Microbial Activity − Indicators & Drivers

Dissertation

for the award of the degree

„Doctor rerum naturalium“ (Dr.rer.nat.)

of the Georg-August-University Göttingen

within the doctoral progam Biodiversity and Ecology

of the Georg-August University School of Science (GAUSS)

submitted by

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from Burghausen

Göttingen, May 2016

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Tag der mündlichen Prüfung: 27.06.2016

To my parents

and my children

Mia & Linus

I. Summary/Zusammenfassung

Biota involved in the carbon (C) cycle play a vital role in C sequestration and allocation in the soil food web. Though, the decomposition of soil organics strongly depends on the availability of soil organic matter (SOM) to microorganisms and enzymes. As rhizosphere and detritusphere are biospheres with very high availability of C, leading to high abundance, species diversity and contrasting functions of microbial communities, they are worth to be called hot spots of microbial functioning.

During the last decades, the interest on research on microbial activity in soil increased. However, there is a lack of comprehensive understanding of the indicators and drivers of microbial activity in rhizosphere and detritusphere. This is especially due to the objective necessity for more than a single measure to determine these factors. For that reason, this thesis aims to investigate microbial activity, its indicators and its drivers.

An arable field experiment with different substrate input (corn rhizodeposits vs. corn litter) was conducted to determine microbial activity by various activity indicators, such as microbial respiration, microbial biomass and enzyme kinetics. To account for decreasing amount and quality of substrates in rhizosphere and detritusphere with soil depth, we exhibited depth gradients of microbial activity indicators. Special attention was paid to several indices of multiple enzymes and their activity (e.g. specific activity, catalytic efficiency and ratios between C- and N-cycling enzymes) down the soil profile. Not only in the field, but also along a climatic gradient we investigated microbial activity. Therefore, the RNA:dsDNA ratio was determined in order to identify the metabolic status of microbes in contrasting soil types. Alongside with the major field and climatic gradient experiment laboratory and greenhouse studies were performed. These addressed the drivers, such as root hairs and protozoan predation, which were identified by isotopic labelling approaches and several indicators of microbial activity. Isotope technologies facilitate the tracing of e.g. C- and N-fluxes to achieve their central purpose of understanding the linkages of biota in terrestrial soil food webs. Soil zymography was used to exhibit the spatial distribution of enzyme activity in the rhizosphere in situ.

Substrates with contrasting availability changed functional properties of the soil microbial community and induced a shift in enzymatic systems. In particular, the rooted surface layer showed increased microbial activity compared to litter-amended and bare fallow

soil. Rhizodeposits are an important primary source of C and energy for soil microorganisms, stimulating their growth and activity. Accounted by the availability of rhizodeposits, hot spots in the surface layer provided preferable habitats for microbes.

Most microbial indicators were affected by the substrate input only in the topsoil. Microbial indicators included the catalytic efficiency of enzymes, which decreased by 2- to 20-fold from top- (< 40 cm) to subsoil (> 40 cm), irrespective of the substrate input. This suggests that the limited amount and quality of substrates at depth is an important constraint on microbial activity.

The RNA:dsDNA ratios towards the indication of the metabolic status of soil microbial communities was subjected to biased RNA quantity due to high clay contents in Chernozems, whereas at intermediate and low clay contents the RNA reflected reliable results.

Protozoa are assumed to be key-players in the C flux from bacteria to higher trophic levels, thereby affecting soil microbial activity. A triple-labelling experiment was conducted to investigate the effects of grazing by Acanthamoeba on C and N fluxes and microbial activity indicators in the rhizosphere and detritusphere. C fluxes and enzyme activities were driven by substrate input and quality and further stimulated by faunal grazing. This revealed that Acanthamoeba grazing contributes to microbial stimulation, especially in the rhizosphere.

To assess the influence of root hairs on microbial activity and rhizosphere priming, a continuous ^{13}C labelling experiment was conducted in a greenhouse. Root hairs induced positive priming during tillering. Without root hairs SOM decomposition was suppressed. Chitinase and ß-xylosidase activities increased during positive priming, indicating decomposition of stable SOM. This clearly showed the strong influence of root hairs on microbial activity during the early stages of plant growth, whereas at later stages the root hairs were a less important driver of microbial activity.

In summary, this thesis extends the understanding of factors affecting microbial activity in soil. It demonstrates that microbial activity can be meaningfully characterized by a careful selection of indicators. The chosen set of indicator is applicable at the landscape scale as well as for process-based investigations at the root scale.

Zusammenfassung

Biota des Kohlenstoffkreislaufs spielen bei der Speicherung und Verteilung des Kohlenstoffs im Bodennahrungsnetz eine herausragende Rolle. Wobei der Abbau organischer Bodensubstanz stark von deren Verfügbarkeit für Mikroorganismen und Enzyme abhängt. Rhizosphäre und Detritusphäre verfügen über außerordentlich große Mengen an organischem Kohlenstoff. Dies macht diese Biosphären zu Hot-Spots mikrobieller Aktivität.

Während der letzten Jahrzehnte stieg das Interesse an der Forschung zu mikrobieller Aktivität im Boden. Dennoch herrscht noch immer kein umfassendes Verständinis von Indikatoren und Triebkräften mikrobieller Aktivität in der Rhizosphäre und Detritusphäre. Dies ist nicht zuletzt der Tatsache geschuldet, dass es mehr als einer einzigen Methode zur Bestimmung dieser Faktoren bedarf. Aus diesem Grund setzt sich diese Arbeit zum Ziel, die mikrobielle Aktivität, ihre Indikatoren und Treiber zu explorieren.

Folglich wurden diverse Indikatoren mikrobieller Aktivität, wie mikrobielle Respiration, mikrobielle Biomasse und Enzymkinetik, an Proben aus einem Feldexperiment mit differierendem Substrat-Eintrag (Mais-Rhizodeposite vs. Mais-Streu) ermittelt. Tiefengradienten der Indikatoren mikrobieller Aktivität dienten der Beurteilung der Auswirkungen sinkender Substratqualität und -quantität in Rhizo- und Detritusphäre mit zunehmender Bodentiefe. Besonderer Fokus lag dabei auf den Indizes unterschiedlicher Enzyme und deren Aktivität (z. B. spezifische Aktivität, katalytische Effizienz und Verhältnisse zwischen Enzymen des Kohlenstoff- und Stickstoff-Kreislaufs) im Tiefenprofil des Bodens. Neben dieser Feldstudie wurde das Verhältinis von RNA zu dsDNA als Indikator mikrobieller Aktivität entlang eines klimatischen Grandienten ermittelt, um den metabolischen Status innerhalb unterschiedlicher Bodentypen zu bestimmen. Die Feldstudien wurden um Experimente in Labor und Gewächshaus ergänzt, in denen mittels Isotopenmarkierungsverfahren die Effekte von Wurzelhaaren und Protisten auf die mikrobielle Aktivität beleuchtet wurden. Diese Methode ermöglichte ein Nachvollziehen des C- und N-Flusses und trug damit zum Verständnis der Verflechtungen der Organismen im terrestrischen Boden-Nahrungsnetz bei. Die räumliche Verteilung der Enzymaktivität in der Rhizosphäre wurde anhand der Boden-Zymographie in situ untersucht.

Durch unterschiedliche Substratverfügbarkeit wurde ein Wandel der funktionellen Eigenschaften der Mikroorganismengemeinschaften und des enzymatischen Systems induziert. Speziell der durchwurzelte Oberbodenhorizont zeigte einen Anstieg der mikrobiellen Aktivität im Vergleich zum Boden mit Streueintrag und der Kontrolle. Rhizodeposite sind eine grundlegende Kohlenstoff- und Energiequelle für Bodenmikroorganismen und stimulieren deren Wachstum und Aktivität. Die Präsenz von Rhizodepositen in Hot-Spots macht diese zu bevorzugten Habitaten für Mikroorganismen. Die Mehrzahl der Indikatoren für mikrobielle Aktivität wurde ausschließlich im Oberboden durch den Substrateintrag beeinflusst. Darunter auch die katalytische Effizienz, die – ungeachtet des Substrateintrags – von Oberboden (< 40 cm) zu Unterboden (> 40 cm) um das 2- bis 20-fache abnahm. Dies ließ auf die Relevanz der mit der Tiefe abnehmenden Menge und Qualität der Substrate im Boden als einflussnehmenden Faktor auf die mikrobielle Aktivität schließen. Das Verhältnis von RNA zu dsDNA spiegelte den metabolischen Status der mikrobiellen Organismengesellschaften in den meisten der beprobten Böden wider. Wohingegen das RNA:dsDNA Verhältnis dieser Indikatoreigenschaft widersprach, lagen erhöhte Tongehalte vor, die nach der Extraktion zu Ungenauigkeiten bei der Bestimmung der RNA-Quantität führten.

Protozoen wird beim Vorgang des Kohlenstoffflusses von Bakterien zu Organismen höherer Trophieebenen eine bedeutende Rolle zugesprochen, was ebenfalls ihren Einfluss auf die mikrobielle Aktivität im Boden unterstreicht. Um diesen Effekten, im Speziellen jenen der Acanthoamoebe auf den Kohlenstoff- und Stickstofffluss, sowie die Indikatoren mikrobieller Aktivität in der Rhizo- und Detritusphäre nachzugehen, wurde ein dreifaches Isotopenmarkierungs-Experiment durchgeführt. Es ergab, dass Kohlenstoffflüsse und Enzymaktivitäten sowohl von Substrateintrag als auch Substratqualität in Rhizo- wie Detritusphäre sowie deren faunistischer Besiedlung abhängen. Daraus erschloss sich, dass die Besiedlung mit Acanthamoeben als potenzielle Triebkraft mikrobieller Aktivität, besonders innerhalb der Rhizosphäre, gedeutet werden kann.

Um den Einfluss von Wurzelhaaren auf die mikrobielle Aktivität und den Priming Effekt in der Rhizosphäre einzuschätzen, wurde ein Experiment im Gewächshaus mit kontinuierlicher Markierung von Boden mit Pflanzenbewuchs und einer Kontrolle ohne Bewuchs mit ^{13}C-Isotopen durchgeführt. Wurzelhaare zeigten sich darin als Initiatoren eines positiven Rhizosphären-Priming Effektes während der Wachstumsphase, wohingegen der

Abbau organischer Bodensubstanz in den Kontrollen gehemmt war. Im Falle der positiven Initialwirkung der Wurzelhaare stiegen zudem die Enzymaktivitäten von Chitinase und ß-Xylosidase an, was auf eine Zersetzung stabiler, organischer Bodensubstanz hinwies. Damit konnte ein deutlicher Effekt von Wurzelhaaren auf die mikrobielle Aktivität im Boden während der Phase des Pflanzenwachtums nachgewiesen werden.

Somit vermittelt diese Arbeit ein weiterführendes Verständnis der auf mikrobielle Aktivität im Boden einwirkenden Faktoren und stellt eine Auswahl von Indikatoren zur Charakterisierung dieser Aktivität vor, die sowohl auf der Landschaftsebene als auch in der prozessorientierten Forschung im Wurzelraum Anwendung finden kann.

Acknowledgements

Mein besonderer Dank gilt meinem Doktorvater Prof. Dr. Yakov Kuzyakov, der es mir ermöglicht hat, meine Promotion in diesem interessanten Forschungsfeld durchzuführen. Ich möchte ihm für seine wertvollen Anregungen und Ratschläge und die hilfreichen Diskussionen während der gesamten Zeit meiner Promotion danken.

Meinem Thesis Commitee: Prof. Dr. Y. Kuzyakov, Prof. Dr. H. Flessa, und Prof. Dr. S. Scheu danke ich für die Betreuung sowie Prof. Dr. Heinz Flessa für die Zweitbegutachtung meiner Promotion. Weiterer Dank gilt den Mitgliedern der Prüfungskommission: Prof. Dr. A. Carminati, Prof. Dr. R. Jörgensen und Prof. Dr. D. Sauer.

Der DFG danke ich für die Finanzierung dieses Projekts im Rahmen der DFG-Forschergruppe FOR 918 "*Carbon flow in belowground food webs assessed by isotope tracers* ".

Ohne die Unterstützung bei der Laborarbeit durch Susann Enzmann und Heike Strutz wäre die Umsetzung der zahlreichen Experimente nicht möglich gewesen. In diesem Zuge danke ich Viola Schade für ihre Mitarbeit. Auch Jan-Carl Siegmund möchte ich danken; er hat mir bei den zahlreichen Probenahmen unter die Arme gegriffen.

Prof. Dr. Evgenia Blagodatskaya danke ich für die fachliche Beratung während der Durchführung meiner Promotion.

Nicht zu vergessen, Dr. Peter Gernandt, der mir die eindrucksvollen Details der Feldbodenkunde näherbrachte. − Die Weltmeisterschaft in Brasilien rückt näher, Coach!

Doch ganz besonders möchte ich Dr. Johanna Pausch danken, die immer ein offenes Ohr für mich hatte und die mit ihrer Unterstützung und ihren Ideen zur erfolgreichen Durchführung dieser Arbeit beigetragen hat. Es war eine sehr schöne Zeit mit Dir und Deiner Familie in Californien. Lass uns das bald wiederholen!

Mein größter Dank gewährt meinem gerade verstorbenen Vater, der immer an mich glaubte und mich motivierte im Leben etwas zu erreichen, sei es beruflich oder privat.

Ich möchte allen Leuten danken, die mich während meiner Doktorarbeit begleitet haben und mir mit Rat und Tat zur Seite standen.

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Abbreviations

^{13}C	Stable carbon isotope with atomic mass 13
^{14}C	Radioactive carbon isotope with atomic mass 14
C	Carbon
CO_2	Carbon dioxide
dsDNA	Double-stranded deoxyribonucleic acid
^{15}N	Stable nitrogen isotope with atomic mass 15
N	Nitrogen
N_2O	Nitrous oxide
P	Phosphor
RGB	Truecolor image
RNA	Ribonucleic acid
RPE	Rhizosphere priming effect
SEM	Standard error of mean
SOM	Soil organic matter

1. Introduction

1.1 Organic carbon in below-ground systems

Increasing concentrations of atmospheric CO_2 have prompted a flood of studies on soil C cycling (e.g. Amundson, 2001), with a strong focus on C sequestration, storage, and stabilization (Janzen, 2006; Lützow and Kögel-Knabner, 2006). However, the fate of C in the belowground system, its exchange between plants (e.g. rhizodeposition) and microorganisms (e.g. C uptake and mineralization) and especially its flux through food webs and the general relationship between soil biodiversity and ecosystem functioning remains poorly known (Bradford and Wood, 2014). The large diversity of organisms and their close interactions, that are central to biogeochemical cycles (Scheu and Setälä, 2002), rely on the amount and availability of organic C and nutrients, which therefore form the basis of soil food webs.

1.2 Soil hot spots: rhizosphere and detritusphere

Rhizosphere and detritusphere are soil microsites with a very high resource availability for microorganisms. They affect their biomass, composition and functions, which make them worth to be called “hot spots” (Marschner *et al.*, 2012; Kuzyakov and Blagodatskaya, 2015). These hot spots are relevant not only from the perspective of SOM availability and C limitation but also from the perspective of abiotic processes which limit microbial activity (Kuzyakov and Blagodatskaya, 2015). The C and nutrient transport in these hot spots is divided into two major energy channels 1) the bacterial energy channel and 2) the fungal energy channel. Bacteria and fungi are important bottom-up drivers (resource quantity and quality) for the structure of soil food webs due to their specific resource utilization during decomposition of SOM (Kramer, 2014).

About 30% of root-derived C is metabolized in form of easily available substrates (low-molecular weight) by the bacterial communities (Holtkamp and Wal, 2011). In the rhizosphere, where the soil volume is influenced by living plant roots, the major source of

easily degradable C are rhizodeposits (Gregory, 2006). Rhizodeposition leads to a proliferation of microorganisms and consequently to changes in the fluxes allocation patterns of C through the decomposer system (Kuzyakov and Cheng, 2001; Kuzyakov, 2002b). Roots release a broad range of compounds, such as sugars, amino and aromatic acids, proteins and enzymes to attract beneficial organisms in the rhizosphere (Badri *et al.*, 2009). Labile compounds released by living roots or by lysis of root cells stimulate microbial activity (Nannipieri *et al.*, 2012) and microbial growth (Panikov, 1995; Oger *et al.*, 2004; Blagodatskaya *et al.*, 2009) in a similar way as exudates (Kuzyakov and Domanski, 2000; Marschner *et al.*, 2004). On the one hand, labile soil C inputs can regulate decomposition of more recalcitrant soil C by controlling the activity and relative abundance of fungi and bacteria (de Graaff *et al.*, 2010). On the other hand, microbial biomass, activity and composition are strongly affected by a diverse micro-, meso- and macro-fauna (Bonkowski et al., 2000; Scheu and Setälä, 2002; Ruess et al., 2016).

The detritusphere is characterized by high concentrations of easily degradable C sources, particularly at the early stages of residue decomposition (Poll *et al.*, 2008; Bastian *et al.*, 2009). Older substrate is mainly polymeric material of low availability for primary decomposers. Litter input is decisive for shaping the fungal communities in soils (Moll *et al.*, 2015). When litter is decomposed, C and nutrient pathways predominantly rely on the enzymatic capability of the major primary decomposers, able to degrade recalcitrant C sources (Dilly and Nannipieri, 2001). Especially, the soil-litter interface shows higher enzyme activities than the surrounding bulk soil (Kandeler *et al.*, 1999). Synergistic action of hydrolytic and oxidative enzymes is assumed when root litter is decomposed (Amin *et al.*, 2014).

1.3 Soil microbial activity indicators (Study 1–4)

Soil microbial communities consist of a wide range of organisms in different physiological states, such as active, viable, living, dormant, passive, dying, or dead (Johnsen *et al.*, 2001; Lennon and Jones, 2011; Blagodatskaya and Kuzyakov, 2013). Active microbes utilize the available susbtrate and therefore maintain biochemical transformations

(Blagodatskaya and Kuzyakov, 2013; Gunina *et al.*, 2014). The fraction of active microbes in hot spots is 2–20 times higher than in the bulk soil (Kuzyakov and Blagodatskaya, 2015). The total microbial biomass consists of only about 0.1–2% of active microorganisms without input of easily available substrates, whereas potentially active microorganisms contribute up to 60% of the total microbial biomass (Blagodatskaya and Kuzyakov, 2013). However, the mechanisms controlling the percentage of microbes being active are poorly defined.

The active state of soil microbes can be recognized by their ability to produce enzymes (Burns, 1982). Studies on enzyme activities have therefore strongly increased during the last decades (Sinsabaugh *et al.*, 1991; Allison and Vitousek, 2005; Marx *et al.*, 2005). The production of extracellular enzymes is regulated by nutrient availability and energy demand (Sinsabaugh *et al.*, 2009). Thus, enzyme activities are reliable microbial activity indicators and are closely interrelated with soil quality (Bending *et al.*, 2004; Paudel *et al.*, 2011).

