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Ressourcenlimitierung von Abbauprozessen:
Die Rolle von Pilzen

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Verfasserin / Verfasser: Jörg Schnecker
Studienrichtung /Studienzweig (lt. Studienblatt): Diplomstudium Ökologie (Stzw)
Betreuer: Prof. Dr. Andreas Richter

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“Far out in the uncharted backwaters of the unfashionable end of the Western Spiral arm of the Galaxy lies a small unregarded yellow sun. Orbiting this at a distance of roughly ninety-eight million miles is an utterly insignificant little blue-green planet …”

(Douglas Adams)
General Introduction

Earth’s terrestrial ecosystems are a major sink for carbon dioxide, which is released by human activities such as fossil fuel burning and land use change. 2477 Gt of carbon are stored in soils and vegetation of these ecosystems (IPCC, 2000). Temperate and boreal forests, which cover approximately 16% of the land mass, contain 29% of the terrestrial carbon stocks (IPCC, 2000). To estimate the function of these ecosystems under a future climate, the basic processes in soil, and the interactions between vegetation and soil have to be thoroughly investigated.

Plants provide the main input of carbon into the soil. In temperate forest ecosystems leaf litterfall, root exudation and root turnover are the main input of assimilated carbon to the soil. On average 50% of the carbon fixed by plants is transported belowground, and up to 25% of this translocated carbon is further on released into the soil (Kuzyakov, 2002; Hobbie, 2006). Carbohydrates and other low-molecular weight substances, exuded by plant roots, establish a highly active microbial community in the soil surrounding the roots, the rhizosphere.

The term “rhizosphere” was first used by Lorenz Hiltner in 1904 and defined as the soil influenced by roots ("Einflusssphäre der Wurzel") (Hartmann et al., 2008). Roots influence the physical and chemical structure of the soil in their direct vicinity. Plant nutrient uptake and release changes in pH and redox potential of the soil (Jones et al., 2009) as well as the altered accessibility of water (Hinsinger et al., 2009) and thus differentiate the rhizosphere and the bulk soil. Rhizosphere effects have been observed up to a distance of 10 mm around the roots (Nichol & Silk, 2001; Vetterlein et al., 2007). The physical and chemical structure prevailing in the rhizosphere, favor other microbial groups compared to the bulk soil and establish a distinct microbial community (Smalla et al., 2001; Kent & Triplett, 2002; Paterson et al., 2007). Because of the
various microsites with individual biotic and abiotic conditions, the rhizosphere is considered to be one of the most diverse habitats (Jones & Hinsinger, 2008). In the rhizosphere as well as in the bulk soil the microbial communities are characterized by the interactions of bacteria, archaea and fungi. The ability of specific fungi to form mycorrhizal associations with roots allows establishing an interface between plants and soil that is of major importance in terms of water and nutrient cycling in forest ecosystems.

The associations of plants and fungi provide advantages for both partners: Plants supply the mycorrhizal fungi with carbohydrates derived from photosynthesis and fungi deliver nutrients such as nitrogen and phosphorus to the plants (Richardson et al., 2009). The fungal mycelium explores the soil more efficient than fine roots and extends the surface for nutrient uptake. Additionally, mycorrhizal fungi are considered to be an effective defense against plant pathogens, and have the ability to actively release nutrients from rock surfaces, mineral particles (Landeweert et al., 2001) and SOM.

The term “Mykorhiza” was first used by Albert Frank (1885). Since then the topic of plant and fungus interaction has been of interest to botanists, soil biologists and ecologists.

Mycorrhizal associations seem to be very old and are thought to have been evolved with the first land plants, approximately 450 to 500 million years ago (Cairney, 2000; Redecker et al., 2000). These first associations of fungi and plants belonged to the arbuscular mycorrhiza type. Ectomycorrhiza, the main mycorrhiza type of woody plants in temperate and boreal climates evolved about 200 million years ago.

Mycorrhizal association can be classified in seven types, depending on involved plant and fungal species and morphological characterizations (Finlay, 2008). The most abundant types are arbuscular mycorrhiza (AM), ericoid mycorrhiza (ERM), and ectomycorrhiza (ECM). Additionally, orchid mycorrhiza, monotropoid mycorrhiza, arbutoid mycorrhiza, and ectendomycorrhiza can occur (Finlay, 2008).
AM is widespread and can be found in most ecosystems. The fungi grow intracellularly and form typical highly branched fungal structures, so called arbuscules (Finlay, 2008).

In contrast to AM, the fungi forming ECM do not penetrate the plant cells. The hyphae grow intercellularly between the epidermal and cortical cells of the roots and form the so-called Hartig net. Additionally, the roots are surrounded by a fungal mantle. The fungi often form an extraradical mycelium to explore the surrounding soil. In forest ecosystems, the biomass of mantle and extraradical mycelium can account for 1000 kg ha⁻¹ (Wallander et al., 2001). ECM are mostly associations between Ascomycota or Basidiomycota with perennial woody plants (Fitter & Moyersoen, 1996). Compared to AM, less plant species form symbioses with ectomycorrhizal fungi. Nevertheless, ECM are the dominant forms of mycorrhiza in forest and woodland ecosystems (Taylor & Alexander, 2005). Globally, about 10,000 fungal species and approximately 8,000 plant species are known to form ectomycorrhizal symbioses (Taylor & Alexander, 2005).

In addition to the functional role of mycorrhizal and saprotrophic fungi and a large variety of different bacteria in decomposition of organic material in forest soils, the (functional) importance of archaea has only been started to be discussed recently.

Archaea are the “newcomers” in biology. Since their first discovery and classification as archaeabacteria (Woese et al., 1978), a new taxonomic system with archaea being one of three domains of life (Woese et al., 1990) has been established. Originally archaea have been considered occupying mainly extreme habitats. However, with the discovery of their abundance in marine ecosystems (Delong, 1992; Fuhrman et al., 1992) this theory was abandoned. Since then, archaea have been found in nearly every habitat possible on earth (Chaban et al., 2006). In soils, archaea may be of great importance in the nitrogen cycle (Nicol & Schleper, 2006) and may outnumber ammonia-oxidising bacteria (Leininger et al., 2006). Recent findings concerning the ability of production and exudation of laccases (Uthandi et al., 2010) even make their involvement in the degradation of SOM possible. In contrast to bacteria, the
diversity of archaea is increased in the rhizosphere compared to bulk soil and archaea seem to prefer roots colonized by ECM over non colonized ones (Bomberg et al., 2003).

