was a worthy successor ("Soweit das Auge reicht, das alles wird mal Dir gehören, mein Sohn.").
PD Dr. Michael Friedrich (MPI für terrestrische Mikrobiologie, Marburg) and his whole group, particularly **Bianca Pommerenke** ("Käffchen, Kippchen?") are very much acknowledged for a productive and instructive collaboration, for giving me the introduction into the whole "molecular-microbial-thing" and the friendly admission and nice atmosphere in the laboratory. I thank **Prof. Dr. Rong Ji** (University Nanjing, China) and **Prof. Dr. Andreas Schäffer** (RWTH Aachen) for the productive and instructive collaboration, for supporting ^{14}C measurements and for giving me insight into the analysis of humic matter in soils. **Prof. Dr. Ellen Kandeler** (Universität Hohenheim) is acknowledged for the productive and instructive collaboration. Many thanks to **Christian Poll** (Universität Hohenheim) for the analyses of PLFAs, for a great time on various conferences and for sharing a small tent with me and Sven in the back of beyond. I thank **Dr. Reinhard Langel** (Kompetenzzentrum Stabile Isotope, Göttingen) for the ^{13}C- and ^{15}N-analysis of thousands of samples and **Dr. Michael Brecht** (TU-Darmstadt) for help in the ^{14}C-laboratory. Many thanks to **Udo Pelger**, **Karl Schuller** and their

excellent crew for keeping my work running by building and repairing whatever I needed and for still lending the department bus to me.

Many thanks to **Mark Maraun** for his help with hundreds of statistical problems, for thoughtful discussions, for sharing various Saturdays in the laboratory listening to the "Bundesliga" simulcasts and for taking always time talking about the most important thing in the world (I will never abandon the hope you sometimes realize that S04 sucks). Thanks to **Jens Illig** for his friendship, for exciting and funny times and for lively discussion at work and also until the early morning in the swamp. Thanks to **Michael Bonkowski** (for his helpful comments on microbial life in the rhizosphere) **Theodora Volovei** (for several entertaining coffee and cigarette breaks and help in the laboratory) and **Gabriele Bayer** (for handling my various travel applications). I thank **Ina Schäfer** (for entertaining breaks at work and interesting gossip from my old building), **Katja Rosenberg** (for sharing the time with me in the laboratory and various pubs in Marburg), and **Nico Eisenhauer** (for the honeymoon in Krakow). Many thanks to the old school members **Knut Kreuzer**, **Michael Heethoff, Michael Laumann** and **Stephan Partsch** for the revision of parts of the manuscript and for a great time, in particular having some all-night "conferences" or stupid trips to water reservoirs. I thank all the other members of the **Scheu group** (whom I can't mention in detail, because the list would be to long) bearing my inspection walkways and always providing me with cookies and / or chocolate bars. I have spent some really great time with all of you during both work and leisure.

Many thanks to **Sabine Butenschön** and **Carsten Paßlack** for mental and as well as financial support.

I am grateful to my parents **Gudrun** and **Johann Butenschön** who gave me all kind of support during my life and for helpful instructions to find and keep the right way.

Teile der vorliegenden Arbeit wurden bisher wie folgt publiziert, bzw. auf Tagungen präsentiert.

Paper

BUTENSCHOEN, O., JI, R., SCHÄFFER, A., SCHEU, S.: The fate of catechol in soil as affected by earthworms and clay (in prep.).

BUTENSCHOEN, O., POMMERENKE, B., ROSENBERG, K., FRIEDRICH, M., SCHEU, S.: Earthworms and the soil microbial food web – Insight into a `black box´ using RNA stable isotope probing (in prep.).

BUTENSCHOEN, O., MARHAN, S., SCHEU, S.: Response of soil microorganisms and endogeic earthworms to cutting of grassland plants – a laboratory experiment (in prep.).

BUTENSCHOEN, O., POLL, C., LANGEL, R., KANDELER, E., MARHAN, S., SCHEU, S.: Fungi and endogeic earthworms – antagonists in stabilization of litter carbon in soils. Submitted to Soil Biology & Biochemistry

Talks

BUTENSCHOEN, O., POLL, C., KANDELER, E., MARHAN, S., SCHEU, S. (2006): Endogeic earthworms reduce the fungal stabilization of litter derived carbon at the soil litter interface, Vortrag, The 8th international Symposium on earthworm ecology (Krakow, Poland).

BUTENSCHOEN, O., MARHAN, S., POLL, C., KANDELER, E., SCHEU, S. (2005): Einfluss endogäischer Regenwürmer auf die Translokation von Kohlenstoff an der Grenzfläche Streu-Boden, Vortrag, Jahrestagung der DBG (Marburg).

POSTER

BUTENSCHOEN, O., MARHAN, S., SCHEU, S. (2006): Effects of earthworms on stabilization and mobilization of soil organic matter - major research achievements of a six year period, Poster, Final Colloquium of the SPP 1090 (Bayreuth).

BUTENSCHOEN, O., JI, R., SCHÄFFER, A., SCHEU, S. (2005): Einfluss der Verfügbarkeit von Ton auf die Stabilisation von Catechol durch endogäische Regenwürmer, Poster, Jahrestreffen des SPP 1090 (Hannover).

BUTENSCHOEN, O., JI, R., SCHÄFFER, A., SCHEU, S. (2005): The fate of catechol in soils affected by earthworm activity and clay content, Poster, 2nd International Conference: Mechanisms of Organic Matter Stabilization and Destabilization in Soils (Asilomar, CA, USA).

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Die vorliegende Arbeit wurde unter Leitung von Prof. Dr. Stefan Scheu am Institut für Zoologie der Technischen Universität Darmstadt durchgeführt und von der Deutschen Forschungsgemeinschaft (DFG) im Rahmen des Schwerpunktprogramms SPP 1090: “Böden als Quelle und Senke für CO_2“ gefördert.

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Hilfsmitteln angefertigt habe. Ich habe noch keinen weiteren Promotionsversuch unternommen.

Darmstadt, den (Olaf Butenschön)

Wenn es um das Wetter geht, kann sich niemand auf seine Wahrnehmung verlassen. Du wachst zum Beispiel morgens auf und bist dir sicher, dass schlechtes Wetter ist. Der Himmel sieht mehr als grau aus. Aber dann, im Lauf des Tages, wird offensichtlich, dass es sich um Hochnebel handelt, Hochnebel, der einfach so verfliegt. Und dahinter ist der Himmel strahlend blau.

A.S.