Not only enzymes reflect microbial activity in soil, but also RNA:DNA ratio indicates the metabolic status of microorganisms. DNA is a biomolecule, which is associated with living organisms and thus serve as a microbial biomass indicator. The positive linear correlation between dsDNA content and total microbial biomass (Anderson and Martens, 2013) as well as the amount of RNA per cell, which is proportional to metabolic activity of microorganisms (Molin and Givskov, 2001), relating the RNA:DNA ratio to microbial performance.

Eco-physiological indices that reflect microbial C mineralization can be generated e.g. by relating physiological performances to the total microbial biomass per unit time (Anderson and Domsch, 1986; Anderson, 2003). For each of these approaches the total microbial biomass needs to be considered, as the capacity of a single species cannot be recognized individually (Anderson and Domsch, 1986).

Since the substrate input and its quality strongly depend on the C-content and the recalcitrance of the organic material, microbial activity and growth strongly differ between ecosystems. To test the effects of different substrate input (rhizodeposits vs. plant litter) on microbial activity, we conducted a manipulated field experiment with maize (Study

1−3). Moreover, microbial activity indicators (RNA:dsDNA) were determined in different soil types with contrasting C contents (Study 4).

1.4 Microbial loop – A driver of microbial activity? (Study 5)

Rhizodeposits and plant residues supply soil microbial communities substrate (Wardle, 1992), that potentially increase decomposition and nitrogen (N) release from SOM (Kuzyakov, 2002b; Chen *et al.*, 2007). Similar to the priming effect, (later termed Chapter I.1.5) protists which are the base of the heterotrophic soil food webs (Darbyshire, 1994), increase the available N pool in soil through the ingestion and destruction of bacterial cells and excretion of ammonia (Stout, 1980). Since N is a limiting nutrient in the rhizosphere (Kuzyakov and Xu, 2013), increased N availability in presence of protists stimulates plant growth through the so-called `microbial loop` network (Clarholm, 1985). Plant growth and root exudation may lead to higher microbial activity and higher SOM decomposition as well as N release (Gerhardson and Clarholm, 1986; Kuikman *et al.*, 1990). In particular, the rhizosphere provides up to 30-fold higher protist densities than the bulk soil (Zwart and Brussaard, 1991; Griffiths and Bardgett, 1997), and is therefore strongly top-down controlled (Bonkowski, 2004). Bacteria dominate the crucial hot spots for nutrient cycling and plant growth. Therefore, protozoan grazing affects not only microbial community composition, but overall ecosystem properties as well (Krome *et al.*, 2009; Rosenberg *et al.*, 2009). Despite the diverse feeding behavior of protists, they are mainly described as bacterial feeders in soil food webs (Ruiter *et al.*, 1995). However, also omnivorous and mycophagous feeding behavior was recently reported (Geisen and Rosengarten, 2015; Geisen *et al.*, 2016). There is still lack of knowledge on enzyme stystems, which occur intra- and extra-cellular of the cells of the targeted organisms (Additional research). Especially, the benefits for plant and microorganisms by mechanisms of protozoan predation are of high interest (Bonkowski and Clarholm, 2012). For this reason, we conducted a microcosm experiment to investigate the effects of protozoan grazing on C flux and enzyme activities in rhizosphere and detritusphere.

1.5 Effects of root hairs on microbial activity and rhizosphere priming (Study 6)

Root exudates and root associated microbial communities affect SOM decomposition in soil, a process termed *rhizosphere priming effect* (Kuzyakov, 2002a; Blagodatskaya *et al.*, 2007; Cheng, 2009). Consequently, root morphology and biomass affect rhizosphere priming (Kuzyakov, 2002a; Marschner *et al.*, 2002; Björk and Majdi, 2007). Root hairs, as an important part of root architecture`, play a crucial role in the rhizosphere (Gahoonia *et al.*, 1997; Gahoonia and Nielsen, 1998). For example, they increase water and nutrient uptake of the plant by the extension of the absorbing root surface (Jungk, 2001). Furthermore, root hairs are involved in multiple biogeochemical cycles (Gilroy and Jones, 2000). However, the effect of root hairs on microbial activity and rhizosphere priming has not been investigated. Therefore, a continuous labelling experiment was conducted in a greenhouse with additional light to determine the effect of root hairs on microbial activity and rhizosphere priming.

1.6 Objectives

In summary, the objectives of the present work were

1) to assess specific microbial growth rates and enzyme activities in the rhizosphere of maize (rhizodeposots vs. SOM) (Study 1).

2) to identify microbial activity by different indicators, such as microbial respiration, microbial biomass and enzyme kinetics in rhizosphere and detritusphere (rhizodeposits vs. litter-amended soil) (Study 2).

3) to evaluate depth gradients of microbial activity indicators, including CO_2 and N_2O, and to compare several enzyme indexes with depth in rhizosphere and detritusphere (Study 3).

4) to elucidate the suitability of the RNA:dsDNA ratio as an indicator of the physiological state of microorganisms in different soil types (Study 4).

5) to investigate the effects of Acanthamoeba grazing on C flux and enzyme activities in rhizosphere and detritusphere (Study 5).

6) to assess the influence of root hairs on microbial activity and rhizosphere priming (Study 6).

7) to examine growth rates during predator-prey interaction and to determine intra- and extra-cellular enzyme properties of protists (Additional research II.7.1).

8) to estimate the fungal activity in model systems in order to calculate the C budget of microorganisms added as preys to the soil based on $\delta^{13}C$ of microbial biomass, DOC, SOM and CO_2 (Additional research II.7.2).

9) to identify the C and N resources fueling microbial-protozoan interactions and plant uptake (N) by adding $^{13}C/^{15}N$ labelled *Lolium perenne* root litter to the system (Additional research II.7.3).

10) to examine microbial activity and rhizosphere priming effect in top- and subsoils by continuous isotopic labelling approach (Additional research II.7.4).

11) to determine the effect of glucose addition on the distribution of leucine-aminopeptidase activity in the rhizosphere in situ by soil zymography (Additional research II.7.5).

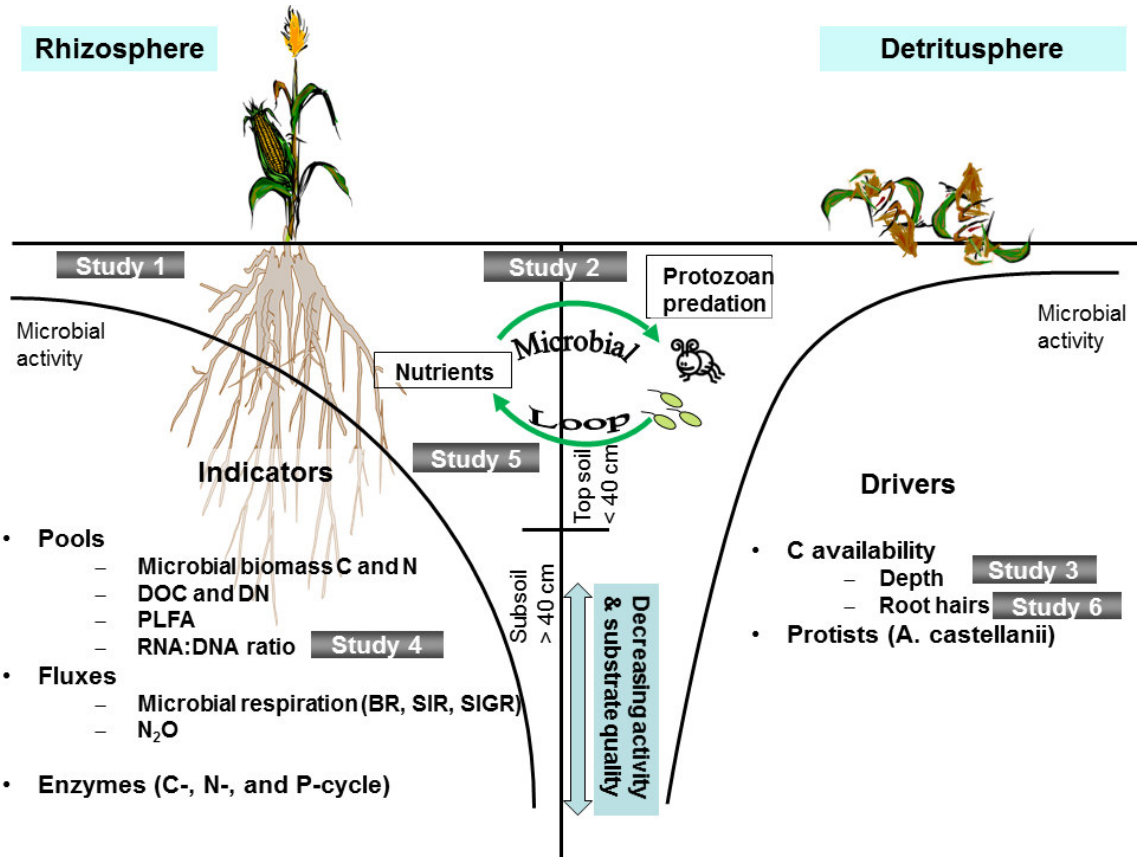

Figure I.1/1 Thematical overview of studies; indicators and drivers of microbial activity in rhizosphere and detritusphere

2. Material and Methods

2.1 Experimental sites and designs

2.1.1 Agricultural field site (Study 1–3)

In spring 2012, 12 experimental field plots (5 m x 5 m) were established on an arable loamy haplic Luvisol located on a terrace plain of the Leine River in central Germany (Holtensen) (Table I.2/1; Pausch, 2012; Kramer *et al.*, 2013). The area features a temperate climate with a long-term annual mean precipitation of 645 mm and an air temperature of 8.7 °C (Kramer *et al.*, 2012). Three treatments – rooted, litter amended and fallow soil – were set up on the experimental plots, with 4 replicates each. We separated the plots from each other by buffer stripes of 2 m and 6 m in row and inter-row, respectively. Soil samples were taken in July, 2012 and 2013 down the soil profile (each 10 cm down to 50 cm and 60−70 cm).

Table I.2/1 Soil properties (±SEM) of the loamy haplic Luvisoil determined before the start of the experiment (Kramer et al., 2012; Pausch et al., 2012). Significant differences are indicated by letters ($P < 0.05$).

Horizon	Depth [m]	Texture clay/silt/sand [% (w/w)]	pH [$CaCl_2$]	SEM	bulk density [g cm^{-3}]	SEM	C_{org} [g kg^{-1}]	SEM	N_{total} [g kg^{-1}]	SEM	C/N
Ap1	0–0.25	7.0/87.2/5.8	6.0 a	0.1	1.38 a	0.0	12.4 a	0.4	1.3±0.0 a	0.0	9.8 a
Ap2	0.25–0.37	7.1/87.8/5.0	6.2 a	0.1	1.61 b	0.0	6.9 b	1.2	0.8±0.1 b	0.1	9.2 a
Btw1	0.37–0.65	7.1/87.7/5.1	6.6 b	0.1	1.55 c	0.0	3.3 c	0.5	0.4±0.0 c	0.0	8.9 ab
Btw2	>0.65	6.8/88.4/4.8	7.0 c	0.1	1.68 b	0.0	1.8 c	1.8	0.3±0.0 c	0.0	6.9 b

Figure I.2/2 Maize planted field plot.

2.1.2 Soils along a climatic gradient (Study 4)

The RNA:dsDNA ratios were determined in top 10 cm-layers of five soils located in european part of Russia: Gleyic Retisol, Luvisol, virgin and arable Chernozem and Haplic Calcisol (IUSS Working Group WRB, 2015). Retisol was sampled at the bottom (accumulative) part of the slope in Tver Region, and the Luvisol at the top (autonomous) part of the slope at the right bank of the Oka River near the town Pushchino in Moscow region. Chernozem was sampled in Russian Federal Nature Preserve "Kamennaya Step" located in Talovsky District in Voronezh Region, in the watershed of rivers Bitug and Khoper. Calcisol was sampled in the Astrakhan region (Figure I.2/3).

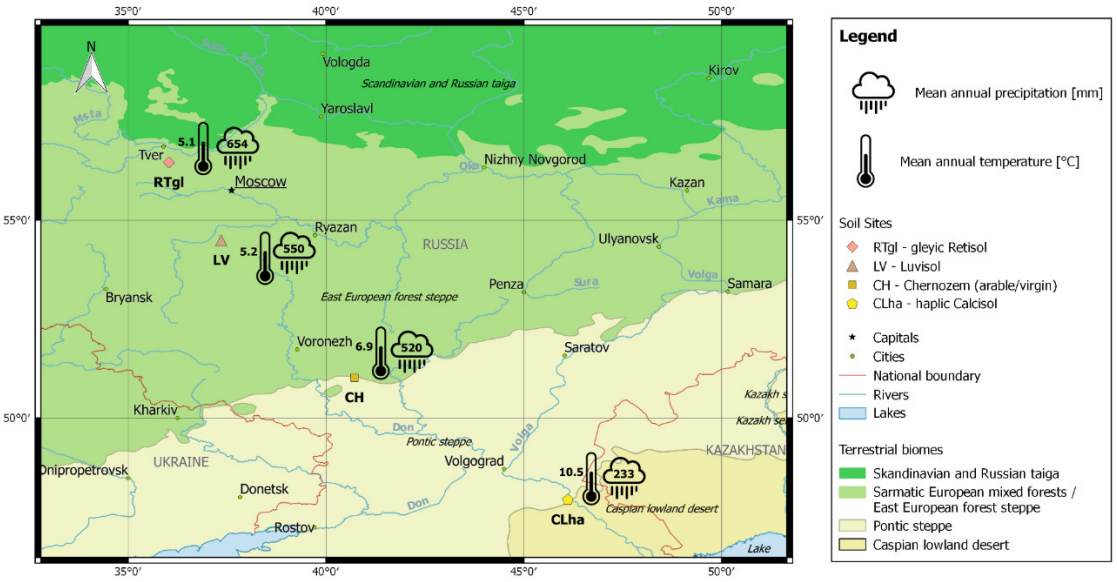

Figure I.2/3 Map of Russia – Area of interest on a scale of 1:4,400,000 including climate data, sample sites and terrestrial biomes. Created with QGIS 2.8.1-Wien (WGS 84, EPSG-Code: 4326) Icon "Thermometer" made by Yannick [http://yanlu.de] from http://www.flaticon.com; Icon "Rain" made by Yihsuan Lu [https://thenounproject.com/Yihsuanlu/] from http://thenounproject.com

2.2 Isotope technologies

2.2.1 Triple-labelling/Growth chamber (Study 5)

The decomposition of plant derived, below-ground C sources results in two major pathways based on 1) root litter, and 2) rhizodeposits (especially exudates). The amount and quality of substrates entering, affect microbial processes in the rhizosphere and detritusphere. Furthermore, soil fauna has important functions in regulating microbial activity and enzymatic substrate utilization. To identify specific drivers of microbial activity, such as increased exudation due to protozoan predation (additional N supply), we established a triple-labelling (^{13}C, ^{14}C and ^{15}N) experiment. This allowed the identification of C resources (rhizodeposited C by ^{14}C and root litter by ^{13}C) that fuel microbial-protozoan interactions in both soil hot spots: rhizosphere and detritusphere. Soil was taken from the same arable field presented above, autoclaved and re-inoculated with a microbial community previously extracted from this soil. The following treatments were established: 1)

no addition of plant C, 2) addition of sterilized ^{13}C /^{15}N-labelled root litter, representing detritusphere 3) growing maize plants, representing rhizosphere. Results on ^{14}C are presented in study 5, those on ^{15}N in the additional research.

Figure I.2/4 $^{14}CO_2$ pulse labelling of maize with belowground soil microcosms

2.2.2 Continuous labelling/Greenhouse (Study 6)

To investigate the effects of root hairs on microbial activity and rhizosphere priming, a ^{13}C-labelling experiment was conducted (Figure I.2/5). Soil samples were taken from the upper 30 cm of a sandy loam (Mollisol) at an arable field site on the campus reserves of the University of California, Santa Cruz. The soil contained 1.18±0.01% organic C and 0.13±0.001% N, had $\delta^{13}C$ and $\delta^{15}N$ values of -26.45±0.07‰ and 7.12±0.02‰, respectively, and a pH value of 5.8. Two barley types, a wild type and a root-hairless mutant called bald root barley (Gahoonia *et al.*, 2001), were grown in a greenhouse and were continuously labelled with ^{13}C depleted CO_2 (Cheng and Dijkstra, 2007).