The total functional spectrum of archaea in the soil and especially in the rhizosphere is far from being fully understood. However, the insights obtained in the last years suggest that archaea are of great importance in nitrogen cycling and might also be involved in carbon processing in the soil to a considerable extend.

Microorganisms in the rhizosphere are highly dependent on plant derived carbon. One important source of carbohydrates for microorganisms are root exudates, which mainly consist of free sugars, amino acids, and organic acids (Jones et al., 2009). The input of low molecular weight compounds into the soil may allow microorganisms to enhance decomposition of SOM, as well as nutrient uptake and transformation (Subke et al., 2004; Dijkstra & Cheng, 2007). The observed enhancements in enzyme production (Weintraub et al., 2007), nitrogen mineralisation (Dijkstra et al., 2009) and soil organic matter (SOM) decomposition (Nottingham et al., 2009) are referred to as priming effect. Fontaine and co-workers (2003) provided a slightly different idea of how priming may enhance SOM degradation. They hypothesized that the input of cellulose, a main component of plant tissue, leads to low glucose release and in turn favors slow growing K-strategist, which are capable of the decomposition of recalcitrant substances in the SOM pool. After the exhaustion of the added cellulose, increased SOM degradation could still be observed (Fontaine et al., 2004). The release of cellulose from dead roots could therefore lead to an enhanced decomposition of stored SOM with a long turnover time. On the ecosystem level this may affect the carbon exchange pattern between the biosphere and the atmosphere.

Manipulating the transport of recent photosynthetates from the plant’s canopy to the soil provides information on microbial processes as well as on the microbes that directly depend on this carbon source. Besides shading and thinning and therefore reducing the CO₂ fixation in plants, tree girdling is often used to stop the carbohydrate transport from the canopy to the roots. Girdling is done by
removing a ten centimeter broad stripe of bark all around the trees. This method prevents the downward directed flow of carbohydrates in the phloem but keeps the upward transport of water intact for a certain time (Zeller et al., 2008). Several month after the initial girdling, dead roots have to be considered as an additional carbon input into the soil (Ruess et al., 2003).

Study aims

In times of ongoing global change an improved understanding of the basic processes underlying the complex cycling of carbon and nitrogen in soils is urgently needed. Specifically, more knowledge is needed to upscale these processes to an ecosystem level and to estimate potential changes under possible future scenarios, including elevated CO₂ uptake by plants, altered water availability or changing light conditions (IPCC, 2007). The rhizosphere is a very active part of the soil and a hot-spot for carbon and nutrient turnover. It’s unique microbial composition and the involvement of mycorrhizal fungi in various processes distinguishes it from the bulk soil.

The overall goal of the present study was to get insight in the microbial community composition in the rhizosphere of a beech forest ecosystem and to relate it to processes such as nitrogen transformations and enzyme activities. We used PLFA analysis of fungi and bacteria, and qPCR of bacterial and archaeal 16S rRNA genes to assess the microbial community composition and to quantify the different microbial groups. The fungal community composition on the roots was determined by cloning and sequencing. Gross nitrogen mineralization and nitrification, as well as potential enzyme activities (glucanase, chitinases, peptidase, peroxidase and phenoloxidase) were determined. A tree girdling experiment was carried out to identify the effects of plant derived carbon on shaping the microbial community composition in the rhizosphere and to estimate their participation in processes relevant at the ecosystem scale. We expected tree girdling to shift the microbial community structure in the rhizosphere from microorganisms depending on recently fixed plant carbon, such as ectomycorrhizal fungi or bacteria feeding on easily
degradeable root exudates, to a community with a stronger ability to decompose SOM. We also expected, that these shifts in the microbial community would lead to lower hydrolytic enzyme activities and higher oxidative enzyme activities caused by reduced supply with easily degradable carbon, but an enhanced availability of complex substances from increased root litter.
References


General Introduction


The effects of reduced root exudation on microbial community structure and function in the rhizosphere of a beech forest

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The following persons contributed analytical data to this work:
Markus Gorfer¹, Frank Rasche² and Florian Stange³

¹University of Natural Resources and Life Sciences, Department of Applied Genetics and Cell Biology, Vienna, Austria (molecular identification of root associated fungi), ²Austrian Research Centers GmbH, Department of Bioresources, Seibersdorf, Austria (qPCR of 16S rRNA gene copy number and the bacterial/archeal amoA gene), ³Bundesanstalt für Geowissenschaften und Rohstoffe, Hannover, Germany (measurements of ¹⁵N in nitrate)
Abstract

Organic carbon, freshly assimilated in the canopy of trees and translocated to the roots and further to the soil, is one of the main C sources for microorganisms in the immediate vicinity of the plants roots, the rhizosphere. In order to investigate the effect of this plant derived carbon on microbial community structure and function in the rhizosphere we interrupted the input of carbon to the soil by physical tree girdling. Fungal, bacterial and archaeal abundance was estimated by PFLA analysis and qPCR of bacterial and archaeal DNA. In addition, several bacterial phyla were quantified by qPCR and root associated fungi were characterized by cloning and sequencing and subsequently categorized in saprotrophic, ectomycorrhizal (ECM) and ericoid mycorrhizal fungi. Potential activities of extracellular enzymes as well as gross nitrogen mineralization and gross nitrification rates were measured to obtain insight in microbial processes. Girdling caused significant reductions of total bacterial (-22%) and fungal (-41.5%) PLFAs, but an increase in archaeal 16S rRNA gene copies (+125%). The fungal PLFA biomarker 18:2ω6,9 alone decreased by 68%. The ratio of saprotrophic to ECM fungi changed from 1.5:1 to 16:1. Nitrification, one of the key processes in the N cycle, increased as a consequence of the girdling treatment and showed a statistically significant positive relationship with sum of bacterial and archaeal amoA gene copy numbers (R²=0.42, p-value<0.05). Exoglucanase and endochitinase activities decreased after girdling whereas peroxidase activities increased. However exochitinase and phenoloxidase activities were not affected. Archaea were the only microbial group which showed a significant positive correlation to the increased peroxidase activities. We conclude that belowground carbon allocation by trees shapes the microbial community structure and processes in a beech forest soil. If the transport of carbon from the plants to the soil is interrupted, the microbial community shifts to the disadvantage of ectomycorrhizal fungi and provides room for microorganisms that may otherwise not be able to compete with fast growing species in the rhizosphere.
and with ECM fungi. In our case archaea and saprotrophic fungi appeared to profit from the changed competitive circumstances.
**Introduction**

In forest ecosystems, soil microorganisms are highly dependent on plant derived carbon. There are two major pathways in which plant derived carbon, which is fixed in the canopy, becomes available for microorganisms in the soil: first, decomposition of plant root or leaf litter, and second exudation of low-molecular-weight compounds by roots. Estimates show, that up to half of the plant’s net primary production can be transferred below-ground (Hobbie, 2006). From the carbon translocated to the belowground compartment, 15 to 25% are exudated into the soil (Kuzyakov, 2002; Hobbie, 2006). Exudates, which mainly consist of free sugars, amino acids and organic acids (Jones et al., 2009), are easily accessible and assimilable for microorganisms. This C is not only used to build up biomass, but also to produce extracellular enzymes, which in turn facilitate decomposition of more complex organic matter. This is often referred to as the rhizosphere priming effect (Weintraub et al., 2007; Dijkstra et al., 2009). Priming is thought to specifically enhance the turnover of recalcitrant soil carbon (Subke et al., 2004; Dijkstra & Cheng, 2007). However, the input of complex but regular substances, in particular cellulose, to the soil, seems to cause stronger SOM degradation than easily available carbon forms (Fontaine et al., 2004). Rhizosphere priming may be of special importance in situations of low nutrient availability, in which slow growing SOM degrading K-strategists have advantages over fast growing microorganisms that feed on low-molecular-weight compounds. In such a situation the input of cellulose from dead roots may specifically favour microbes able to degrade SOM leading to the release of nitrogen, which can be taken up by plants (Fontaine et al., 2003).

One way to study the influence root exudates on soil microbial community composition and function is to manipulate the transport of carbon to the soil. Recent girdling experiments have shown that such a reduction of plant derived carbon dramatically reduces soil respiration and alters the microbial community (Andersen et al., 2005; Goettlicher et al., 2006; Yarwood et al., 2009). These changes also trigger alterations in the nitrogen cycling in the soil. Dannenmann and co-workers (2006; 2009) reported that the concentration of nitrate was
increased in soils with reduced carbon input, caused by a decreased immobilisation and an increased gross nitrification. These changes may ultimately lead to a change in the C to N ratio in the soil, and subsequently to an alteration of the microbial community at the expanse of fungi (Hogberg et al., 2007).

The rhizosphere, i.e. the soil in the immediate vicinity of the roots, is highly active and dynamic (Bais et al., 2006) and the interface where nutrient and signal exchange between plants and soil microbes takes place. The microbial community in the rhizosphere has been shown to be influenced by the roots and to differ from the adjacent bulk soil community (Smalla et al., 2001; Paterson et al., 2007). The exchange of nutrients between soil and plants in many ecosystems is mediated by mycorrhizal fungi, which thus belong to and extend the rhizosphere.

Mycorrhizal fungi can account for more than 30% of the microbial biomass in the soil (Hogberg & Hogberg, 2002). The total biomass of mycorrhizal fungi, including mantles and the extraradical mycelium, can account for up to 1 t ha\(^{-1}\) in forest ecosystems (Wallander et al., 2001). Mycorrhizal fungi provide not only the interface between plants and soil, but are also a major sink for carbon and nutrients (Hobbie, 2006). In exchange for carbohydrates, mycorrhizal fungi support their plant partner with nutrients, especially in temperate and boreal forests under low nutrient availability (Aikio & Ruotsalainen, 2002; Read & Perez-Moreno, 2003). ECM fungi are thus among the first to be affected by changes in the supply of easily degradable assimilates (Druebert et al., 2009).

The goal of the present study was to assess the effect of belowground carbon allocation by trees on the microbial community structure and on soil microbial processes in a temperate beech forest. Since the major fluxes of carbon and nutrients between plants and microbes in temperate forests are occurring at the tiny interface between roots (including the mycorrhizal network) and soils our study focuses on the rhizosphere.

We hypothesized that:
i. tree girdling will shift the microbial community structure in the vicinity of roots, from microorganisms depending on recently fixed plant carbon, such as ectomycorrhizal fungi or bacteria feeding on easily assimilable root exudates, to a community with a stronger ability to decompose recalcitrant substrates, and that

ii. the changes of the microbial community composition will be translated into lower activities of hydrolytic enzyme and associated processes, because of a decreased rhizosphere priming and subsequently into higher activities of oxidative enzymes.

To test these hypotheses we conducted a tree-girdling experiment in a mature beech forest near Vienna, Austria. We assessed the microbial community structure in the rhizosphere by analysis of PLFA pattern and qPCR of bacterial and archaeal 16S rRNA. Additionally we cloned and sequenced DNA of fungi living directly on or in the roots and categorized them into saprotrophic fungi, ectomycorrhiza and ericoid mycorrhiza. Gross nitrogen mineralization and nitrification rates and a set of six extracellular enzymes activities were analysed to characterize processes the rhizosphere.
Material and Methods

Site description

The experimental site for this study was an approximately 65 years old beech forest (Hordelymo-Fagetum) in Klausenleopoldsdorf, Lower Austria, situated 40 km southwest of Vienna, on a North-North-East-facing slope, approximately 510 m above sea level. The main soil type at the site is a dystric cambisol over Flysh. Mean annual precipitation accounts for around 800 mm and mean annual temperature is around 8.2°C. The surrounding area has been studied extensively throughout the last decade for vegetation and soil parameters as well as trace gas fluxes (Hahn et al., 2000; Stange et al., 2000; Hackl et al., 2004; Hackl et al., 2005; Kitzler et al., 2006).