Figure I.2/5 Two barley (*Hordeum vulgare* L.) types, a wild type (cv. optic; WT) and a root-hairless mutant grown in a greenhouse with continuous ^{13}C labelling device

2.2 Microbial activity indicators

Microbial activity indicators, used to address microbial activity in soil are described as follows:

Table I.2/2 Short description of soil microbial activity indicators and enzyme indexes

Microbial activity indicators	**Description**	**References**
Basal respiration	The basal respiration (BR) of soils is defined as the respiration without addition of organic substrate to soil at 22°C, which originates from the turnover of SOM. It is taken as an indicator of microbial activity and C turnover in soil. The rate of basal respiration reflects both the amount and quality of substrate.	Anderson and Domsch, 1978
SIR	Substrate-induced respiration (SIR) is the measurement of soil respiration in the presence of an added substrate. The initial maximal respiration rate induced by glucose is proportional to the size of the primal soil microbial biomass. SIR is a black-box method, not differentiating between distinct groups of microorganisms (e.g. bacteria vs. fungi). An advantage is the lower detection limit compared to CFE method, in case of scarce C sources.	Anderson and Domsch, 1978; Kaiser *et al.*, 1992; Lin and Brookes, 1999
BR:SIR ratio	The ratio between BR and SIR was used as a relative measure of microbial respiration. The ratio indicates the distribution between r and K transition in the substrate-responsive microbial biomass.	Odum, 1985; Stenström, 2001
SIGR	Quantification of the fraction of actively growing biomass, which is capable for immediate growth on added substrate. The model simulates the transition process of soil microorganisms from sustaining to the active state, i.e. lag phase and exponential phase of growth, due to the inclusion of the physiological state concept.	Panikov, 1995; Blagodatsky *et al.*, 2000; Wutzler *et al.*, 2012
Metabolic quotient	The metabolic quotient (CO_2 release/soil microbial biomass; q_{CO2}) reflects the availability of C used by the microorganisms and is inversely related to microbial efficiency.	Anderson and Domsch, 1986, 2010; Anderson, 2003

Table I.2/2 Short description of soil microbial activity indicators and enzyme indexes

Microbial activity indicators	Description	References
N_2O	Fungal and bacterial denitrification is a heterotrophic processes coupling the reduction of NO_3^-/NO_2^- with the oxidation of an electron donor, often organic C. Through stepwise reductions, denitrification ends with N gases, i.e., N_2O and/or N_2, depending on microbial taxa and environmental conditions.	Menyailo *et al.*, 2002; Henderson *et al.*, 2010; Chen *et al.*, 2015
Microbial biomass C	The soil microbial biomass responds much more quickly than most other soil fractions to changing environmental conditions such as changes in substrate inputs and is a more sensitive indicator of changing soil conditions than, e.g., the total SOM content.	Brookes *et al.*, 1985; Wu and Joergensen, 1990; Joergensen *et al.*, 2011
Microbial biomass N	Nitrogen made available through protein depolymerization and is rapidly taken up by microbes. The balance between protein depolymerization, N mineralization and nitrification reflects the degree of microbial N limitation.	Brookes *et al.*, 1985; Kaiser *et al.*, 2011; Wild *et al.*, 2015
β-glucosidase	β-glucosidase is acting in the cleavage of cellobiose into glucose molecules.	Deng and Tabatabai, 1994; Tabatabai, 1994
β-xylosidase	β-xylosidase is responsible for the breakdown of hemicelluloses.	German *et al.*, 2011
β-cellobiohydrolase	β-cellobiohydrolas is responsible for consecutive stages of cellulose degradation.	Marx *et al.*, 2005; German *et al.*, 2011
Chitinase	Chitin is composed of linked amino sugar subunits and occurs associated to other structural polymers such as proteins or glucans, which often contribute more than 50% of the mass in chitin-containing tissue.	Gooday, 1990; Beier and Bertilsson, 2013
Acid phosphatase	Acid phosphatase mineralizes organic P into phosphate by hydrolyzing phosphoric (mono) ester bonds under acidic conditions.	Eivazi and Tabatabai, 1977
Leucine-aminopeptidase Tyrosine-aminopeptidase	Activities of leucine-and tyrosine-aminopeptidase are responsible for the hydrolysis of L-peptide bonds.	Kourtev *et al.*, 2003; Štursová and Baldrian, 2011

Table I.2/2 Short description of soil microbial activity indicators and enzyme indexes

Enzyme indexes	**Description**	**References**
Specific enzyme activity	Ratio of potential activity to microbial biomass	Trasar-Cepeda *et al.*, 2008; Stone *et al.*, 2014
Catalytic efficiency	Catalytic properties of enzymes (V_{max}/K_m)	Koshland, 2002; Moscatelli *et al.*, 2012; Loeppmann *et al.*, 2016
V_{max} ratio of C- to N- and C- to P-cycling enzymes	Relative activities of C vs. N and C vs. P acquiring enzymes	Sinsabaugh *et al.*, 2008
Proportions of C- to N- and C- to P-cycling enzymes	Proportional enzyme activities of C vs. N and C vs. P acquiring enzymes	Moorhead *et al.*, 2013; Hill *et al.*, 2014
Vector length	Relative C vs. nutrient acquisition	Moorhead *et al.*, 2013, 2016
Vector angle	Relative P vs. N limitation	Moorhead *et al.*, 2013, 2016

3. Main results

The methodological novelty and outcome of studies 1−6 are presented in Table I.3/3. We elucidated microbial processes in two soil hot spots: rhizosphere and detritusphere, and determined various microbial activity indicators with soil depth (Table I.3/4). Moreover, we exhibited the RNA:dsDNA ratio in different soil types along a climatic gradient (Figure I.2/3). The drivers of microbial activity were investigated on smaller scales (growth chamber and greenhouse).

Table I.3/3 Synthesis of methodological innovations and main innovative results of Studies 1–6

Study	Aims	Type of study	Methodological innovations	Main innovative results
1	Determination of specific microbial growth rates and enzyme activities in rhizosphere and bare fallow soil	Field	V_{max}:dsDNA ratio as an alternative to specific enzyme activities, constituted a convenient microbial activity indicator	Similar specific microbial growth rates and microbial biomass contents were demonstrated for rooted vs. root-free soil when we compared the two soil layer. Active microbial biomass increased by 17-fold in the rhizosphere at 10–20 cm depth compared to the upper 10 cm
2	Identification of microbial activity by different indicators, such as microbial respiration, microbial biomass and enzyme kinetics with contrasting substrate availability	Field	Metabolic respiratory response methods in combination with enzyme kinetics were determined	Substrates with contrasting availability (rhizodeposits vs. plant residues) changed functional properties of the soil microbial community and induced a shift in enzyme systems

Table I.3/3 Synthesis of methodological innovations and main innovative results of Studies 1–6

Study	Aims	Type of study	Methodological innovations	Main innovative results
3	Assessment of depth gradients of microbial activity indicators, including CO_2 and N_2O in rhizosphere and detritusphere.	Field	Enzyme indexes down the soil profile based on enzyme kinetics and vector analyses	The catalytic efficiency of enzymes decreased 2- to 20-fold from top- (< 40 cm) to subsoil (> 40 cm), irrespective of the substrate input.
4	Testing the capability of RNA:dsDNA ratio as an indicator of microbial activity	Soil types along a climatic gradient	Broad range of soil types	RNA:dsDNA ratios towards the indication of the metabolic status of soil microbial communities adheres to biased RNA quantity due to high clay contents.
5	Effects of Acanthamoeba grazing on C flux and microbial activity indicators in rhizosphere and detritusphere	Growth chamber	Sophisticated experimental setup under sterile conditions, triple labelling experiment	C fluxes and enzyme activities were driven by substrate input and quality in the rhizosphere and detritusphere and further stimulated by faunal grazing.
6	Effect of root hairs on microbial activity and rhizosphere priming	Greenhouse + additional lightning	Continuous ^{13}C-labelling of barley	Root hairs induced positive priming during tillering. Without root hairs SOM decomposition was suppressed. In case of positive priming, the chitinase and ß-xylosidase activities increased indicating decomposition of stable SOM.

Shifts in microbial growth strategy, upregulation of enzyme production and increased microbial respiration demonstrated strong root effects in maize planted soil (Study 1). In the rhizosphere the specific microbial growth rates decreased by 42% at 10–20 cm depth compared to the surface-layer. This suggests the dominance of highly active but slower growing microbes with depth, reflecting also their slower turnover. This shift in enzyme

systems with depth due to resource scarcity and lower substrate quality is decisive for microorganisms to benefit from their costs of energy investments (Allison *et al.*, 2011; Stone *et al.*, 2014).

The availability of C and nutrients in the soil affected the efficiency of enzymes mediating the catalytic reaction, especially in the presence of roots. Substrates of contrasting quality (e.g. root exudates, plant residues) changed functional properties of the soil microbial community and induced a shift in enzyme systems (Study 2). The microbial N demand increased in the rhizosphere (Study 1, 2, 3), which boosted proteolytic enzyme activities (e.g. leucine-aminopeptidase) and hampered microbial growth in rooted soil (Study 1).

Accordingly, N_2O dissimilation and dissolved N were reduced in the rhizosphere compared to the detritusphere. This again reflects increased N uptake into microbial cells by direct substrate incorporation or after extracellular proteolytic degradation of the substrate (Study 3). In case additional N was available in the rhizosphere due to protozoan excretion of ammonia (Study 5) or due to the amendment of mineral N, most enzyme activities increased (Additional research).

Most microbial activity indicators were higher in the rhizosphere than in bare fallow soil (Table I.3/4). This increase in microbial activity was significant only in the topsoil, whereas in the subsoil, both rhizosphere and detritusphere were comparable to fallow soil (Study 3). In general, the RNA:dsDNA ratios showed soil-specific patterns. However, caution should be paid to the RNA extraction, as the RNA content was strongly affected by the clay content of the soil (Study 4).

We investigated the potential drivers of microbial activity by isotopic labelling and various activity indicators. Higher uptake of root-derived C into the microbial biomass with amoebaean predation than without amoeba, reflected preferred substrate utilization of freshly plant-derived C sources due to enhanced root exudation (Study 5). In the presence of amoeba the plant uptake of ^{15}N was higher in the shoots relative to the roots (Additional research). The higher N investments to the shoots showed that the plant benefits by the additional N pool in the soil through protozoan excretions. Consequently, the plant increases its growth and enhances root exudation. Microbial activity indicators such as

enzyme systems, which are essential factors of microbial decomposition mechanisms in soil, implied differential susceptibility of microbes on Acanthamoeba grazing.

Root hairs, as potential drivers of microbial activity affected enzyme activities at tillering stage (Study 6). The SOM-derived CO_2 was higher for the barley wild type (with root hairs) than for the hairless barley mutant, while root-derived CO_2 did not differ between the two types. This was in accordance to higher chitinase and ß-xylosidase activities reflecting enhanced microbial mineralization and extracellular enzyme degradation of recalcitrant C sources.

Table I.3/4 Microbial activity indicators on contrasting substrate amount and substrate quality

Microbial activity indicators	Rhizosphere	Detritusphere	Depth (rhizosphere and/or detritusphere		Study	Site
	compared to bare fallow soil		From 0–10 to 10–20 cm	From top- to subsoil		
Fluxes						
Basal respiration	↑	–	↓	↓	2	field
SIR	↑	–	↓	↓	2, 3	field
BR:SIR ratio	–	–	–	n.d.	2	field
SIGR	–	n.d.	↓	n.d.	1	field
Metabolic quotient	–	–	–	n.d.	2	field
N_2O	↓	–	↓	↓	3	field
Pools						
Microbial biomass C	↑	–	↓	↓	2, 3	field
Microbial biomass N	↑	–	↓	↓	2, 3	field
Dissolved organic C	–	–	↓	–	2, 3	field
Dissolved N	↑/↓*	–	↓/–*	↓	2, **3	field
Enzyme activities						
β-glucosidase	↑	–	↓	↓	2, 3, 5	field, growth chamber
β-xylosidase	–/↑*	–/↑*	–	↓	3, *5	field, growth chamber
β-cellobiohydrolase	–	↑	↓	↓	2, 3	field
Chitinase	↑	↑	n.d.	n.d.	1, 5	growth chamber
Acid phosphatase	↓/↑*	↓/–*	↓	↓	2,3,**5	field, growth chamber
Leucine-aminopeptidase	↑	–	↓	↓	1, 3, 5	field, growth chamber
Tyrosine-aminopeptidase	–/↑*	–/↑*	↑	↓	1, 3, *5	field, growth chamber

n.d.: not determined, respectively not shown in the studies; ↑: increase (P<0.05); ↓: decrease (P<0.05); –: not significant relative to fallow soil

Drivers of microbial activity were reflected by most of the indicators measured (Table I.3/5), for example, by the enhanced incorporation of root-C and the increased enzyme activities during protozoan predation (Study 5). Highest plant density confirmed with highest beta-cellobiosidase in top- and subsoil but this pattern was not consistent among all enzymes (Additional research).

Table I.3/5 Soil microbial activity drivers and their sensitivity

Drivers	**Microbial indicators**									**Soil type and Study**
	Fluxes		**Pools**				**Enzymes´properties**			
	Respiration	N_2O	CFE-derived MBC	dsDNA	RNA	EOC	EN	Vmax	Vmax/Km	
Decreasing depth	↓	↓	↓	↓	↘	↘	↘	↓	↓	Luvisol Study 3
Amoeban and Colembolan predation	↘	↗	↓	↓	↘	–/↓	–	↑	↓	(Autoclaved) Luvisol Study 5 + Add. research
Root hairs	↑	↗	–	↑	↑	–	–	↓/↑	↘	Mollisol Study 6
Plant density	↑	↗	↑	↗	↗	↑	↑	↑	↘	Mollisol + Add. research
Climatic gradient	↘	↗	↓	↓	-	↘	↘	↘	↘	Chernozem, Retisol, Luvisol, Calcisol Study 4

↓ = strong decrease; ↑ = strong increase; ↘ = weak decrease; ↗ = weak increase; orange arrows indicated suggestions

4. Conclusions

Available C sources such as root C and litter C stimulated not only microbial activity, but also turnover of MBC. Most of the tested microbial activity indicators indicate microbial C and nutrient demand in rhizosphere and detritusphere. For example, the proportions of multiple enzyme activities and catalytic efficiencies reflected both stoichiometric and C quality effects on decomposer communities. The combination of methods such as the quantification of RNA and DNA, microbial respiration, and enzymes allowed to draw a detailed picture of microbial activity in rhizosphere and detritusphere.

In conclusion, the substrate availability and the stimulation of microbes by micro-fauna were identified as important drivers of microbial activity in the rhizosphere. Especially, root hairs appeared as crucial driver of microbial activity during the tillering, whereas at head-emergence stage this effect disappeared. Special emphasis should be placed on potential mechanisms linking root morphology and microbial activity with rhizosphere priming effects.

Overall this thesis contributes to disentangle complex and highly dynamic microbial food web interactions in soil. The presented results underline the role of microorganisms as critical links in the C and nutrient transfer in rhizosphere and detritusphere as well as in deeper soil layers.

5. Contributions to the included manuscripts

The PhD. thesis comprises 5 published papers and 1 paper which is in preparation. The co-authors listed on the manuscripts contributed as follows:

Study 1: Substrate quality affects microbial- and enzyme activities in rooted soil

Status: Published in *Journal of Plant Nutrition and Soil Science* (2015). DOI: 10.1002/jpln.201400518 (Focus Issue: Soil hot spots)

S. Loeppmann:	60% (experimental design, accomplishment of experiment, laboratory analyses, data preparation and interpretation, manuscript preparation)
M. Semenov:	15% (laboratory analysis, data preparation)
E. Blagodatskaya:	15% (discussion of experimental design and results, comments to improve the manuscript)
Y. Kuzyakov:	10% (comments to improve the manuscript)

Study 2: Substrate quality affects kinetics and catalytic efficiency of exo-enzymes in rhizosphere and detritusphere

Status: Published in *Soil Biology and Biochemistry* (2016). **92**: 111–118

S. Loeppmann:	70% (accomplishment of experiment, field sampling, laboratory analyses, data preparation and interpretation, manuscript preparation)
E. Blagodatskaya:	10% (discussion of results, comments to improve the manuscript)
J. Pausch:	10% (comments to improve the manuscript)
Y. Kuzyakov	10% (comments to improve the manuscript)

Study 3: Enzyme properties throughout the soil depth – A matter of substrate quality in rhizosphere and detritusphere

Status: Published in *Soil Biology and Biochemistry* (2016). **103**: 274–283

S. Loeppmann: 65% (accomplishment of experiment, sampling, laboratory analyses, data preparation and interpretation, manuscript preparation)

E. Blagodatskaya: 15% (discussion of results, comments to improve the manuscript)

J. Pausch: 10% (sampling, comments to improve the manuscript)

Y. Kuzyakov 10% (comments to improve the manuscript)

Study 4: Towards to physiological status of soil microorganisms determined by RNA:dsDNA ratio

Status: In preparation for submission to *Ecological Indicators*

S. Loeppmann: 50% (laboratory analysis, data preparation and interpretation, manuscript preparation, comments to the manuscript)

M. Semenov: 30% (accomplishment of experiment, sampling, laboratory analysis, manuscript preparation)

E. Blagodatskaya: 10% (discussion of results, comments to improve the manuscript)

Y. Kuzyakov: 10% (comments to improve the manuscript)

Study 5: Effects of Acanthamoeba grazing on carbon flux and enzyme activities in rhizosphere and detritusphere

Status: In preparation for submission to *Plant and Soil*

S. Loeppmann: 50% (accomplishment of experiment, sampling, laboratory analyses, data preparation and interpretation, manuscript preparation)

F. Clissmann: 15% (accomplishment of experiment, sampling, comments to the manuscript)

A. Gunina: 10% (laboratory analysis, comments to the manuscript)

J. Pausch: 10% (discussion of results, comments to the manuscript)

R. Koller: 5% (comments to improve the manuscript)

M. Bonkowski: 5% (comments to improve the manuscript)

Y. Kuzyakov: 5% (comments to improve the manuscript)

Study 6: Effect of root hairs on rhizosphere priming

Status: Published in *Soil Biology and Biochemistry* (2016)

J. Pausch: 45% (accomplishment of experiment, sampling, laboratory analyses, data preparation and interpretation, manuscript preparation)

S. Loeppmann: 25% (sampling, laboratory analyses, data preparation, discussion of results, comments to improve the manuscript)

A. Kühnel: 10% (sampling, laboratory analysis)

J. Forbush: 10% (sampling, laboratory analysis)

Y. Kuzyakov: 5% (comments to improve the manuscript)

W. Cheng: 5% (comments to improve the manuscript)

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II. Manuscripts

1. Substrate quality affects microbial- and enzyme activities in rooted soil

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Published in *Journal of Plant Nutrition and Soil Science* (2016) 179, 39–47 (Focus Issue: Soil hot spots)

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Abstract

The rhizosphere reflects a sphere of high substrate input by means of rhizodeposits. Active microorganisms and extracellular enzymes are known to be responsible for substrate utilization in soil, especially in rooted soil. We tested for microbial- and enzyme activities in arable soil, in order to investigate the effects of continuous input of easily available organics (e.g. root-exudates) to the microbial community. In a field experiment with maize, rooted and root-free soil were analyzed and rhizosphere processes were linked to microbial activity indicators, such as specific microbial growth rates and kinetics of six hydrolytic extracellular enzymes: β-glucosidase, β-cellobiohydrolase, β-xylosidase, acid phosphatase, leucine- and tyrosine-aminopeptidase.

Higher potential activities of leucine-aminopeptidase (2-fold) for rooted vs. root-free soil, suggested increased costs of enzyme production, which retarded the specific microbial growth rates. Total microbial biomass determined by the substrate-induced respiration technique and dsDNA extraction method was 23% and 42% higher in the rooted surface-layer (0–10 cm) compared to root-free soil, respectively. For rooted soil, potential enzyme activities of β-glucosidase were reduced by 23%, acid phosphatase by 25% and increased by 300% for β-cellobiohydrolase at 10–20 cm depth compared to the surface-layer. The actively growing microbial biomass increased by 17.4-fold in rooted soil in the 10–20 cm layer compared to the upper 10 cm. Despite the specific microbial growth rates showing no changes in the presence of roots, these rates decreased by 42% at 10–20 cm depth compared to the surface-layer. This suggests the dominance in abundances of highly active but slower growing microbes with depth, reflecting also their slower turnover. Shifts in microbial growth strategy, upregulation of enzyme production and increased microbial respiration indicated strong root effects in maize planted soil.

Key words: Microbial activity, microbial biomass, specific enzyme activity, specific microbial growth rates, dsDNA

1.1 Introduction

The rhizosphere is considered as one of the most important microbial hotspots, more precisely a hot sphere in soil as it is characterized by high microbial abundance and activity due to high amounts and diversity of easily available substrates (*Hinsinger* et al., 2005; *Walker* et al., 2003). There are three main sources of substrate input to the rhizosphere: 1) root exudates released from intact cells, 2) lysates of sloughed-off cells and root tissue, and 3) mucilage (*Gregory,* 2006; *Neumann* and *Römheld*, 2007). These forms of root derived C is frequently termed rhizodeposition. Root exudates are readily available sources of C and energy for microbes (*Haichar* et al., 2008; *Paterson,* 2003; 2007).