Experimental setting

To stop carbon fluxes from the canopy to the roots and further to the soil, beech trees were girdled at breast height by cutting off 10 cm broad rings of bark and phloem. This method allows further water uptake of the plants in the xylem (Zeller et al., 2008). Three 20 m x 20 m girdling plots were installed in April 2006, from which the inner 10 m x 10 m was used for sampling. Two subplots were installed in each of the girdling plots. Six 5 m x 5 m plots served as controls. The plots including 1 m of the surrounding area were frequently freed from under storey plants, mainly beech seedlings. At the end of July 2007 soil and root samples were taken using steel cylinders with a height of 11 cm and an inner diameter of 8 cm. For the sampling, litter on the ground was removed and the A horizon was used for further processing. Five soil cores were taken from each plot or subplot, respectively. Roots were manually removed from the soil, by gently shaking the roots. Soil sticking closely to the roots (i.e., less than 2 mm around the root) was collected separately. This soil is called “rhizosphere soil” hereafter. The remaining soil was further sieved and the remaining roots sorted-out by hand picking. Soil samples were pooled according to plots. Roots were thoroughly washed and divided into fine roots (< 1 mm in diameter), roots
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> 1 mm diameter and visibly dead roots. Fine roots were cut into pieces approximately 1.5 cm long. After measuring their weight, subsamples were pooled according to the plots. From each plot 0.5 g were stored in 50% ethanol (Merck, Germany) for counting mycorrhiza tips, 0.2 g were put in RINAlater (Sigma-Aldrich, Austria) for molecular analysis and 2 g extracted for PLFA analysis. The remaining root biomass was dried to determine the water and carbohydrate content.

Microbial Community structure

Molecular Identification of Root Associated Fungi

For DNA isolation up to 500 mg fine root material (< 2 mm) was collected and total DNA isolated with the DNeasy Plant Mini Kit (Qiagen). Fungal ITS- and partial LSU-region were amplified with ITS1F (CTTGGTCATTAGGAGGAAGTAA) (Gardes & Bruns, 1993), which is specific for fungi, and the universal eukaryotic primer TW13 (GGTCCGTGTTTCAAGACG) (Taylor & Bruns, 1999). The LSU region serves for higher order identification of fungi with no highly homologous ITS reference sequences in public databases. All PCR reactions were run in triplicate on a T3 Thermocycler (Biometra) with an annealing temperature of 54 °C and 30 cycles. Due to low DNA amounts in samples G1 and G2 a second nested amplification step was performed with primer pair ITS1 (White et al., 1990)/TW13 and the same cycling conditions as for the first amplification step. Replicate PCR products for each sample were pooled before ligation into plasmid pTZ57R/T (Fermentas). From each clone library 96 clones were picked at random and the plasmid inserts sequenced with vector primers M13fwd and T7. Sequencing was done by AGOWA GmbH. Sequences were assembled and edited with Vector NTI10 software (Invitrogen). Mended contig sequences were submitted to a nucleotide BLAST Search (Altschul et al., 1990). BLAST searches were performed separately with parts of the sequence corresponding to the ITS and partial LSU region, respectively. In order to check for chimaeric sequences, the two taxonomies were compared for consistency. Reference hits were scrutinised concerning their reliability (e.g. sequences from strains from
collections like CBS were preferably taken as reliable references). In cases in which sequences could not reliably be identified to a certain taxonomic level, the lowest common affiliation of reliable reference sequences was taken.

Phospholipid fatty acids
Phospholipid fatty acids were analysed using a modified procedure described by Frostegard et al. (1991). 2 g of fresh soil were extracted with a mixture of citric acid buffer, chloroform and methanol. The separation of neutral lipids and phospholipids was performed on silica columns (Supelco, nr. 505048) by sequential elution with chloroform, acetone and methanol. Phospholipids were converted to fatty acid methyl esters (FAMEs) by alkaline methanolysis, after adding methyl-nonadecanoate (19:0) as an internal standard. Dried FAMEs were dissolved in isooctane and analysed by gas chromatography (HP G1530A) on a DB23 column (Agilent). We used a qualitative standard containing a mixture of bacterial FAMEs (Supelco, nr. 47080-U). The internal standard (19:0) peak served as a reference to calculate the concentration of FAMEs.

For Gram-positive bacteria the biomarkers i15:0, a15:0, i16:0, i17:0 and a17:0 were used. The fatty acids 18:1ω7, cy17:0, 16:1ω7, 16:1ω9, cy18:0, cy19:0 and 16:1ω5 were assigned to Gram-negative bacteria. The sum of the above mentioned markers together with 18:1ω5, 17:0, 15:0, 17:1ω6, 17:1ω7 were used as estimates for total bacterial biomass. Fungi were assessed by three biomarkers (18:2ω6,9, 18:1ω9, 18:3ω3,6,9), already used in other experiments (Hill et al., 2000; Leckie, 2005; Högberg & Read, 2006; Joergensen & Wichern, 2008). Additionally the biomarker 10Me16:0 was assigned to actinomyceta. Protozoa were represented by the fatty acid 20:2ω6,9.

Quantitative PCR
Amplicons from each investigated taxonomic group and functional gene were generated for standard preparation. PCR cocktails containing template DNA,
PCR reaction buffer (Invitrogen), MgCl₂, each oligonucleotide (total bacteria (Eub338:Eub518 (Lane, 1991; Muyzer et al., 1993)), total archaea (Ar109f:Ar912r (Lueders & Friedrich, 2000)), alpha-proteobacteria (Eub338:Alf685 (Lane, 1991)), beta-proteobacteria (Eub338:Bet680 (Lane, 1991; Overmann et al., 1999)), acidobacteria (Acid31F:Eub518 (Muyzer et al., 1993; Barns et al., 1999)), verrucomicrobia (VMB537f:VMB1295r (O’Farrell & Janssen, 1999)), bacterial amoA gene (amoA-1f:amoA-2r, (Rotthauwe et al., 1997)), archaeal amoA gene (Arch-amoAF:Arch-amoAR (Francis et al., 2005)), each dNTP, BSA, dimethyl sulfoxide (DMSO) for archaeal amoA gene and Taq DNA polymerase (Invitrogen), were produced. Invisorb® Spin PCRapid kit (Invitek, Berlin, Germany) was used to purify amplicons. These amplicons were ligated into the StrataClone™ PCR cloning vector pSC-A (Stratagene, La Jolla, CA, USA). StrataClone™ SoloPack® competent cells (Stratagene) were then transformed with the ligation products. To isolate plasmid DNA, the Plasmid Miniprep Kit (Bio-Rad Laboratories, Hercules, CA, USA). The PCR cocktail for quantitative PCR, contained iQ SybrGreen Supermix (Bio-Rad Laboratories), each primer (primer sets were the same as described above), BSA, and the template DNA for taxonomic groups and functional genes. Except the bacterial amoA gene, all qPCRs of functional genes were supplemented with DMSO. An iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad Laboratories) was used to run the PCR reactions. PCR amplicon quantities were analysed by using the iCycler Optical System Software Version 3.1 (Bio-Rad Laboratories).

Microbial processes

Gross N Mineralisation and Nitrification

To determine gross nitrogen mineralization and nitrification ¹⁵N pool dilution experiments were performed by a modified method after Barrett & Burke (2000). The method in detail is described in Kaiser et al. (2005). In short, 2 times each of 1.5 g of fresh sieved rhizosphere soil were used for determination of the N mineralisation and nitrification rates. The samples were labeled with (¹⁵NH₄)Cl, 0.25 mM, 10 atom% (Isotec, USA) and K¹⁵NO₃, 0.25 mM, 10 atom% (Isotec,
USA) for mineralization and nitrification, respectively and incubated for 4 h and 24 h. Afterwards, the samples were extracted with 15 mL 2M KCl (Sigma-Aldrich, Austria) and filtered using ash-free filter paper (Whatman, Germany). These extracts were transferred to 50 mL glass bottles, MgO (Sigma-Aldrich, Austria) was added to convert \( \text{NH}_4^+ \) to \( \text{NH}_3 \), which was trapped on filter discs soaked with 15 µL 2.5 M \( \text{KHSO}_4 \) (Sigma-Aldrich, Austria) and wrapped in PTFE tape. After incubation for 5 days at room-temperature under constant agitation, the acid traps were dried over concentrated \( \text{H}_2\text{SO}_4 \) in a dessicator for 48 h. The filter discs were then transferred into tin capsules and the 15N content measured by EA-IRMS. Enrichments in nitrate for gross nitrification measurements were determined by the Spinmas method (Russow, 1999). Nitrogen mineralisation and nitrification rates were calculated with a modified formula after Bengtson et al. (2006).

**Enzymes**

Potential exoenzyme activities in the soil were determined by a modified version of the methods described by Marx et al. (2001) and Saiya-Cork et al. (2002). The procedure is described in detail in Kaiser et al. (2010). Enzyme activities were measured within 48 h after sample collection. For the measurement of exochitinase, endochitinase and exoglucanase 4-methylumbelliferyl (MUF) labelled substrates (MUF-N-acetyl-\( \beta \)-D-glucosamide, MUF-\( \beta \)-D-N,N’,N”-triacetylchitotrioside, MUF-cellobioside, respectively) were used, whereas 7-amino-4-methyl coumarin (AMC) was used as substrate for peptidase activity. L-3,4-dihydroxyphenylalanin, DOPA (Sigma-Aldrich, Austria) was used to determine peroxidase and phenoloxidase activity. Fresh sieved soil samples (1 g) were mixed suspended in sodium acetate buffer, adjusted to pH 5.5 with acetic acid (Merck, Germany) and treated with an ultrasonicator (Sonopuls HD2070, Bandelin, Germany) for 30 s. The enzyme activity measurements were started by mixing the soil suspensions with the appropriate MUF or AMC substrate and incubation at room temperature for 140 min in black microtiterplates (Greiner bio-one, Austria). The incubations were stopped with 10 µL 1 M NaOH (J. T. Baker, Germany). Emission was measured at an excitation wavelength of 365 nm and an emission of 450 nm with a TECAN infinite M200.
fluorimeter. Determination of phenoloxidase and peroxidase was performed after Sinsabaugh et al. (1999) by mixing aliquots of the soil suspension with DOPA (20 mM). Samples were shaken for 10 min and centrifuged. The supernatant was transferred to transparent microtiter-plates (Greiner bio-one, Austria). To the wells, designated for peroxidase measurement, 10 µL 0.3% H₂O₂ (Merck, Germany) was added. The absorption was measured photometrically at 450 nm after incubation for 20 h in the dark. To obtain peroxidase activity, values for phenoloxidase activities were subtracted from values for peroxidase activities.

**Plant roots**

*Carbohydrates*

Starch content of roots was estimated after enzymatic digestion as described elsewhere (Richter *et al.*, 2009). Briefly, 20 mg of root powder was washed with cold 50% ethanol, and subsequently twice with 80% ethanol at 60°C. The residue was then dried under reduced pressure (SpeedVac, Concentrator 5301, Eppendorf, Austria) and incubated with (i) heat stable α-amylase (from *Bacillus licheniformis*, 500 U; Sigma-Aldrich, Austria) at 85°C and (ii) with amyllogucosidase (from *Aspergillus niger*, 10 U; Roche, Austria) at pH 4.6 and at 55°C. The resulting solution was then extracted with chloroform (Sigma-Aldrich, Austria) and centrifuged. Aliquots from the water phase containing starch derived glucose were diluted 1:5 with water and injected into an anionexchange chromatography system (Dionex ICS-3000, PA1 column, 50 mM NaOH as eluent) to determine glucose concentrations.