The release of labile compounds (including enzymes) by living roots or by lysis of root cells stimulates microbial activity (*Nannipieri* et al., 2012) and microbial growth (*Blagodatskaya* et al., 2009; *Oger* et al., 2004; *Panikov,* 1995) in the similar ways as rhizodeposits (*Kuzyakov* and *Domanski*, 2000; *Marschner* et al., 2004). The release of root exudates and other rhizodeposits is ongoing, and is localized in soil (*Pausch* and *Kuzyakov*, 2011). Consequently, localization of easily available C produces hotspots of microbial abundance and activities, frequently termed as the "rhizosphere effect" (*Lynch*, 1997; *Sørensen,* 1997).

It is thought that the production of extracellular enzymes is regulated by nutrient availability and energy demand (*Sinsabaugh* et al., 2009). Therefore, extracellular enzyme activities in the rhizosphere are generally higher compared to root-free soils, similarly to total microbial biomass and microbial activity measured as respiration or growth rates (*Badalucco* and *Nannipieri*, 2005). Roots and associated mycorrhizal community are known to be major producers of β-glucosidases and acid phosphatases (*Conn* and *Dighton*, 2000). Despite soil enzymes being partly of plant origin, the microorganisms are the main source of enzymes mediating the cycling of main nutrients (C, N, P and S) (*Aon* et al., 2001) and thus, enzyme activity is frequently proportional to microbial biomass (*Frankenberger* and *Dick*, 1983). Hence, overall greater microbial biomass and higher enzyme activity can be predicted not solely in the rhizosphere but in a whole soil

layer with high root density, e.g. in rooted soil as compared with soil without plants, e.g. in bare fallow soil.

The upper 30 cm contain 70–90% of the root biomass of maize (*Amos* and *Walters*, 2006), where available C sources induce activity of numerous microbial groups, which are usually limited by N. Nutrient limitation for roots and microorganisms in the rhizosphere is far greater than in root-free soil. This leads to strong competition between roots and microorganisms for nutrients (*Kuzyakov* and *Xu,* 2013; *Paterson*, 2003). Hence, the rhizosphere is not only a hotspot of microbial activity, but also a hotspot of plant-microbial interactions including competition, resulting not only in acceleration but under specific conditions also in retardation of microbial growth (*Blagodatskaya* et al., 2014b).

As microbial communities allocate resources to enzyme production in relation to substrate availability and growth requirements to reduce costs and maximize their resource returns (*Allison* and *Vitousek*, 2005), we hypothesized that specific microbial growth rates increase in rooted soil compared to the fallow control. We suggest plant induced lower inorganic N contents in the soil compared to fallow control increase peptidases activities (*Stursova* et al., 2006). We further hypothesized that enzyme activity per unit microbial biomass (e.g. specific activity) would increase from 0–10 to 10–20 cm, reflecting greater microbial allocation to C-cycling enzyme production depending on decreased C availability (*Allison* et al., 2011).

These hypotheses were tested in a multi-factorial field manipulation experiment with soil sampled under maize (rooted soil) and bare fallow at two depths (0–10 and 10–20 cm). Potential enzyme activities and soil microbial biomass were measured. Microbial growth were determined by kinetic approach, due to substrate-induced respiratory response of microorganisms, enabling estimation of total and growing biomass of the glucose-consuming part of microbial community (*Panikov*, 1995; *Panikov* and *Sizova*, 1996). We used substrate-induced respiration (SIR) (*Anderson* and *Domsch*, 1978) and substrate-induced growth respiration (SIGR) of microbial cells. By combining these methods we were able to investigate microbial activity in the rhizosphere in order to elucidate the effects of rhizodeposits on microbial activity.

1.2 Materials and Methods

1.2.1 Study site

The experimental agricultural field is located on the terrace plain of the river Leine in the north-west of Göttingen (Lower-Saxony), Germany (51°33´N, 9°53´E; 158m NN). The area has a temperate climate with a long-term annual mean precipitation of 645 mm and an air temperature of 8.7 °C. The dominant soil types are Luvisols.

In spring 2012, 12 experimental field plots (5 x 5 m) were established and separated from each other by buffer stripes of 2 m and 6 m in row and inter-row, respectively. Two treatments, rooted (P) and root-free (F) soil were set up on the experimental plots, with 4 replicates each. For rooted soil, hybrid maize (*Zea mays L*, Codisco/TMTD 98% Satec) was sown in April 2013 on 4 plots at a density of twelve plants per square meter. In addition 4 plots remained unplanted as a bare fallow control. The fallow control plots were shaded with blinds (mechanical shading 50% and 80%; Accura NTV oHG, Heidenheim). To accomplish similar environmental conditions between the plots, the shading level represented a mean leaf area index of plants during the vegetation period.

1.2.2 Sampling and preparation

In July 2013, we sampled the soil at two depths (0–10 cm, 10–20 cm) for each plot. The field moist soil samples were frozen at –18°C until the analyses. Freezing is known to influence the enzyme activities of extracellular hydrolytic enzymes (*Gianfreda* and *Ruggiero*, 2006; *Lee* et al., 2007). Following the study of *German* et al. (2011) we considered, however, that freezing would not affect the comparability of rooted versus root-free soil as all soil samples were frozen and treated similarly.

Prior to the analyses, the soil samples were thawed in the refrigerator, sieved (< 2 mm) and fine roots and other plant debris were carefully removed with tweezers. The sieved field moist soil samples were pre-incubated for 72 h at 22°C. Soil sub-samples of each plot and depth were dried at 105 °C (24 h) to determine the soil carbon (C_t), nitrogen (N_t)

and moisture contents. The moisture contents of the soil samples ranged from 14% for rooted to 18% for fallow soil. Prior analyses the moisture content was adjusted to 60% of the water holding capacity (WHC). No significant differences were detected in pH, C_t, or N_t content of rooted and root-free sampled soil.

The measurements of microbial respiration, such as SIR and SIGR were used to determine microbial biomass and active microbial biomass as well as microbial growth rates in rooted vs. root-free soil to exhibit the responses of microbes to root exudation. Additionally, we determined the dsDNA-extracted microbial biomass C for validation. The potential hydrolytic extracellular enzyme activities were determined in order to elucidate enzyme production strategies of microorganisms due to substrate decomposition.

1.2.3 dsDNA extraction and quantification procedure

Total soil DNA was extracted by the FastDNA® SPIN kit for soil (MP Biomedicals, Germany). Extraction procedure was carried out according to the manufacturer's protocol with 0.5 g of pre-incubated soil. The method of DNA isolation involved bead beating procedure and binding of DNA to the silica matrix. Before extraction, soils were placed into a freezer overnight to ensure higher DNA yields. Thereafter, soils were added to lysing tubes, treated with lysis buffer, subjected to bead beating in the FastPrep® instrument and processed by protein precipitation solution. DNA was bound to a silica matrix, washed, and eluted in DNase-free water.

The quantity of dsDNA extract was determined by preparing a 150-fold dilution of the extract in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Aliquots of 0.1 ml were then transferred to 96-well microplates (Brand pureGrade, black). For labeling the dsDNA a 200-fold dilution of the dsDNA fluorescence stain PicoGreen® (Molecular Probes, Life Technologies, Germany) was prepared in plastic containers. The dye (0.1 mL) was added to each well with diluted DNA extract (final 300-fold dilution) and left to react at room temperature protected from light for 2 min. Fluorescence intensity was measured with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland) of excitation 485 nm, emission 525 nm and measurement time 1.0 s. Afterwards, the dsDNA yield was determined immediately after extraction and expressed

as µg dsDNA g^{-1} dry weight of soil. The dsDNA of bacteriophage lambda (Molecular Probes, Life Technologies, Germany) was used as a standard. Samples for the standard curve were prepared in TE-buffer in the same way as the experimental samples (*Blagodatskaya* et al., 2014a). Conversion factor from dsDNA into microbial-biomass C (F_{DNA}) of 5.02 was used (*Anderson* and *Martens*, 2013). Microbial biomass was calculated as:

$$C_{mic}\ (\mu g\ g^{-1}\ soil) = F_{DNA} \times dsDNA\ (\mu g\ g^{-1}\ soil) \quad (1)$$

1.2.4 Enzyme assays

By the use of 4-methylumbelliferone-β-D-cellobioside, 4-methylumbelliferone-β-D-glucoside, 4-methylumbelliferone-phosphate, 4-methylumbelliferone-7-β-D-xyloside, L-leucine-7amino-4-methylcoumarin hydrochloride and L-tyrosine-7amino-4-methylcoumarin, the enzyme activities of β-cellobiohydrolase (exo-1,4-β-glucanase, EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.2), β-xylosidase (EC 3.2.2.27) and leucine-/tyrosine-aminopeptidase (EC 3.4.11.1) were determined, respectively. Half a gram field moist soil was added to 50 ml sterile water in autoclaved jars. Aliquots of 50 µl were withdrawn and dispensed in 96-well microplates (Brand pureGrade, black) while stirring the suspension. Buffer (80 ml) was added (0.1 M MES buffer, pH 6.1 for carbohydrases and phosphatase, 0.05 M TRIZMA buffer, pH 7.8 for leucine-/tyrosine-aminopeptidase) (*Marx* et al., 2005). Finally, 100 µl of series concentrations of substrate solutions (20, 40, 60, 80, 100, 200, 400 µmol substrate g $soil^{-1}$) were added to the wells. Plates were kept at 21 °C, agitated and measured fluorometrically (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland). Fluorescence was converted into an amount of MUB (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin), according to specific standards, which had been prepared in sub-samples from the various soil suspensions. The kinetic parameter, V_{max}, was estimated using non-linear regression techniques (Michaelis-Menten kinetics) (*Marx* et al., 2005). Each field replicate was measured as an analytical triplicate.

1.2.5 Substrate-induced growth respiration and calculation of growth parameters

The substrate induced growth respiration (SIGR) method was conducted in a climate chamber (16 °C). Therefore, 23 g of each pre-incubated and moistened (WHC 60%) soil sample was incubated in a microcosm after addition of the substrates and nutrients (*Blagodatsky* et al., 2000). The amended substrate mixture contained glucose (10 mg g^{-1}) and mineral salts, e.g. 1.9 mg g^{-1} $(NH_4)SO_4$, 2.25 mg g^{-1} K_2HPO_4 and 3.8 mg g^{-1} $MgSO_4$-$7H_2O$. Instead of talcum, a glucose solution was applied. Glucose was used, because it is one of the abundant components of root exudates (*Whipps* and *Lynch*, 1983; *Derrien* et al., 2004). Substrate concentrations, sufficient for unlimited exponential growth of microorganisms, were estimated in preliminary experiments in which different amounts of glucose and nutrients were added. The amount of mineral salts was selected so that the added substrate did not change the pH of soil (< 0.1). (*Blagodatskaya* et al., 2007). After addition of the substrate-nutrient mixture and stirring with a common, handheld kitchen blender, the soil samples were immediately placed into 24 flasks (394 cm^3) (*Anderson and Domsch*, 1978). A gas chromatograph (GC 6000 VEGA series 2, Carlo Erba instruments, UK) was modified for automatic sampling, measuring and calibration. The soil samples were kept in closed systems (microcosm) under quasi-stationary conditions and the evolved CO_2 was measured every 120 minutes.

According to *Wutzler* et al. (2012) equation (2) is effectively a three parameter equation when accepting the following assumptions. During unlimited growth, λ may be accepted as a basic stoiciometric constant of 0.9 (*Panikov* and *Sizova*, 1996). And second Y_{CO2} assumed to be constant 1.5 during the experiment (*Blagodatsky* et al., 2000). After *Panikov* (1995), the growth associated respiration is allowed to change with changing activity of microbial biomass, where μ_{max} is maximum specific growth rate, e.g. potential maximum of fully active cells, r_0 is the initial physiological state ($0 < r_0 < 1$), x_0 is the initial microbial biomass. However, we used the dsDNA derived microbial biomass C contents to reduce the parameter of equation (2) and calculated the specific growth rates.

$$p(t) = x_0 (1-r_0) \left(\frac{1}{\lambda} - 1 \right) \frac{\mu_{max}}{Y_{CO2}} + x_0 r_0 \frac{1}{\lambda} \frac{\mu_{max}}{Y_{CO2}} Exp^{\mu_{max} t} \quad (2)$$

In the beginning the curve is often dominated by adapting the physiological state of the microbial cells (bacteria and fungi), called the lag-phase. During this phase often only a weak increase in microbial biomass (*Wutzler* et al., 2012) and a linear increase in microbial respiration is observed (data not shown). The lag-phase (t_{lag}) was elucidated as the time interval from substrate amendment to the moment when the increasing rate of growth-related respiration (B) became as high as the rate of respiration uncoupled from the growth of microorganisms (A).

$$t_{lag} = \frac{(A/B)}{\mu_{max}} \qquad (3)$$

The following unlimited exponential growth phase is dominated by a growing, active microbial biomass. More complete theoretical background and details on equations derivations were described elsewhere (*Blagodatsky* et al., 2000; *Panikov,* 1995; *Wutzler* et al., 2012).

1.2.6 Substrate-induced respiration

The substrate-induced microbial respiration (SIR) method provides a parameter for the potentially active microbial biomass without any growth of microbial cells based on respiration measurements following the addition of glucose and mineral salts as it is already explained for SIGR. The same amount of soil was incubated in flasks (1098 cm^3) for 5 h after addition of the substrates. Gas samples (15 ml) were taken hourly and the C concentrations were analyzed by gas chromatography (GC 6000 VEGA series 2, Carlo Erba instruments, UK). We obtained the CO_2 concentrations and calculated the CO_2 flux rates. The data were corrected by the specific gas flux factor and multiplied with the headspace volume. Afterwards, the CO_2 fluxes were related to the dry weight of the soil and time during the incubation experiment (*Anderson* and *Domsch*, 1978).

C_{mic} was determined, using the initial rate of substrate-induced respiration (SIR) (*Anderson and Domsch* 1978; *Anderson* and *Joergensen*, 1997) and recalculated according to the conversion factor of *Kaiser* et al., (1992).

$$C_{mic}\,(\mu g\ g^{-1}\ soil) = 30 vCO_2\,(\mu L\ g^{-1}\ soil\ h^{-1}) \qquad (4)$$

1.2.7 Salt-extractable and total N

Moist soil (7.5 g) was extracted with 30 ml of 0.05 M K_2SO_4 for 1 h (Bruulsema and Duxbury, 1996) by overhead shaking (40 rev min-1). The soil suspension was centrifuged for 10 min at approx. 2500 x g. Afterwards, the supernatant was filtered through Rotilabo-rondfilters (type 15A, Carl Roth GmbH & Co.KG). The N-contents of the K_2SO_4 extracts were measured using a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena). The total N-contents were measured using a elemental analyzer (NA1110, CE –instruments, Rodana, Milano, Italy).

1.2.8 Statistical analyses

The means of four field replicates with standard errors are presented in tables and figures. A t-test was applied to characterize the effects of roots and soil depths. When significant effects were identified, a multiple post-hoc comparison using the Holm-Sidak method ($P<0.05$) was performed.

Parameter optimization was restricted to the applied model Equation (2) as indicated by maximum values of statistic criteria: r^2, the fraction of total variation explained by the model defined as ratio of model weighted sum of squares to total weighted sum of squares. Outliers were identified by the ROUT method, based on the False Discovery Rate (FDR), where Q got specified, which was the maximum desired FDR (*Motulsky* and *Brown,* 2006). The data of potential enzyme activities were treated in the same way.

1.3 Results

1.3.1 The Rhizosphere effect

Microbial biomass C (dsDNA derived) in the surface-layer was 42% higher in rooted versus root-free soil. This was confirmed by higher activities of β-glucosidase (4.7-fold) and leucine-aminopeptidase (2-fold) as well as by SIR (23%) in the rooted surface-layer compared to root-free soil. Higher specific enzyme activities (potential enzyme activity per DNA content) were observed for leucine-aminopeptidase and β-glucosidase in rooted compared to root-free soil (Table II.1/7). Microbial biomass C based on the DNA content (Eq. 1, Figure II.1/6) showed the same trends as that assessed by SIR for rooted and root-free soil in the first 10 cm depth (Eq. 4, Figure II.1/7). No significant differences were detected for the maximum specific growth rates (μ_{max}) and β-xylosidase between rooted and root-free soils. Total N- and salt-extractable N-contents reduced for rooted soil compared the fallow control (Figure II.1/10). Especially the K_2SO_4-extractable N-contents decreased by 26% in the first 10 cm and 53% at 10–20 cm depth for rooted vs. root-free soil.

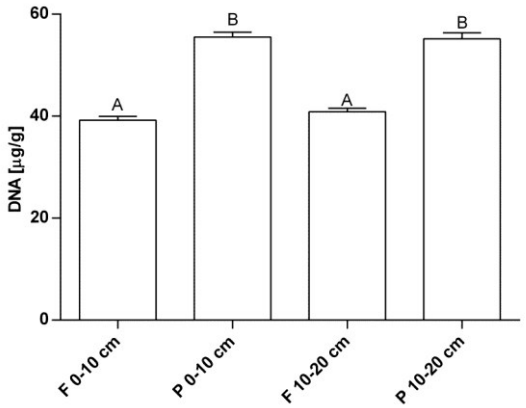

Figure II.1/6 Extractable dsDNA contents (DNA, ±SEM) in root-free (F) and rooted (P) soil at two depths (0–10 and 10–20 cm). The dsDNA-Cmic contents were calculated using a factor of 5.02 (Anderson and Martens, 2013). Significant root effects are indicated by different capital letters ($P<0.05$).

Extractable dsDNA contents (DNA, ±SEM) in root-free (F) and rooted (P) soil at two depths (0–10 and 10–20 cm). The dsDNA-Cmic contents were calculated using a factor of 5.02 (Anderson and Martens, 2013). Significant root effects are indicated by different capital letters (P<0.05).