For analysis of sugars (glucose, fructose, sucrose, raffinose) hot water extracts were prepared by incubation of 60 mg of dry powder with 1.5 mL deionised water at 85°C for 30 minutes. The supernatant after centrifugation was then used for analysis. The samples were diluted 1:20 and measured by anion-chromatography as described above.
Mycorrhizal root colonization

Mycorrhizal root colonization was expressed as mycorrhizal root tips per meter root length and later on calculated as tips per gram dry weight. To measure root length, the roots were evenly dispersed on a Petri dish (9 cm in diameter), and their length was determined with a line intersect method at 30 x magnification (Newman, 1966). Mycorrhizal root colonization was obtained by counting all mycorrhized root tips in four quadrats of the Petri dish making up 11% of the total area of the Petri dish.

Statistical analyses

All statistical analyses were performed with R 2.7.2. For correlations the Itm package for R was used.
Results

Microbial community structure

The microbial community structure was assessed by PLFA analysis and by quantitative PCR of archaeal and bacterial DNA. Tree girdling significantly reduced bacterial 16S rRNA by 22% and the amount of PLFA biomarkers by 16.8% (Figure 1). The sum of the fungal PLFA biomarkers, 18:1ω9, 18:2ω6,9 and 18:3ω3,6,9 decreased by 41.5% after girdling and the fungal biomarker 18:2ω6,9, decreased by 68% (Figure 1a). Archaea were the only organisms that significantly increased by girdling (Figure 1b). No other group of organism, showed a statistically significant increase caused by the treatment.

DNA sequencing of fungal clones associated with fine roots revealed that in control plots the ectomycorrhizal fungi accounted for 41.5% of all clones whereas in girdling plots only 5.3% of the clones found where ECM fungi (Figure 2). The clones from ECM in control plots could be assigned to nine different fungal orders, nearly half of which were in the order Russulales. In contrast to the control the ECM clones from the girdling plots could only be assigned to three different orders. Russulales were only one percent of all clones in girdling samples compared to 19.7% in the controls. From the total 28 saprotrophic orders, only 8 orders were found both girdling and control plots. The most abundant order was Agricales with 29.2% in control and 42.2% in girdling plots. Girdling increased Rhytismatales from 5.4% to 23.5%. Coniochaetales and an unidentified order within the Dothideomycetes were not present in control plots but made up 7.1% and 5.1% of the total clones in girdling plots. Helotiales decreased from 11.1% to 5.2% and Corticiales increased from 0.5% to 3.8% in girdling plots.

Microbial Processes

Gross mineralisation rates were slightly lower after girdling, although the differences were not significant. Nitrification rates, in contrast increased as a
consequence of the girdling treatment (Figure 3). Also the amount of bacterial and archaeal amoA gene copies increased by 158% and 226% respectively (data not shown). A correlation of the log transformed amounts of total amoA gene copies and the nitrification rates showed a statistically significant relationship ($R^2=0.42$, p-value<0.05) (Figure 3).

Potential exoenzyme activities were influenced by girdling in different ways (Figure 4). While exochitinase, peptidase and phenoloxidase activities were not affected, exoglucanase and endochitinase activities decreased, whereas peroxidase activities increased by 27% as a consequence of the girdling.

To link the microbial community structure to the processes the exoenzyme activities and the mineralization and nitrification rates were tested for correlations with the PLFA biomarkers (Table 1) and the amount of 16S rRNA gene copies obtained from qPCR (Table 2). The fungal biomarker 18:2ω6,9 was the only one which correlated with all processes that were significantly changed by the girdling treatment. It was positively correlated with exoglucanase and endochitinase activities and negatively correlated with peroxidase activity and nitrification rates. Archaea quantified by qPCR correlated positively with peroxidase and negatively with exoglucanase and endochitinase activity (Table 2). However none of the used biomarkers was statistically related to phenoloxidase activities or to mineralization rates. The only biomarker that showed a positive statistical relationship to the nitrification rates was i15:0, a marker for Gram-positive bacteria, which makes up approximately 24% of all markers for Gram-positive bacteria.

**Plant roots**

Reduced carbon transport from the canopy to the roots led to a depletion in the sugars and starch pools of root. Glucose, fructose and sucrose as well as starch contents were 80% to 90% lower in roots of girdled trees 14 month after treatment (Table 3). Similar results could also be found in coarse roots (data not shown). This decline in carbohydrates in fine roots highly correlated with the amount of root tips that were colonized by mycorrhizal fungi (Figure 5).
Discussion

Tree girdling changed the microbial community mainly by reducing the microbial biomass, but also by shifting the community composition to the disadvantage of fungi, particularly of mycorrhizal fungi (Figure 1). The reduced supply of roots with low-molecular-weight carbohydrates also led to a depletion of sugars and starch pools in the roots to about 10% of controls (Table 3). Drubert et al. (2009) reported significant reductions of soluble carbohydrates and starch in fine roots of girdled beech trees already 10 weeks after girdling. Root carbohydrates may be used to supply the ectomycorrhizal partner at times when no current photosynthetates are available. Our data indicate that the carbohydrate pool was fully exhausted one year after girdling (remaining sugars are possible necessary to maintain cellular metabolism), and that the supply of plant carbon to ectomycorrhizal fungi was negligible. This is also evident in the strong correlation between carbohydrates and the number mycorrhizal root tips per g dry mass (Figure 5).