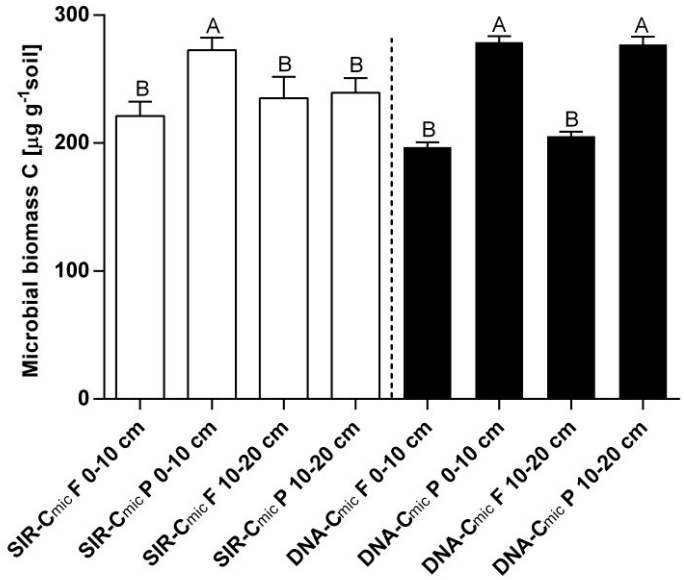

Figure II.1/7 Comparison of substrate-induced respiration (SIR)-C_{mic} contents and dsDNA-C_{mic} contents (C_{mic}, ±SEM) for root-free (F) and rooted (P) soil at two depths. Significant root effects comparing SIR-C_{mic} contents and DNA-C_{mic} contents are indicated by different capital letters (P<0.05).

1.3.2 Effects on microbial indicators with soil depth

The effect of depth on microbial parameters was more pronounced in rooted versus root-free soil. Microbial biomass C decreased by 14% with depth in rooted soil (Table II.1/6; SIR derived). The β-glucosidase activity was reduced by 23% and acid phosphatase by 25% for the rooted soil at 10–20 cm depth compared to the surface-layer (Figure II.1/8). The β-cellobiohydrolase activities in rooted soil almost tripled for rooted soil at 10–20 cm compared to 0–10 cm depth. No clear pattern with depth for C-cycling specific enzymes was determined (Table II.1/6). Despite the microbial specific growth rates were

independent on the root presence, these rates significantly slowed down for 42% in the lower layer compared to the first 10 cm depth (Figure II.1/9).

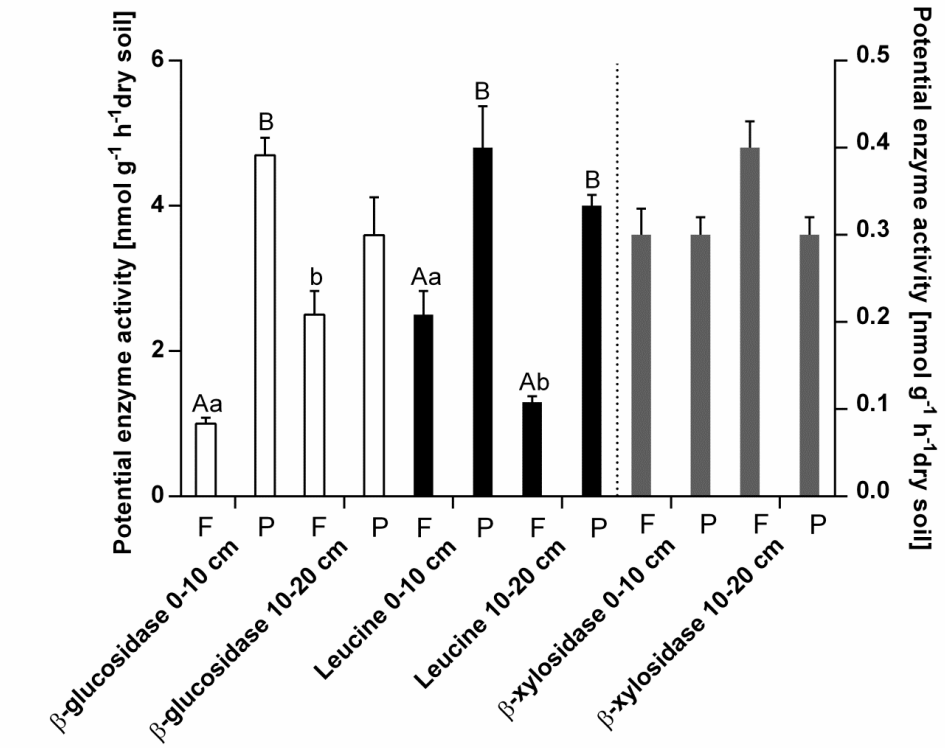

Figure II.1/8 Potential hydrolytic exo-enzyme activities (V_{max}; ± SEM, n = 12) for root-free (F) and rooted (P) soil at two depths (0–10 and 10–20 cm) are presented. Significant root effects are indicated by different capital letters. Lower-case letters signed significant root effects with depth (P<0.05).

Table II.1/6 Respiration parameters, active growing microbial biomass and cell masses are given for rooted and root-free soil at 0–10 and 10–20 cm soil depth. Significant effects of roots are indicated by different capital letters (P<0.05). Lower-case letters signed significant root effects with depth (P<0.05).

	SIR	Active growing C_{mic}		Lag time	Total cell mass
Soil	**µg CO2-C g^{-1} h^{-1}**	**µg C g^{-1}**	**%**	**h**	**µg g^{-1}**
Rooted 0-10 cm	9.1±0.2	0.2±0.01a	0.1	27	619±10
Root-free 0-10 cm	7.4±0.5	0.3±0.03	0.2	23	437±9
Rooted 10-20 cm	8.0±0.2	3.5±0.3Ab	1.3	18	616±12
Root-free 10-20 cm	7.8±0.3	0.6±0.07B	0.3	30	456±8

The maximum specific microbial growth rates (μ_{max}) varied between 0.11±0.015 and 0.19±0.03 h^{-1} (Eq. 2) overall the soil samples (Figure II.1/9). The actively growing microbial biomass did not exceed 1.3% of total biomass and was highest in rooted soil at 10–20 cm depth. Active part doubled with depth for root-free and increased by 17.5 times for rooted soil with the depth. In rooted soil microorganisms started to grow 12 h earlier at 10–20 cm depth compared with root-free soil (Table II.1/6).

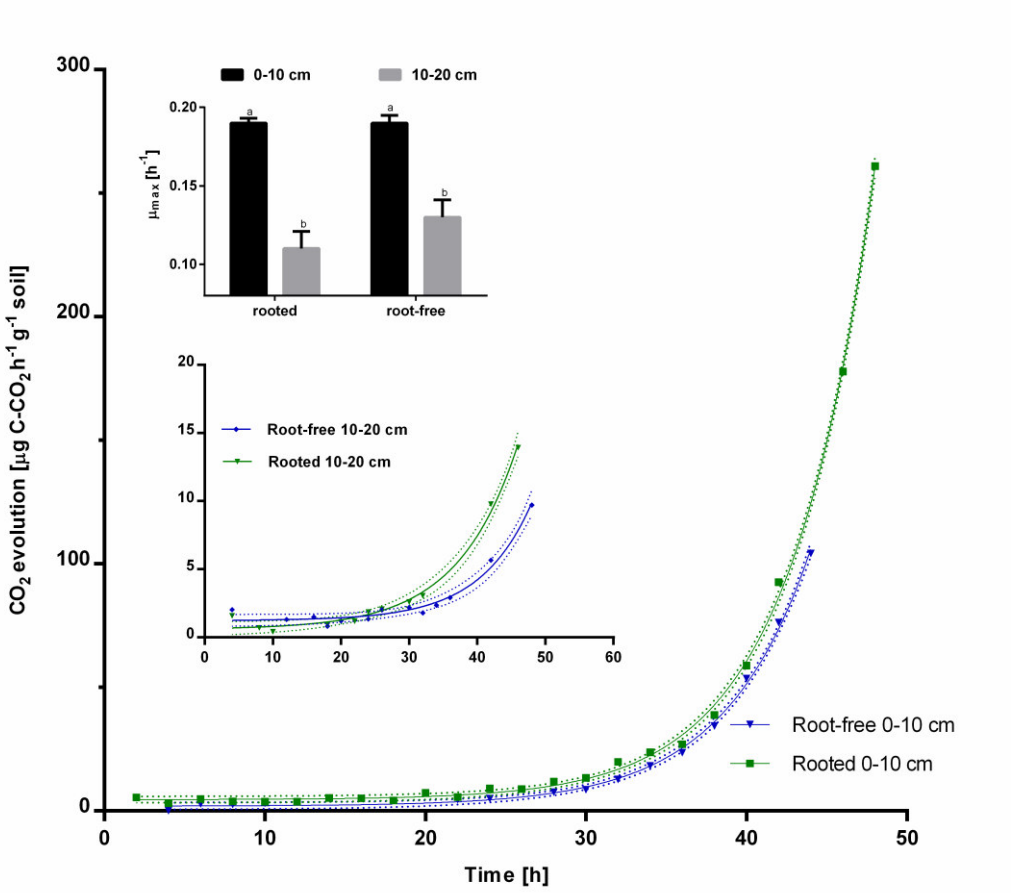

Figure II.1/9 Maximal specific growth rates (μ_{max}, ± SEM) are presented for rooted and root-free soil at 0–10 and 10–20 cm soil depth. Significant effects are assessed by Mann-Whitney test (P<0.05) and indicated by different capital letters. Lower-case letters signed significant root effects with depth (P<0.05). A confidence band (95%) was calculated for rooted and root-free soil to be aware of outlier.

Table II.1/7 Specific enzyme activities (potential enzyme activity per dsDNA content) (± SEM) are calculated for rooted and root-free soil at 0–10 and 10–20 cm soil depth. Significant root effects are indicated by different capital letters (P<0.05). Lower-case letters signed significant root effects with depth (P<0.05).

	Potential enzyme activity to dsDNA ratios [nmol h^{-1} mg^{-1} dsDNA]					
Soil	**Tyrosine**	**Acid phos-phate**	**β-xylo-sidase**	**Leucine**	**β-cellobiohydro-lase**	**β-gluco-sidase**
Root-free 0-10 cm	23.0 ± 0.4a	107.1 ± 1.2aA	7.7 ± 0.1	64.8 ± 1.6aA	132.6 ± 2.5aA	25.5 ± 0.4aA
Rooted 0-10 cm	18.0 ± 0.3a	64.8 ± 0.9aB	5.4 ± 0.1	86.4 ± 1.9aB	16.2 ± 0.1aB	84.6 ± 0.9aB
Root-free 10-20 cm	31.8 ± 0.5b	85.6 ± 1.3bA	9.8 ± 0.1	31.8 ± 0.4bA	58.7 ± 1.9bA	61.1 ± 1.5b
Rooted 10-20 cm	29.0 ± 0.3b	48.9 ± 0.6bB	5.4 ± 0.1	72.5 ± 0.7bB	48.9 ± 1.5bB	65.2 ± 1.8b

1.4 Discussion

1.4.1 Microbial biomass, growth and activity in the rhizosphere and root-free soil

The abundance of roots clearly enhanced microbial biomass by increased rhizodeposition. 30–60% of the photosynthetically fixed C can be translocated to the roots and up to 40% of the fixed C can be lost by rhizodeposition (*Kuzyakov* and *Domanski*, 2000; *Neumann* and *Römheld*, 2007). At our field site about 50% of the roots were allocated to the upper 10 cm (*Pausch* et al., 2013). The decreasing root biomass with depth led to lower rhizodeposition (*Pausch* et al., 2013), which reflected a positive correlation (*Van der Krift* et al., 2001). As a consequence of lower root biomass and rhizodeposits, microbial turnover increased and specific growth rates retarded at 10–20 cm depth compared to the surface-layer (*Blagodatskaya* et al., 2014b).

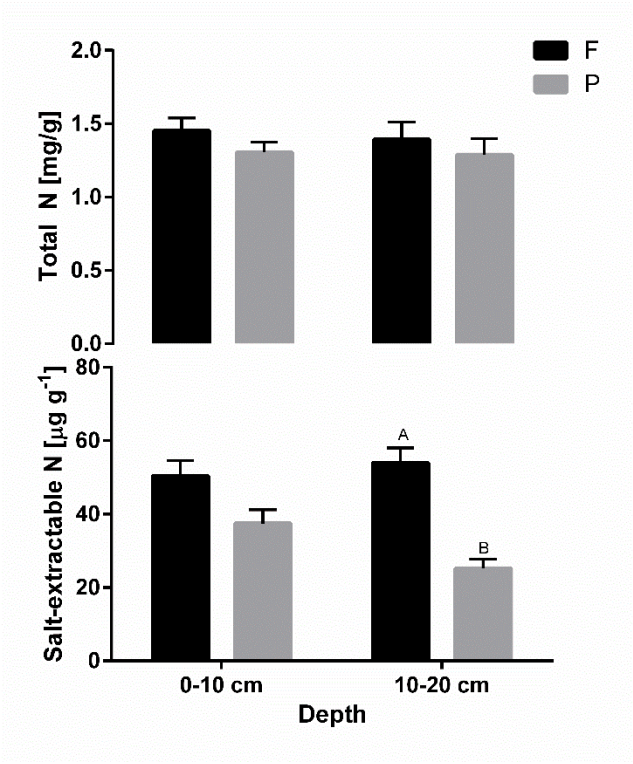

Figure II.1/10 Total nitrogen and salt-extractable nitrogen (N, ± SEM) are presented for rooted (P) and root-free soil (F) at 0–10 and 10–20 cm soil depth. Significant root effects are indicated by different capital letters ($P < 0.05$).

The decreased input of easily decomposable substrates by rhizodeposition with depth may induce stronger competition for substrates between microorganisms, especially for N (*Fontaine* et al., 2003; *Fischer* et al., 2013; *Paterson*, 2003), which resulted in lower microbial biomass at 10–20 cm depth (*Badalucco* and *Nannipieri*, 2005) or in slower growth (*Merckx* et al., 1987). Slower growth rates but greater fraction of active biomass and higher activity of cellulases at 10–20 cm depth versus the first 10 cm depth indicated the shift in abundances to slow-growing oligotrophic microorganisms (*Blagodatskaya* et al., 2014a, 2014c). This was related to the high abundance of cellulolytic enzymes, which was possibly associated with dead roots. The decrease of microbial specific growth rates with the depth could be a consequence of growth limitation by the depletion of N (*Helal und Sauerbeck*, 1986; *Merckx* et al., 1987). The competition for N between microbe-microbe or plant-microbe interactions in rooted soil (*Badri* et al., 2009; *Bowen* and *Rovira*,

1999; *Paterson*, 2003) may have shifted the microbial community structure from r-selected to slow-growing (K-selected) microorganisms (*Blagodatskaya* et al., 2009; *Fierer* et al., 2007).

The 23% greater microbial respiration response in rooted versus root-free soils indicated a high fraction of potentially active microorganisms (*Blagodatskaya* and *Kuzyakov*, 2013). Microbial cells, which maintain a potentially activity status (*Bodegom*, 2007), are able to immediately utilize an occasional substrate input (*De Nobili* et al., 2001). This was confirmed by a shorter lag-period for rooted soil at 10–20 cm depth, reflecting a high microbial affinity to the respective substrate input.

1.4.2 Potential and specific enzyme activities in rooted soil

The increased activities of leucine-aminopeptidase for rooted soil compared to the fallow control indicated the higher energy investments of microbes in producing proteolytic exoenzymes (e.g. leucine-aminopeptidase) in order to utilize N-bonded molecules. This suggests that lower contents of inorganic N are available for microbes in rooted soil (*Stursova* et al., 2006). Conformingly, the salt-extractable N- and total N-contents were reduced in the rooted sphere, especially at 10–20 cm depth. Due to reduced accessibility to proteolytic degradation immobilized enzymes often demonstrate higher stability compared with free extracellular enzymes (*Allison*, 2006). Plant roots as sinks for excess N enhanced continued mineralization driven by microbes, but shifted interaction of enzymatic systems (*Pinton* et al., 2007).

As the increased costs for enzyme production reduce the fitness of microbes, because those resources cannot be invested for reproduction (*Allison* et al., 2011), the specific microbial growth rates were retarded in rooted vs. root-free soil (*Blagodatskaya* et al., 2014b).

The leucine-aminopeptidase and β-glucosidase activities decreased for rooted soil at the first 20 cm depth, which was in line with the studies of *Steinweg* et al., (2013) and *Taylor* et al., (2012). However, the β-cellobiohydrolase activity increased and β-xylosidase activity stayed constant in rooted soil at the first 20 cm depth. Enzymatic systems of β-

cellobiohydrolases and β-glucosidases showed a contra-balanced behavior, especially in the surface-layer. For substrate utilization it is suggested that soil microorganisms use glucan (cellulose) as the preferred substrate irrespective of the type of residue (*Amin* et al., 2014, *Leitner* et al., 2012).

In 10–20 cm depth, the ratios of β-xylosidase and β-glucosidase to dsDNA contents raised for root-free soil indicated lower availability of C sources for enzymatic C utilization compared to the first 10 cm (*Stone* et al., 2014). This is in accordance to the specific activities of C-cycling enzymes, reported to increase with depth and reflecting greater microbial allocation to C-cycling enzyme production depending on decreased C availability (*Allison* et al., 2011).

1.5 Conclusions

The applied combination of approaches: analysis of the double-stranded DNA contents, enzyme activities and respiration kinetics, gave quantitative insights in microbial traits in rooted vs. root-free soil. Strong rhizosphere effects were elucidated for most of the measured microbial activity indicators. Thus rooted soil had greater microbial biomass, potential enzyme activity rates and substrate-induced respiration compared to root-free soil. Similar specific microbial growth rates and dsDNA-derived microbial biomass contents were demonstrated for rooted vs. root-free soil when we compared the two soil layer. However, the active microbial biomass increased strongly in the rhizosphere at the 10–20 cm depth.

The demand for N by microbes and maize plants clearly affected the potential and specific enzyme activities in the rooted sphere of an arable soil. Thus, the competition for that resource induced strong microbial- and plant-interactions, which boosted proteolytic enzyme activities (e.g. leucine-aminopeptidases) and hampered microbial growth in rooted soil. We conclude that the rhizosphere, namely rooted soil served as an area for microbes and extracellular enzymes, which strongly depended on the present substrates in the rooted zone of maize plants.

Acknowledgements

We thank Susann Enzmann, Heike Strutz and Viola Schade for their intensive help during the field and laboratory work. This study was supported by the German Research Foundation (DFG) within the Research Unit FOR 918“Carbon Flow in Belowground Food Webs assessed by Isotope Tracers”. We thank for the organization of the DBG workshop: “Soil processes – is the whole system regulated at hotspots? – From micro-scales to the pedon”!

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2. Substrate quality affects kinetics and catalytic efficiency of exo-enzymes in rhizosphere and detritusphere

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Published in *Soil Biology and Biochemistry* (2016), 92: 111–118

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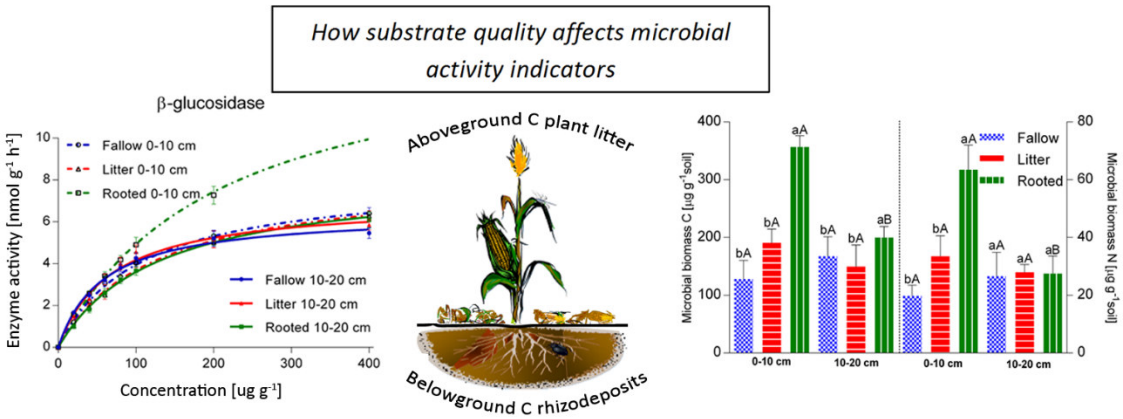
Research highlights

- Living roots increased microbial biomass by 179% and microbial respiration by 100% in rooted topsoil compared to fallow soil.
- The catalytic efficiency (Vmax/Km) of acid phosphatase was higher in rhizosphere and detritusphere compared to fallow.
- The Km reduction of all enzymes in 10–20 cm versus the upper 10 cm indicated increasing substrate affinity with depth.
- The catalytic efficiency increased from 0–10 to 10–20 cm for β-glucosidase, acid phosphatase and β-xylosidase.

Graphical abstract



Abstract

Microbial and enzyme functioning depends on the quality of substrates, which strongly differ in bare soil and in the hotspots of microbial activity such as the rhizosphere and detritusphere. We established a field experiment to determine the effects of contrasting substrate quality, namely, soil organic matter, maize shoot litter (detritusphere) and maize rhizodeposits (rhizosphere) on microorganisms and their extracellular enzymes in an arable soil. Kinetic parameters (V_{max} and K_m) of four hydrolytic extracellular enzymes: β-cellobiohydrolase, β-glucosidase, acid phosphate and β-xylosidase were analyzed in 0–10 and 10–20 cm to elucidate the effects of substrate content on substrate affinity and catalytic efficiency (V_{max}/K_m). Living roots increased microbial biomass by 179% and microbial respiration by 100% compared to fallow soil. Lower enzyme affinities to substrates (e.g. 93% for β-glucosidase) in rooted soil pointed to the domination of r-strategists, which are favored in the decomposition of labile organics common in the rhizosphere. No differences in catalytic properties of cellulolytic enzymes were detected between bulk and litter-treated soil, indicating the recalcitrance of organics in both treatments. The rhizosphere and detritusphere effects on enzyme kinetics were negligible in 10–20 cm, except β-glucosidase. The reduction of K_m of all enzymes in 10–20 cm versus the upper 10 cm indicated increasing substrate affinity with depth. Nonetheless, the catalytic efficiency increased from 0–10 to 10–20 cm (e.g. up to 420% for acid phosphatase), reflecting changes in properties and functioning of enzymatic systems. This pointed to a shift towards a more K-selected microbial community with higher affinity and more efficient substrate utilization. It also indicated the microbial adaptation to decreasing substrate contents with depth by altered enzyme functioning. Overall, the catalytic properties of cellulolytic enzymes were much more strongly affected by plants (substrate quality in the rhizosphere and detritusphere compared to bare fallow) than by depth (substrate content).

Key words: Enzyme kinetics, enzyme affinity, performance constant, substrate-induced respiration, microbial biomass

2.1 Introduction

Microbial community composition in soils is governed by substrate quantity, quality and input regularity. Microhabitats with high substrate input but contrasting quality such as rhizosphere and detritusphere differ in their dominating microbial species (Kandeler et al., 2001; Marschner et al., 2004, 2012; Kuzyakov and Blagodatskaya, 2015). As a result of the differences in microbial key players, the decomposition pathways of organic compounds are significantly different in the rhizosphere and detritusphere.

In the rhizosphere, low molecular weight organic substrates, such as root released exudates, lysates and mucilage may accelerate microbial growth (Neumann and Römheld, 2007). The microbes become more active and thereby, produce more intracellular and extracellular enzymes compared to bare fallow (Burns, 1978, 1982). Hence, rhizodeposition directly couples plant and microbial activities in the root channel. Besides the regular input of rhizodeposits, root-litter is abundant as a substrate source for microbial decomposition. However, the small differences in C availability due to the root-litter do not affect the impact of root-exudation on soil organic matter decomposition (de Graaff et al., 2014).

The detritusphere is characterized by high concentrations of easily degradable C sources, particularly at the early stages of residue decomposition (Bastian et al., 2009; Poll et al., 2010). As a result, the remaining substrates in the detritusphere are mainly polymeric plant residues of low degradability, for example cellulose and hemicelluloses (Nannipieri et al., 2012). Besides fast microbial uptake, the diffusion of soluble C and advective transport is frequently responsible for the depletion of the water-soluble C compounds (Gregorich et al., 2003; Poll et al., 2008). The recalcitrant plant-originated compounds require cascades of enzymes causing slower decomposition (Theuerl and Buscot, 2010). Their mineralization involves the action of several cellulases (e.g. β-cellobiohydrolase, β-glucosidase) to produce oligomeric cellobiose and to further degrade it to monomeric glucan (Nannipieri et al., 2012). The β-xylosidase is an exo-cellular enzyme involved in

the degradation of the major polymeric constituents of plant litter by degrading the hemicellulose xylan (linear polysaccharide β-1,4-xylan) into its readily available compounds xylose and other carbohydrates (Sinsabaugh and Moorhead, 1994).

The quantity and quality of plant litter inputs to the soil (both above- and belowground) influences substrate availability for microbes. This may control microbial community structure, and alter enzyme systems (Paul and Clark, 1996; Horwath, 2007). It remains unclear which factor – substrate quantity or quality – is mainly responsible for the catalytic properties of enzymes hydrolyzing plant organics.

We compared the rooted and the litter-treated soil to a bare fallow soil, suggesting lower microbial biomass and microbial respiration due to lower C availability for the litter-treated and the fallow soil. Easily available substrates, such as root exudates, are quickly consumed by microorganisms with enzymes of low substrate affinity (typical for fast-growing r-strategists), reflecting higher K_m values (Fierer et al., 2007; MacArthur and Wilson, 1967). The slow-growing K-strategists with enzymes of high substrate affinity (lower K_m) are better adapted for growth on poorly degradable substrates (e.g. on the litter channel) (Blagodatskaya et al., 2009; Dorodnikov et al., 2009). Therefore, in hotspots with contrasting substrate quality, the shift in species domination may result in production of iso-enzymes, i.e. enzymes with the same function but different catalytic properties (Khalili et al., 2011) reflected in the enzyme kinetics (Marx et al., 2001). Furthermore, hydrolytic exo-enzymes in contrasting locations in the soil (i.e. immobilized vs. free) may change intrinsic enzyme properties, such as K_m values (Paulson and Kurtz, 1970; Rao et al., 1996).

There is a lack of studies comparing kinetic parameters of enzymes in hotspots of microbial activity such as the rhizosphere and detritusphere. Therefore, we measured the substrate affinity (K_m) and catalytic efficiency (V_{max}/K_m) (Gianfreda et al., 1995; Moscatelli et al., 2012) of 4 extracellular enzymes (β-cellobiohydrolase, β-glucosidase, acid phosphatase, β-xylosidase) in the vicinity of living roots of maize (rhizosphere) and maize litter (detritusphere).

According to evolutionary-economic principles the microbial communities allocate resources to enzyme production in relation to substrate availability and growth requirements to reduce costs and maximize their resource returns (Allison and Vitousek, 2005). The metabolic energy required for protein synthesis and excretion, as well as the C and nutrient content of the enzymes themselves are considered as costs of enzyme production in soils. The resource benefits of enzyme production can be invested in reproduction effort of microorganisms (Allison et al., 2011). Available forms of N and P are suggested to suppress the production of N- and P-acquiring enzymes and stimulate the microbial allocation to C-degrading enzymes (Allison et al. 2011; Sinsabaugh and Moorhead, 1994).

We hypothesized, that the kinetic parameters (V_{max}, K_m) of hydrolytic enzymes are different in microbial communities formed in soil hotspots as compared with bulk soil. To prove the effects of the substrate contents, the soil was sampled from 0–10 and 10–20 cm depths reflecting similar quality but lower input of substrate with depth. We further hypothesized that decreasing substrate content with depth increases the substrate affinity and catalytic efficiency.

To test these hypotheses we determined the parameters of microbial respiration (Anderson and Domsch, 1985; Anderson and Joergensen, 1997; Cheng and Coleman, 1989) and of enzyme kinetics (Nannipieri et al., 2012; Sinsabaugh, 2010), as indicators of organic C mineralization and substrate-specific utilization (Kourtev et al., 2002). This was done in the rhizosphere, detritusphere and soil from a bare fallow. This is the first study, combining such general microbial activity indicators as respiration with specific indicators as the kinetics of extracellular hydrolytic enzymes. This enables elucidating the effect of two contrasting C sources – rhizodeposits and plant litter – on the functioning of microbial communities under field conditions.

2.2 Materials and methods

2.2.1 Study site and sampling

This study was conducted at an experimental agricultural site in the north-west of Göttingen (Lower-Saxony), Germany (51°33´N, 9°53´E; 158m NN). The area has a temperate climate with a long-term annual mean precipitation of 645 mm and an air temperature of 8.7°C. The dominant soil types are loamy haplic -Luvisols, partly with slight stagnic properties (Table I.2/1).

In spring 2012, 12 experimental plots (5 x 5 m) were established in the field and separated from each other by buffer stripes of 2 m and 6 m in row and inter-row, respectively. Three treatments, – rooted, litter amended and fallow soil – were set up on the experimental plots, with 4 replicates each.

All experimental plots were tilled with a chisel plough to a depth of 10 cm (tillage date: 12th of April 2012). For the rooted treatment, hybrid maize (*Zea mays L.*, Codisco/TMTD 98% Satec) was sown on 4 plots at a density of 12 plants m^{-2} (sowing date: 16th of April 2012) N fertilizers (ammonium nitrate urea solution: 110 kg N ha^{-1} and NP fertilizer (di-ammonium phosphate: 110 kg N ha^{-1}) were amended to all treatments, shortly before and after sowing the maize. For the litter treatment 4 plots received 0.8 kg m^{-2} dry maize residues with a C -content of about 44%. Litter application took place in 10 cm soil depth in early June at the start of the crop growth period to ensure the same conditions for the herbivore and detritivore community in the soil. In addition 4 plots remained unplanted as a fallow control. All treatments were kept free from vegetation by manually removing weeds. The obtained differences in the enzyme systems between 0–10 cm and 10–20 cm were thus established within a relatively short period. The shading level represented a mean leaf area index of plants during the vegetation period to accomplish comparable environmental conditions between the plots.

In July, the soil was sampled at 0–10 cm and 10–20 cm on each plot. Soil sub-samples from each plot and depths were dried at 105°C (24 h) to determine the soil moisture content. The water contents of the sampled soil ranged from 28% for fallow to 25% for the

rooted soil, which was significantly lower than the fallow control ($P < 0.001$). All soil samples were frozen at −18°C until the analyses. Prior to the analysis the soil samples were thawed at 4°C. After thawing the soil samples were sieved (< 2 mm) and fine roots and other plant debris were carefully removed with tweezers and the soil was pre-conditioned at 22°C for 72 h. Afterwards, the moisture contents of the soil samples were adjusted to 60% of water holding capacity (WHC) for analyses. No significant differences were detected in pH, C_t, or N_t contents of rooted, litter-treated and fallow soil.

2.2.2 Analyses

The experiments were conducted with the 4 plot replicates for each treatment. Enzyme activities, microbial biomass and CO_2 data were expressed as means ± standard errors of means (±SEM).

Soil microbial biomass

Soil microbial biomass C (C_{mic}) was estimated using the chloroform fumigation extraction (CFE) method described by Brookes et al. (1985) and Vance et al. (1987). Non-fumigated, moist soil (7.5 g) was extracted with 30 ml of 0.05 M K_2SO_4 for 1 h (Bruulsema and Duxbury, 1996) by overhead shaking (40 rev min^{-1}). The same amount of soils was fumigated with ethanol-free chloroform (80 ml) first and then extracted in the same way. The fumigation was carried out in desiccators at room temperature for 24 h. The soil suspension of the fumigated and the non-fumigated samples was centrifuged for 10 min at approx. 2500 x g. Afterwards, the supernatant was filtered through Rotilabo-rondfilters (type 15A, Carl Roth GmbH & Co.KG). The organic C-content of the K_2SO_4 extracts was measured using a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena).

Microbial biomass C and microbial biomass N were calculated by dividing the microbial C or N flush (E_C; E_N), i.e. the difference between extracted C or N from fumigated and non-fumigated soil samples, with a k_{EC} or k_{EN} factor of 0.45 (Joergensen, 1996; Wu et al., 1990).

Basal and substrate-induced microbial respiration

Microbial respiration was determined by substrate-induced respiration (SIR) based on CO_2 efflux after adding glucose and mineral salts (Anderson and Domsch, 1985; Anderson and Joergensen, 1997). The SIR method was conducted in a climate chamber (22°C). Thereby, 23 g (dry weight) of each soil sample was incubated in flasks for 4 h after addition of the substrate. The amended substrate mixture contained glucose (10 mg g^{-1}), talcum (20 mg g^{-1}) and mineral salts, i.e. 1.9 mg g^{-1} $(NH_4)SO_4$, 2.25 mg g^{-1} K_2HPO_4 and 3.8 mg g^{-1} $MgSO_4$-$7H_2O$ (Blagodatsky et al., 2000). Gas samples (15 ml) were taken hourly and the CO_2 concentrations were analyzed by gas chromatography (GC 6000 VEGA series 2, Carlo Erba instruments, UK). The basal respiration (BR) was measured in the same way as the SIR without any substrate amendment and a sampling time interval of 2 h.

To obtain CO_2 flux rates, the slopes of hourly measured CO_2 concentrations were corrected by the specific gas flux and multiplied with the headspace volume (1098 cm^3). We then related the CO_2 fluxes to the soil carbon content and incubation duration. The metabolic quotient (q_{CO2}) indirectly reflects the microbial maintenance expenses, availability and efficiency of microbial substrate utilization and was determined by the ratio of BR to C_{mic} (Anderson and Domsch, 1990).

Enzyme assays

We used fluorogenic methylumbelliferone-based (MU) substrates to measure the enzyme activities of β-cellobiohydrolase, β-glucosidase, acid phosphates and β-xylosidase were measured (Marx et al., 2001). The following 4-Methylumbelliferone derivatives were used as substrates: EC 3.2.1.21, 4-MU-β-D-glucoside; EC 3.2.2.27, 4-MU-β-D-xylopyranoside; EC 3.2.1.91, 4-MU-β-D-cellobioside; EC 3.2.1.30, 4-MU-phosphate. Half a gram of moist soil was added in 50 ml sterile water in autoclaved jars and was dispersed by an ultrasonic disaggregator (50 J s^{-1} for 120 s (De Cesare et al., 2000). Aliquots of 50 µl were withdrawn and dispensed in 96-well microplates (Brand pureGrade, black) while stirring the suspension. In addition to four field replicates we used three analytical replicates for each soil sample and each substrate. Fifty microliter of 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.1) was used as buffer (German et al., 2011). The substrates were pre-solved in 300 µl Dimethyl-sulfoxide (DMSO) and were further diluted by MES to 1 mM a working solution. Finally, 100 µl of series concentrations of substrate

solutions (20, 40, 60, 80, 100, 200, 400 µmol substrate g soil^{-1}) were added to the wells. Plates were kept at 21°C, agitated and measured fluorometrically (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland).

The substrate-dependent rate of reaction (v) mediated by hydrolytic enzymes, followed Michaelis-Menten kinetics (Marx et al., 2001, 2005; Nannipieri et al., 2012; Sinsabaugh, 2010).

$$v = (V_{max} \times [S]) / (K_m + [S]) \qquad (1)$$

Plotting the initial velocity of reaction (v) against increasing concentrations of substrate ([S]) yields a rectangular hyperbola. Based on experimental data, the calculation enables characterizing the specific enzyme-substrate reaction by 2 kinetic parameters: 1) V_{max}, the maximal velocity of enzyme catalysis that theoretically is attained when the enzyme has been saturated by an infinite concentration of substrate, and 2) K_m, the Michaelis constant, which is numerically equal to the concentration of substrate for the half-maximal velocity (Marx et al., 2005). V_{max} is responsible for decomposition rates at saturating substrate concentrations; the K_m reflects the enzyme affinity to the substrate. We calculated the catalytic efficiency factor (catalytic efficiency/specificity constant/performance constant), known as the ratio between V_{max} and K_m (Gianfreda et al., 1995; Koshland, 2002; Moscatelli et al., 2012). The catalytic efficiency reflects the total enzyme catalytic process combining enzyme–substrate complex dissociation (V_{max}) and the rate of enzyme–substrate complex formation (K_m) (Cornish-Bowden, 1995; Koshland, 2002).

Significant effects of soil treatments were assessed by ANOVA at $P < 0.05$. The parameters of the equation were fitted by minimizing the least-square sum using GraphPad Version 6 software (Prism, USA). The three analytical replicates of enzyme activity curves were used for each of four replicated soil samples at two depths (0–10 and 10–20 cm). Parameter optimization was restricted to the applied model equation as indicated by maximum values of statistic criteria: r^2, the fraction of total variation explained by the model defined as the ratio of model weighted sum of squares to total weighted sum of squares. Outliers were identified by the ROUT method, based on the False Discovery Rate (FDR),

where Q was specified, which was the maximum desired FDR (Motulsky and Brown, 2006).

2.3 Results

2.3.1 Microbial biomass C and N, extractable organic C and extractable N

Living roots strongly increased microbial C and N (Figure II.2/11 a, b). C_{mic} and N_{mic} contents in the upper 10 cm of the rooted soil were 178% and 222% higher than the fallow treatment. The average microbial biomass C content in fallow soil was 128 (±46) µg C_{mic} g^{-1} soil, and the microbial biomass N was 20 (±5) µg N_{mic} g^{-1}. The litter application did not affect C_{mic} and N_{mic} compared to the fallow. Living roots increased microbial biomass C and N only in the upper 10 cm, whereas below 10 cm the root effect was negligible.

Furthermore, the salt-extractable organic C (EOC) and salt-extractable N (EN) contents doubled in the rooted soil compared with the litter-amended plots, indicating abundant easily available organics in the upper 10 cm (Figure II.2/11 c, d). The EOC and EN contents increased through planting compared to litter-amended and fallow soil solely in the upper 10 cm. Rhizodeposition increased C_{mic}, N_{mic}, EOC and EN for rooted soil compared to litter-amended and fallow soil only in the first 10 cm.