The fungal biomarker 18:2ω6,9 was strongly reduced in the rhizosphere soil as a consequence of the girdling treatment as has previously been observed for the bulk soil by others (Hogberg et al., 2007; Yarwood et al., 2009; Kaiser et al., 2010). The drastic reduction of ectomycorrhizal clones in comparison to saprotrophic fungi along with the loss of six ectomycorrhizal fungal orders (Figure 2) underline the influence of plant derived carbon on ectomycorrhiza. Similar findings were reported in a boreal forest after girdling despite differences in vegetation and sampling time (4 years compared to about 1 year in this study) (Yarwood et al., 2009). However, we were unable to find any effect of girdling on the amount of specific bacterial phyla quantified by qPCR or of bacterial groups by PLFA analysis (Figure 1). Archaea were the only group found to increase in abundance by the reduction of root exudation (Figure 1). The increase in archaeal 16S rRNA gene copy numbers along with the increase of archaeal amoA genes could explain, at least to a certain extend, the drastically enhanced gross nitrification (Figure 3), and further underline the importance of this microbial group for the nitrogen cycle (Leininger et al., 2006;
Nicol & Schleper, 2006). Another microbial group, which is also held responsible for nitrification, is the group of β-proteobacteria (Kowalchuk & Stephen, 2001; Dell et al., 2008). The amount of 16S rRNA gene copies of β-proteobacteria remained unchanged (Figure 1), although an increase in bacterial amoA was observed. This may be interpreted as (i) bacterial phyla other than β-proteobacteria may increase in activity, (ii) nitrifier of the β-proteobacteria being more active under reduced carbon supply or increased ammonium availability, or (iii) the proportion of β-proteobacteria, able to nitrify, on all β-proteobacteria increased,

Another reason for the elevated nitrification rates might simply be the increased amount of ammonia in the bulk soil of girdling plots (Kaiser et al., unpublished results). In a girdling experiment by Weintraub et al. (2007) the concentration of ammonia was also elevated and was thought to be caused by a reduced plant uptake of ammonia. The loss of 45% of fine roots Kaiser et al. (2010) at the studied site, the decrease of fungal PLFA biomarkers by 41.5%, and the reduction of bacterial 16S rRNA gene copies by 22% could favour archaea by reducing the competition for ammonia with fungi and bacteria and consequently lead to higher nitrification rates.

We measured potential extracellular enzyme activities to address the question if girdling affected the functional rhizosphere community and to estimate possible changes in decomposition processes in the rhizosphere. The reduction of exoglucanase and endochitinase activities (Figure 4) were significantly related to the amount of root tips colonised by mycorrhizal fungi and to the fungal biomarker 18:2ω6,9 (Table 1), indicating that ectomycorrhizal fungi may be the main producers of these enzymes in the rhizosphere and therefore may be responsible for a significant proportion of cellulose and chitin decomposition.

Peroxidase and phenoloxidase activities are the key enzymes needed for the degradation of humified and lignin-containing organic matter in soils (Sinsabaugh et al., 2005). The degradation of such compounds and the decomposition of SOM in general is thought to be a function of slow growing K-strategist which compete with fast-growing r-strategists, mainly living on easily assimilable carbon compounds from root exudates (Fontaine et al., 2003).
Since in our experiment the input of low-molecular-weight compounds by root exudates was interrupted, we expected that r-strategists would lose their competitive advantage, yielding an increased abundance of K-strategists. In another study Fontaine and coworkers (2004) have shown that the input of cellulose led to a priming effect, resulting in increased decomposition of SOM. Cellulose, which is a main constituent of plant roots, may have also been responsible for enhanced production of oxidative enzymes in our experiment, since the living root biomass was reduced by 45% (Kaiser et al., 2010) in our study 14 months after girdling. In our study the phenoloxidase activities remained largely unchanged, while peroxidase activities increased significantly (Fig. 4). Correlations of peroxidase activities with microbial biomarkers, revealed only negative correlations with the investigated fungi and bacterial, but a significantly positive relationship with archaea ($R^2=0.36, p<0.05$). Uthandi et al. (2010) recently discovered the production and exudation of a laccase (a phenoloxidase) by a halophilic archaeon, indicating that archaea might indeed play a role in SOM decomposition. However more research is needed to confirm these abilities and to find out if archaea are capable of the production of peroxidases. It is, however, questionable if mesophilic archaea alone could have been responsible for the observed increase of peroxidase activities. Since there is no explicit PLFA biomarker for saprotrophic fungi and the sequencing of fungal ITS regions only provided relative values, specialized fungi, which are known to be able to produce phenoloxidase and peroxidases could have been increased, despite an overall reduction in fungi.

Although it is recently discussed if ECM are capable of a saprotrophic lifestyle (Courty et al., 2007; Cullings et al., 2008; Baldrian, 2009), our results suggest that mycorrhizal fungi strongly depend on the carbon supply by plants. Even if they are facultative saprotrophs, the decrease of root exudates reduces their competitive abilities against free-living specialized fungi and other microorganisms. The negative correlation of mycorrhizal PLFA biomarker with peroxidase activities suggest that ECM did not participate to a higher degree in the degradation of SOM in girdling plots. The reduction of hydrolytic enzymes
as a consequence of girdling can be interpreted as the loss of a continuous priming effect (Subke et al., 2004; Dijkstra & Cheng, 2007).

In summary this tree girdling experiment provided information on the microbial community structure in the rhizosphere of a beech forest ecosystem, including all three domains of life. While fungal abundance and especially that of ectomycorrhizal fungi was drastically reduced by the girdling treatment, bacteria in general were less affected and no significant differences were found between girdling and control plots for the bacterial phyla we analyzed. Archaea however clearly increased in girdling plots, suggesting that they are not very competitive in the intact rhizosphere. Under control conditions plant exudates clearly favored microorganisms, with a high capacity to produce hydrolytic enzymes. A reduction of belowground carbon allocation by plants, along with increased input of dead organic matter (i.e., root litter) led to enhanced production of oxidative enzymes and to an increase of less competitive organisms, such as archaea. The implications of these findings are far reaching: in the face of ongoing climate change and ever-increasing atmospheric CO₂ concentrations, the belowground carbon allocation of trees may change, altering microbial community composition and thus decomposition processes in the rhizosphere, which in turn may feed-back on the biospheric-atmospheric CO₂ exchange.
References