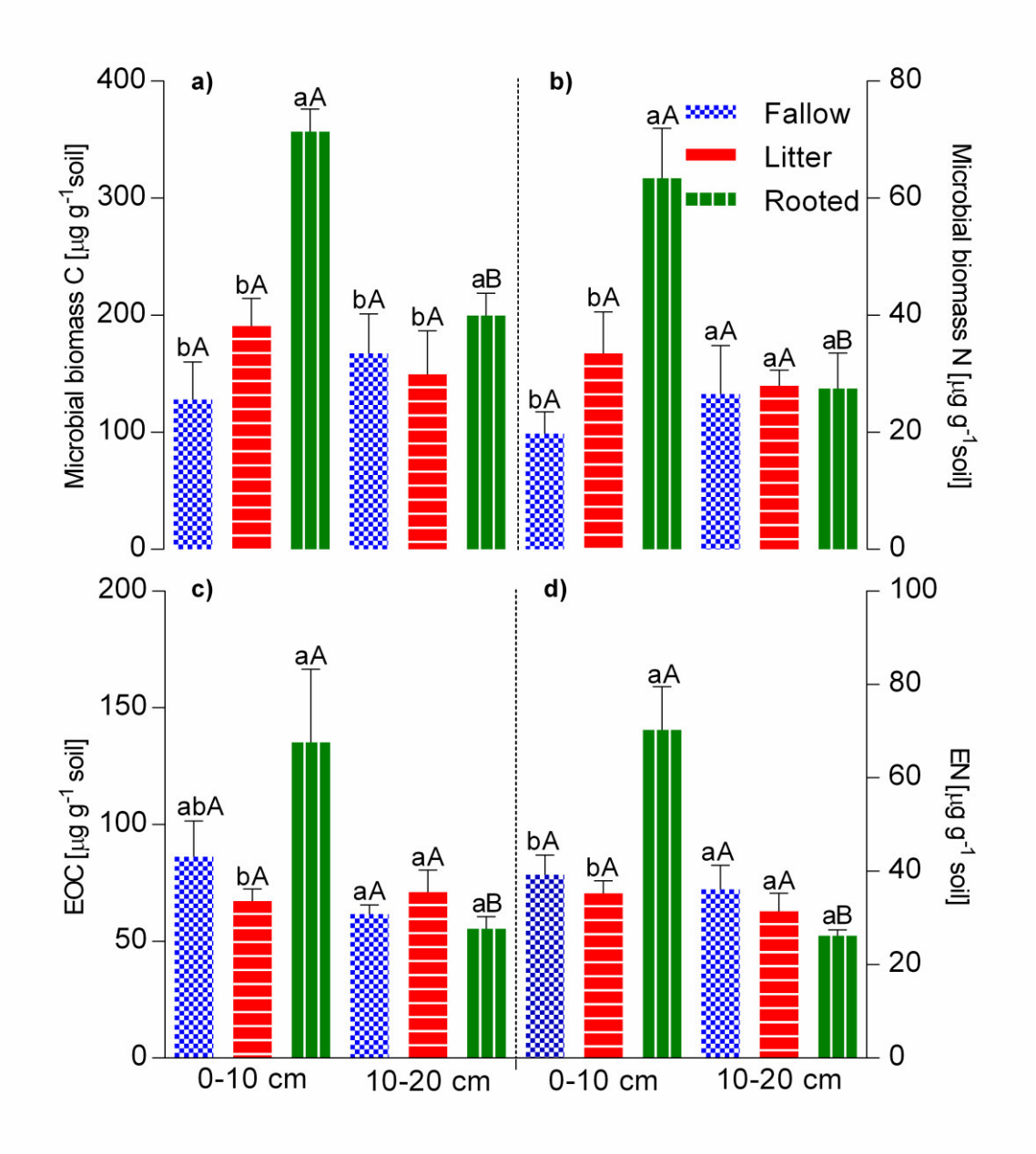


Figure II.2/11 a) Microbial biomass C (C_{mic}), b) extractable organic carbon (EOC), c) microbial biomass N (N_{mic}) and d) extractable nitrogen (EN) (±SEM) for fallow, litter-amended and rooted soil at two depths (0–10 and 10–20 cm). Significant treatment

effects are assessed by ANOVA ($P<0.05$) and indicated by different lower-case letters. Capital letters denote significant soil treatment effects with depth.

2.3.2 Basal respiration and substrate-induced respiration

The basal respiration (BR) (80±20 µg CO_2-C g^{-1} C_{org} h^{-1}) of rooted soil in the upper layer was twice as high relative to litter-amended and fallow soil (Figure II.2/12 a). In 10–20 cm, BR of the planted soil showed significantly ($P<0.05$) reduced rates compared to the upper 10 cm.

The largest SIR values were measured for rooted soil in 0–10 cm. A 2-fold greater CO_2 production rate (0.66±0.07 mg CO_2-C mg^{-1} g^{-1} C_{org} h^{-1}) was determined for rooted compared to litter-amended and fallow soil (Figure II.2/12 b). The effect of planting disappeared in the 10–20 cm layer and showed comparable BR and SIR values for the litter-amended and fallow soil. For litter-amended soil the BR:SIR ratio was lower than for rooted soil, especially in 10–20 cm (Figure II.2/12 c). The decomposition of easily available organics in the rhizosphere clearly increased microbial respiration (BR and SIR) relative to litter-treated and fallow soil in the surface layer.

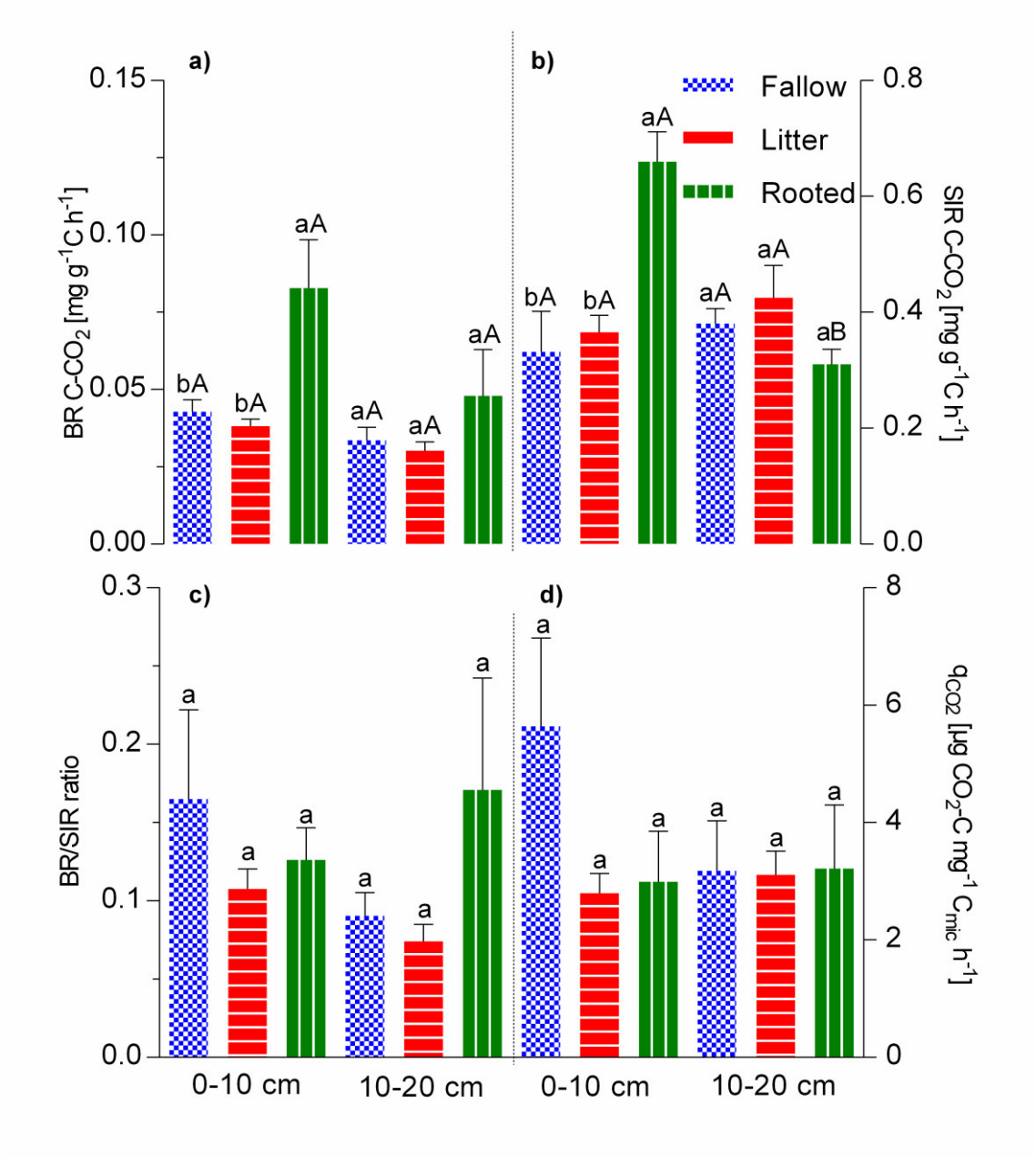


Figure II.2/12 a) Basal respiration (BR), b) substrate-induced respiration (SIR), c) respiratory quotient (BR:SIR ratio) and d) metabolic quotient (q_{CO2}) (±SEM) for fallow, litter-amended and rooted soils at two depths (0–10 and 10–20 cm). Significant

treatment effects are assessed by ANOVA (P<0.05) and indicated by different lower-case letters. Capital letters denote significant soil treatment effects with depth.

2.3.3 Enzyme kinetics

Living plants strongly stimulated the β-glucosidase activity in the upper 10 cm resulting in the highest maximal reaction rate (V_{max}) compared to litter-amended and fallow soil (Figure II.2/13 a; Table II.2/9). The V_{max} of β-glucosidase and β-cellobiohydrolase increased 2-fold for rooted soil in the upper layer at substrate saturation compared to fallow soil (Figure II.2/13 a, b). This indicates high production of glycolytic enzymes by the microbes. The rates of β-glucosidase, acid phosphatase and β-cellobiohydrolase reduced for rooted soil ($P < 0.05$) from 0–10 to 10–20 cm depth (Figure II.2/13 a, b, c).

Table II.2/8 Kinetic parameters (V_{max} and K_m; ±SEM) of extracellular hydrolytic enzymes at 0–10 cm and 10–20 cm for fallow, litter-amended and rooted soils. Lower-case letters indicate significant differences (P<0.05) of V_{max} and K_m by ANOVA with post-hoc Tukey multiple comparison of different treatments at same depth, whereas capital letters indicate the comparison with depth.

Depth	Treatment	β-cellobiohydrolase					β-glucosidase				
		V_{max}	SEM	K_m	SEM	V_{max}/K_m	V_{max}	SEM	K_m	SEM	V_{max}/K_m
[cm]		[nmol g^{-1} h^{-1}]		[µmol g^{-1}]			[nmol g^{-1} h^{-1}]		[µmol g^{-1}]		
0–10	Fallow	1.6b	0.1	26.1b	4.9	0.06	8.1b	0.3	105.9b	9.0	0.08
	Litter	2.1b	0.1	49.3b	8.2	0.04	8.3b	0.4	124.9bA	13.5	0.07
	Rooted	3.4aA	0.4	97.3aA	22.8	0.03	15.0aA	0.8	204.5aA	21.5	0.07
10–20	Fallow	2.2	0.2	50.7	11.9	0.04	6.4b	0.3	57.3b	6.5	0.11
	Litter	2.4	0.2	37.8	7.8	0.06	7.0b	0.4	68.5bB	9.9	0.10
	Rooted	1.9B	0.1	44.7B	6.2	0.04	8.2aB	0.5	129.0aB	14.8	0.06

Table II.2/8 Kinetic parameters (Vmax and Km; ±SEM) of extracellular hydrolytic enzymes at 0–10 cm and 10–20 cm for fallow, litter-amended and rooted soils. Lowercase letters indicate significant differences (P<0.05) of Vmax and Km by ANOVA with post-hoc Tukey multiple comparison of different treatments at same depth, whereas cap-ital letters indicate the comparison with depth.

Depth	Treatment	Acid phosphatase					β-xylosidase				
		V_{max}	SEM	K_m	SEM	V_{max}/K_m	V_{max}	SEM	K_m	SEM	V_{max}/K_m
[cm]		[nmol g^{-1} h^{-1}]		[µmol g^{-1}]			[nmol g^{-1} h^{-1}]		[µmol g^{-1}]		
0–10	Fallow	7.7aA	0.4	164.2aA	19.3	0.05	0.54A	0.03	92.6A	13.7	0.006
	Litter	5.8bA	0.3	79.5bA	12.0	0.07	0.46	0.03	71.9	10.2	0.006
	Rooted	7.2acA	0.4	98.5bA	13.8	0.07	0.45	0.04	81.5	15.5	0.006
10–20	Fallow	4.6B	0.2	22.5B	4.8	0.21	0.39B	0.01	35.4B	4.1	0.011
	Litter	4.4B	0.2	15.8B	3.3	0.28	0.43	0.02	42.1	6.8	0.010
	Rooted	4.8B	0.2	24.3B	4.7	0.20	0.54	0.02	50.6	5.7	0.011

For the rooted soil we determined about 2- and 3-fold higher K_m values for β-glucosidase and β-cellobiohydrolase, respectively, compared to that of the fallow control in 0–10 cm. This reflects a lower affinity to the substrate. The K_m decreased with depth for all analyzed enzymes except for β-cellobiohydrolase where K_m increased in the deeper soil layer of the fallow soil (Table II.2/9). For acid phosphatase we recorded a 6-fold reduction of K_m from 0–10 cm to the 10–20 cm depth (fallow soil, Table II.2/9). The consistent decrease of K_m with depth indicated the reduction of substrates in deeper the soil layer.

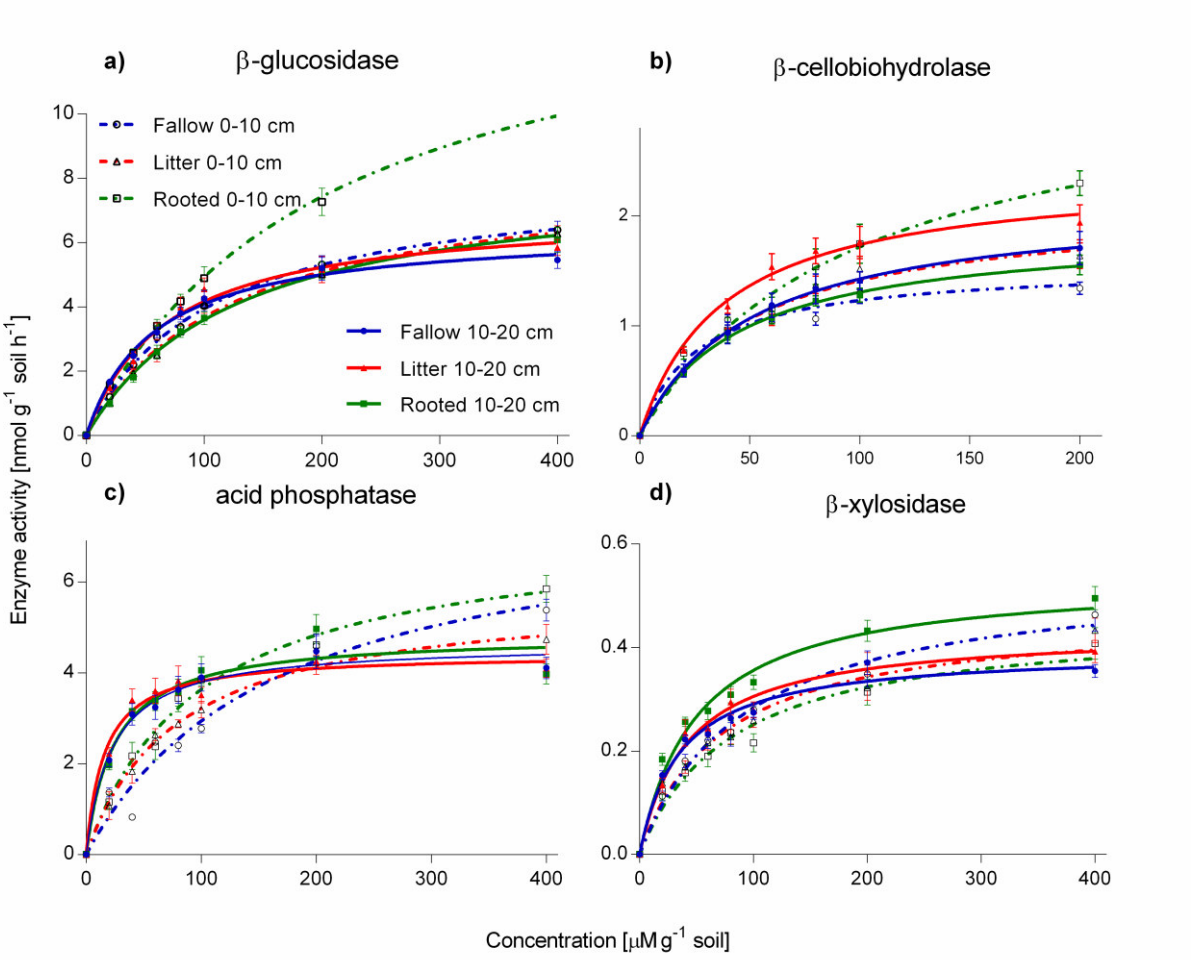


Figure II.2/13 Enzyme kinetics (±SEM): a) β-glucosidase, b) β-cellobiohydrolase, c) acid phosphatase and d) β-xylosidase. The blue color indicates the fallow, litter-amended and rooted soils. Statistics are given in Table II.2/9.

The catalytic efficiency (V_{max}/K_m) of acid phosphatase was higher in rhizosphere and detritusphere compared to fallow soil in the upper 10 cm, with significantly higher enzyme affinity to the substrate (Table II.2/9). Furthermore the V_{max}/K_m ratio increased with depth for β-glucosidase, acid phosphatase and β-xylosidase, and was maximal for acid phosphatase.

The two-way ANOVA for V_{max} and K_m with treatment (substrate quality) and depth (substrate content) as main factors revealed that the effects of both roots and depth were enzyme-specific (Figure II.2/14). The strongest effect for substrate quality was revealed for

V_{max} of β-glucosidase, β-cellobiohydrolase and acid phosphatase, explaining 44, 22 and 11% of variation, respectively. The β-cellobiohydrolase and β-xylosidase were also strongly affected by substrate quality and depth interactions. For K_m of β-glucosidase, 48% of the variation could be explained by the substrate quality factor (Figure II.2/14). Overall, the cellulolytic enzymes were more strongly affected by substrate quality, whereas for phosphatase and xylanase the depth effect was most important.