Figure 1. Microbial community structure assessed by analysis of (a) PLFA pattern and (b) by qPCR of total archaea, bacteria and selected bacterial phyla. Black bars denote controls, grey bars girdling plots. Error bars indicate 1 SE of the mean (n=6). Stars indicate significant differences derived from t-tests for PLFAs and Mann-Whitney tests for qPCR (*: P<0.05, **: P<0.01, ***: P<0.001)
Figure 2. Relative contribution of functional types of fungi to the total fungal community at the root surface. Numbers denote mean percentages (n=3) of the total clones of each treatment. A total of 258 clones were sequenced for control plots and 216 clones for girdling plots.
Figure 3 Gross nitrogen mineralisation and gross nitrification rates. Black bars denote control, grey bars girdling plots; error bars indicate 1 SE (n=5). Stars indicate significant differences (Mann-Whitney tests; **, P<0.01). The insert shows a correlation of gross nitrification rates and the log transformed archaeal and bacterial amoA gene copy number (R²=0.42, P<0.05). Black cycles are control, grey cycles are girdling plots.
Figure 4 Potential extracellular enzyme activities. 4-Methylumbelliferyl (MUF) labelled substrates (MUF-cellobioside, MUF-N-acetyl-β-D-glucosamidase, MUF-β-D-N,N′,N″-triacetylchitotrioside) were used to measure exoglucanase, endochitinase and exochitinase activity, respectively. The substrate to measure peptidase activity was L-leucine 7-amino-4-methyl coumarin (leucine-AMC) and for phenoloxidase and peroxidase activity it was L-3,4-dihydroxyphenylalanin (DOPA) without or with additions of hydrogen peroxide. Peroxidase and phenoloxidase activities have to be multiplied by 100 to obtain the measured values. Black bars denote control, grey bars girdling plots; error bars indicate 1 SE (n=6). Stars indicate significant differences from t-tests, except for peroxidase, which was tested with a Mann-Whitney test (*: P<0.05).
Figure 5 Correlation of the amount of mycorrhizal root tips with (a) the total amount of sugars in fine roots ($R^2 = 0.48$, $P < 0.05$) and (b) with starch content of the fine roots ($R^2 = 0.52$, $P < 0.01$). Black cycles denote control, grey cycles girdling plots.
**Table 1** Correlation of PLFA biomarkers and microbial processes in the rhizosphere (potential enzyme activities, gross N mineralization and nitrification rates) and with mycorrhization (tips per g living root). Values shown are $R^2$ if $P<0.05$. Correlations with $P<0.01$ are in bold. Grey cells indicate negative correlations.

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Table 2 Correlation of 16S rRNA gene copy numbers and potential enzyme activities. Values shown are R² for all significant cases (P<0.05). Correlations with P<0.01 are in bold. Grey cells indicate negative correlations.

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Table 3 Carbohydrate content of fine roots of beech trees in control and gridling plots. The decrease caused by girdling and a correlation with the amount of mycorrhized root tips is also depicted. Amounts of carbohydrates are presented as means ± 1 SE (n=6).

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<th>Decrease (%) (in girdling plots)</th>
<th>Correlation with mycorrhizal root tips per g dry root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Girdling</td>
<td>R²</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.43 ± 0.53</td>
<td>0.71 ± 0.07</td>
<td>84</td>
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<tr>
<td>Fructose</td>
<td>5.69 ± 0.69</td>
<td>0.56 ± 0.10</td>
<td>90</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5.74 ± 0.83</td>
<td>0.27 ± 0.06</td>
<td>95</td>
</tr>
<tr>
<td>Total Sugars</td>
<td>15.86 ± 1.89</td>
<td>1.54 ± 0.23</td>
<td>90</td>
</tr>
<tr>
<td>Starch</td>
<td>1.15 ± 0.19</td>
<td>0.14 ± 0.01</td>
<td>88</td>
</tr>
</tbody>
</table>
Zusammenfassung

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mikrobielle Gemeinschaft und die Prozesse in der Rhizosphäre eines Buchenwaldes durch die Bäume geformt wird. Wenn der Transport von Kohlenstoff von den Pflanzen in den Boden unterbrochen wird, verändert sich die Rhizosphärengemeinschaft zu Ungunsten der Ectomykorrhizapilze. Dies bietet allerdings langsam wachsenden Organismen, die unter Umständen auch dem Reich der Archaeen angehören, die Möglichkeit sich zu etablieren.
Curriculum Vitae

Jörg Schnecker
Bonygasse 9-13/2/15
Wien, 1120

Date of birth  02/01/1984, Amstetten

Nationality  Austria

Education
2008-present  Master Thesis at the Department of Chemical Ecology and Ecosystem Research (Supervisor: Prof. Dr. Andreas Richter)
Title: “Resource limitation of Decomposition: The role of fungi”

2003-present  University of Vienna, Biology/Ecology

2002-2003  Alternative civilian service at the Austrian Red Cross


Work experience
August 2009  Attended the field course “Ecology of Siberia - from steppe to tundra” which took place in western Siberia
2009  Experience in an Isotope laboratory by developing and performing a pool dilution method for phosphorus mineralization within the FWF project MICDIF at the Department of Chemical Ecology and Ecosystem Research, University of Vienna
<table>
<thead>
<tr>
<th>Year/Details</th>
<th>Description</th>
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<tr>
<td>2008-2009</td>
<td>Laboratory analysis of plant litter and soil samples for miscellaneous projects at the Department of Chemical Ecology and Ecosystem Research, University of Vienna</td>
</tr>
<tr>
<td>September 2008</td>
<td>Mycorrhizal enzyme analysis within the scope of the Diploma Thesis, with Dr. Karin Pritsch at the Helmholtz Zentrum München</td>
</tr>
<tr>
<td>September 2007</td>
<td>Laboratory analysis of soil samples as well as field work for miscellaneous projects at the Department of Forest Ecology and Soils, Federal Research and Training Center for Forests, Natural Hazards and Landscape (BFW)</td>
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<tr>
<td>July/ August 2007</td>
<td>Research within the RELIS – Project (Resource limitation of microbial decomposition of soil organic mater) at the Department of Chemical Ecology and Ecosystem Research, University of Vienna</td>
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</table>

**Languages**
- German and English (fluent)

**Additional Qualification**
- Driving licence
- Skilled paramedic
- Skills in MS Office and basic knowledge in Computing and IT

**Civic duties**
- Paramedic at the Red Cross since 2002; still regular shifts