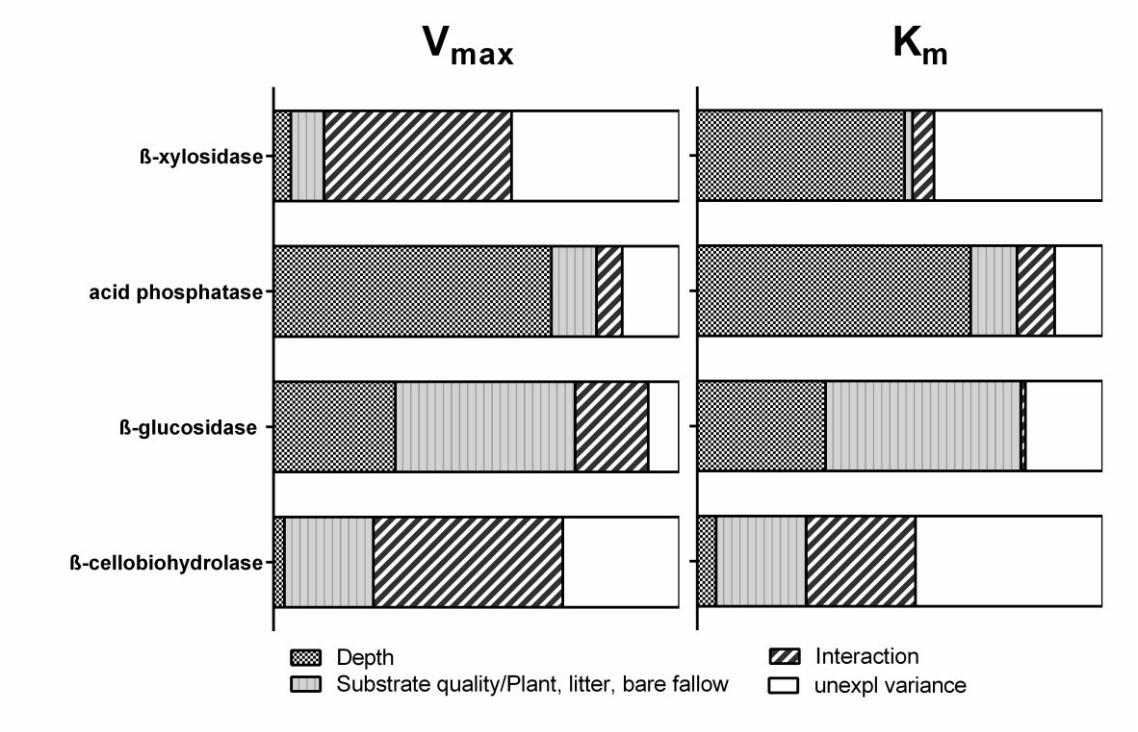


Figure II.2/14 Contribution of the two factors substrate quality and substrate content and their interactions (two depths: 0–10 and 10–20 cm) for variation of V_{max} and K_m. Results of two-way ANOVA.

The enzyme activities for β-glucosidase and β-cellobiohydrolase were greater in rooted plots relative to litter-treated and fallow ones. Moreover, all measured enzyme affinities to the substrates increased strongly from 0–10 cm to the 10–20 cm depth, indicating a shift in enzymatic systems.

2.4 Discussion

2.4.1 Effects of roots

We determined that the strong effects of living plants in the upper 10 cm yielded 179, 222, 100 and 100% higher values for C_{mic}, N_{mic}, BR, and SIR, respectively, compared to the fallow soil (Figure II.2/11, Figure II.2/12). This indicates that microbial proliferation was strongly related to the quantity and quality of substrates available for growth. Meta-analysis of microbial biomass and respiration data from various studies showed 62% and 80% higher values in rhizosphere soil compared to bulk soil, respectively (Finzi et al., 2014). For rooted soil we found a 44% reduction of microbial biomass content from 0–10 to 10–20 cm, reflecting the role of root exudates. Maize roots are concentrated in the upper soil layers (Amos and Walters, 2006). At our field site about 50% of the roots were allocated to the upper 10 cm (Pausch et al., 2013). Since rhizodeposition is positively correlated to root biomass (Van der Krift et al., 2001), the decreasing root biomass led to lower rhizodeposition with depth (Pausch et al., 2013). Accordingly, EOC, N_{mic}, EN contents and microbial respiration were reduced from 0–10 to 10–20 cm for rooted soil by 59, 57 and 63%, respectively. This reduction corresponded with the decrease in the relative amounts of water -soluble C with increasing distance to wheat roots (Merbach et al., 1999; Hafner et al., 2014). Remarkably, EN was lowest for rooted soil compared with litter-amended soil and fallow soil in 10–20 cm. We suggest that a reduced rhizodeposition in 10–20 cm – and thus less EOC and EN – promotes substrate competition between microbes and plants (Blagodatskaya et al., 2014b; Fontaine et al., 2003; Kuzyakov and Xu, 2013). The Michaelis constant (K_m) doubled for β-glucosidase and tripled for β-cellobiohydrolase in the rhizosphere compared to fallow soil, indicating lower enzyme affinity to the substrate. Easily available substrates such as glucose (as a component of root exudates) stimulated fast -growing r-strategists in the upper 10 cm (Blagodatskaya et al., 2009; Pianka, 1970). This agrees with the increased SIR (Figure II.2/12).

The great reduction of K_m for acid phosphatase, β-glucosidase and β-xylosidase with depth (Table II.2/9, Figure II.2/13) means high rates of reaction already present at very

low substrate concentrations (Davidson and Janssens, 2006; Davidson et al., 2006). Remarkably, all tested enzymes reflected this strong effect of decreasing substrate content with depth. This confirms that the activities of extracellular enzymes are mainly a function of the amount of available substrate (Kandeler et al., 1999) and of the microbial biomass present to potentially synthesize them (Geisseler and Horwath, 2009). Due to reduced rhizodeposition in the lower layer, the strong competition for easily degradable C sources favors the K-selected microbes, which are reported to be more competitive under resource limitation even in the rhizosphere (Blagodatskaya et al., 2014b). Accordingly, we determined that the catalytic efficiency of all measured hydrolytic enzymes (except β-cellobiohydrolase, fallow soil; β-glucosidase, rooted soil) increased from 0–10 to 10–20 cm, again indicating a shift to K-selected microbes (Table 2, Figure3). Therefore, a shift in microbial strategy towards higher substrate affinities of enzyme systems suggested a change in substrate content (Blagodatskaya et al., 2009). Such physiological adaptations of microorganisms to substrate content and quality are more important for efficient substrate utilization than the microbial community structure (Stone et al., 2014). Extracellular enzyme systems adapted to the altered substrate supply resulted in a change of catalytic efficiency and in a corresponding shift in the functional structure of the microbial community. Thus, a lower catalytic efficiency indicated the dominance of zymogenous microbial communities (r-strategists) in 0–10 cm depth as compared with 10–20 cm depth, where the K-strategists relatively dominated (Table 2) (Blagodatskaya et al., 2009; Panikov, 1995).

High variations in the unexplained variance of the measured enzymes (Figure II.2/14), reflected highly enzyme-specific determining factors. Increased probability of explained variance for β-glucosidase and acid phosphatase pointed to strong impacts of quantity and quality of the substrate.

2.4.2 Effects of litter

C_{mic}, EN, EOC, BR and SIR values were similar for the litter-amended and fallow soil (Figure II.2/11, Figure II.2/12). This further confirmed that total C_{mic} does not change

after litter addition (Potthoff et al., 2008). Basal and substrate-induced respiration indicated a more efficient C mineralization for the detritusphere than the rhizosphere, suggesting a lower microbial turnover in the detritusphere. Empirical studies of N mineralization have agreed upon a threshold of litter C:N ratio (e.g., 20–40) below which microbial growth will not be N limited. As such, microbes may shift the equilibrium production of enzymes to favor C-acquiring ones (Tian and Shi, 2014). The β-cellobiohydrolase activity, however, was highest in the litter-amended soil in 10–20 cm compared to rooted and fallow plots (Figure II.2/13). This can be due to the quality of the amended maize leaves, which mostly consist of nonlignified primary cell walls, thus making the cellulose and hemicellulose less resistant to enzymes. In contrast, the abundant root-litter in the maize planted treatment, which is rich in secondary cell walls (Amin et al., 2014). The litter C:N ratios frequently show a negative relation to cellulose and β-glucosidase activities (Leitner et al., 2012; Tian and Shi, 2014). This is in line with the comparatively low C:N ratio (21.5) of the used maize litter (Potthoff et al., 2005; 2008) and the increased response of β-cellobiohydrolase activity (Blagodatskaya et al., 2014a). Thus, litter C:N is a good indicator for the total amount, but not for the dynamics of soil enzyme activity (Tian and Shi, 2014). Nonetheless, the reduced substrate affinity (Table 2) for litter-amended soil throughout all tested extracellular enzymes were in line with a lower BR:SIR ratio, especially in 10-20 cm. This points to a shift in substrate availability and thus to changes in the efficiency of C and N utilization.

When comparing the litter-treated with rooted soil, the microbial community develops according to substrate quality and regularity of the input. Therefore, slow-growing microorganisms with more efficient metabolism are usually developed on low available plant residues as compared with easily decomposable root exudates. Thus, the C-cycling hydrolytic exo-enzymes demonstrated slower decomposition rates in litter amended soil, but similar or higher catalytic efficiencies compared to rooted soil. This may reflect a lower waste metabolism of microorganisms in plant litter-treated soil.

2.5 Conclusions

The β-glucosidase, β-cellobiohydrolase and acid phosphatase were strongly affected by substrate quality, which differed in the rhizosphere and detritusphere of maize. Thus, the contrasting substrate quality of living roots and shoot litter created hot spots for the microorganisms, which produced extracellular enzymes for their distinct needs. A pronounced effect of roots was determined in the upper 10 cm caused by rhizodeposition, which maintained an increased microbial biomass C and N, EOC, EN, microbial respiration as well as enzyme activities in the rhizosphere compared to the detritusphere and bare fallow. This effect disappeared in 10–20 cm due to lower contents of easily available substrates, reflecting a lower root biomass. A clear increase of enzyme affinity in 10–20 cm compared to the first 10 cm, pointed a shift towards a more K-selected microbial community.

We conclude that the availability of C and nutrients in the soil clearly affected the metabolic respiratory response as well as the efficiency of enzymes mediating the catalytic reaction, especially in the presence of roots. Substrates with different availability (e.g. root exudates, plant residues) changed functional properties of the soil microbial community and induced a shift in enzymatic systems. These changes are crucial for microorganisms to benefit from the costs of energy investments, caused by a stronger competition for resources.

Acknowledgements

We would like to thank Susann Enzmann, Heike Strutz and Viola Schade for their intensive help during the field and laboratory work. This study was supported by the German Research Foundation (DFG) within the Research Unit “Carbon Flow in Belowground Food Webs assessed by Isotope Tracers”. The contribution of E. Blagodatskaya was supported by the Russian Scientific Foundation (project Nr. 14-14-00625).

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3. Enzyme properties down the soil depth – A matter of substrate quality in rhizosphere and detritusphere

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Published in *Soil Biology and Biochemistry* (2016), 103, 274−283

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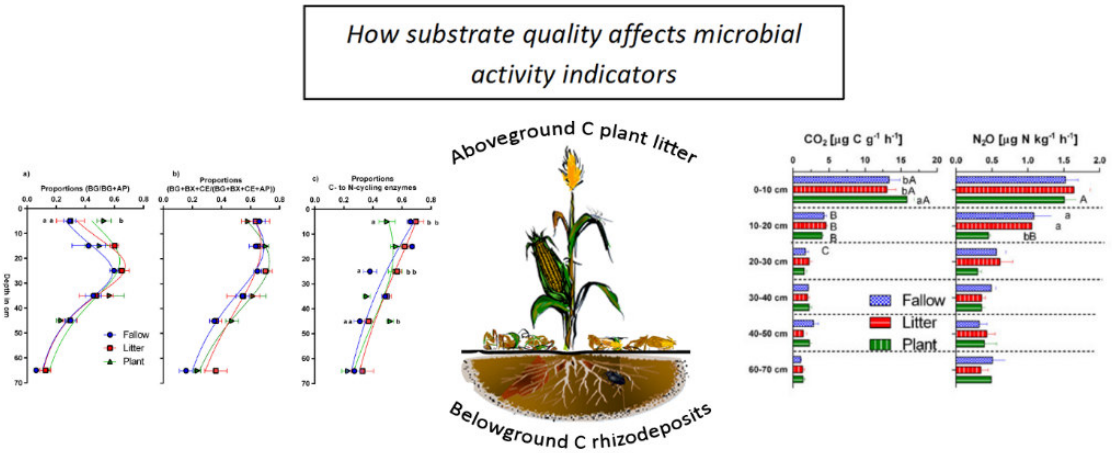
Research highlights

- A field experiment with maize or maize litter amendment was conducted
- Soil profiles were characterized by various microbial activity indicators
- Rhizodeposition decreased relative V_{max} and proportion of C- to N-cycling enzymes
- Strong N limitation increased proteolytic enzyme activities in planted topsoil
- The substrate effect (rhizodeposits vs. litter) was relevant only in the topsoil

Graphical abstract



Abstract

The decomposition of soil organic matter depends strongly on its availability to microorganisms and their enzymes. The rhizosphere and detritusphere are microbial hot spots due to additional substrate input, leading to high abundance, specific species diversity and functional diversity of microbial communities. However, rhizosphere and detritusphere differ in substrate quality, localization, and duration of input. We hypothesized that the contrasting substrate availability between rhizosphere and detritusphere affects the activity of microorganisms and associated enzymes. Organic carbon (C) from the rhizosphere and detritusphere decreases with soil depth and, consequently, microbial hot spots become rarer and competition for C and nutrients increases. In deeper soil (> 40 cm depth) the amount and quality of substrates is expected to decrease and, therefore, the effect of contrasting substrate input to disappear. Plant N uptake is expected to induce lower N contents in the rhizosphere of maize compared to the detritusphere and bare fallow. These hypotheses were tested in a factorial field experiment with 1) maize-planted, 2) maize litter-amended, and 3) bare sites. Enzyme kinetic parameters (Vmax, Km, Ka), extractable organic C and microbial biomass C were compared in soil affected from rhizosphere and detritusphere throughout the profile to 70 cm depth, to assess microbial C and nutrient limitations. A decrease in enzyme activity with depth due to resource scarcity and lower substrate quality appeared in planted and litter-amended soil. N limitation in planted soil increased the activity and substrate affinity of proteolytic enzymes to provide for microbial N demand through SOM decomposition. This was in line with lower Vmax ratios (Vmax for C-cycling enzymes divided by Vmax for N-cycling enzymes) in planted relative to litter-amended topsoil. The catalytic efficiency of enzymes decreased 2- to 20-fold from top- (< 40 cm) to subsoil (> 40 cm), irrespective of the substrate input. Substrate quality in the rhizosphere and detritusphere affected enzyme activities only in the topsoil, whereas a sharp decline of C input with depth led to similar activities in the subsoil. Most of the enzyme indexes reflected shifts in allocation of C and nutrients in the rhizosphere and detritusphere. The presented results underline the role of microorganisms as critical links in the C and nutrient transfers in the rhizosphere and detritusphere.

Key words:

Catalytic efficiency, specific enzyme activity, enzyme indexes, denitrification, microbial respiration, substrate quality

3.1 Introduction

Enzymes in soil catalyze nearly all important transformations in the carbon (C), nitrogen (N), phosphorus (P) and sulfur (S) cycles (Aon et al., 2001; Wallenstein and Burns, 2011). Decomposition of organics is strongly dependent on microbes and enzymes, which are especially abundant in the rhizosphere and detritusphere – two main microbial hot spot environments in soil. The rhizosphere is characterized by high density and quality of substrates for microorganisms (Garbeva et al., 2008; Marschner et al., 2012, 2001), and plants provide a variety of C and energy sources from their roots (Gregory, 2006; Paterson, 2003; Paterson et al., 2007). The detritusphere contains large amounts of cellulose, hemicelluloses and lignin, as main components of plant residues (Kandeler, 1999; Marschner et al., 2012; Nannipieri et al., 2012).

The microbial C:N:P ratios (ecological stoichiometry) are frequently used to indicate how allocation of C and nutrients by microorganisms influence microbial demands on soil pools (Cleveland and Liptzin, 2007; Heuck and Spohn, 2015; Sinsabaugh et al., 2015). Nowadays it is widely accepted that microbial C:N:P stoichiometry affects microbial mineralization of C sources (Mooshammer et al., 2012). Microbial respiration (CO_2) and N_2O production are well known indicators describing microbial activities in soil (Blagodatskaya et al., 2014). In combination with the ratios of commonly measured enzyme activities (Table II.3/9), these indicators provide insights into the microbial community that is investing energy for microbial fitness (Sinsabaugh et al., 2012, 2008; Tapia-Torres et al., 2015). The production of extracellular enzymes is regulated by nutrient availability and energy demand (Sinsabaugh et al., 2009). Thus, enzyme activities are reliable microbial activity indicators and are closely interrelated with soil quality (Bending et al., 2004; Paudel et al., 2011).

Most enzyme studies are restricted to the topsoil, despite the fact that microbial substrate utilization takes place throughout the whole soil profile (Sinsabaugh et al., 1993; Sinsabaugh and Moorhead, 1994; Vranova et al., 2013). Furthermore, only the potential enzyme activity is considered in most studies, whereas rates of enzyme-substrate complex dissociation and enzyme-substrate complex formation are neglected (Koshland, 2002).

Therefore, it is of great interest to study how microbial functioning and enzyme systems vary throughout the soil profile.

As interactions between substrate composition, microbial competition, and nutrient availability are complex, we established a factorial field manipulation experiment including maize-planted, maize litter-amended and bare fallow sites. These sites differed (1) in sources of different substrate quality (root-derived vs. litter-derived vs. none) and (2) in the distribution of substrates with depth. Both substrate quantity and quality strongly decrease with soil depth (Fierer et al., 2003a), because most roots are localized in the topsoil, so the rates of C input to subsoil are low (Fierer et al., 2003 b). Therefore, the subsoil microbial communities differ in composition and activity from the surface communities (Blume et al., 2002; Fierer et al., 2003a; Fritze et al., 2000).

We combined substrate-induced emission of carbon dioxide (CO_2) and nitrous oxide (N_2O) with kinetics of the enzymes β-glucosidase (BG), β-cellobiohydrolase (CE), β-xylosidase (BX), acid phosphatase (AP), and leucine- (LE) and tyrosine- (TY) aminopeptidases to disentangle the effects of substrate quality and substrate amount on microbial activity along the depth gradient. Several approaches for integrating the various enzyme activities into unified indexes were compared (Table II.3/9) (Hill et al., 2014; Moorhead et al., 2016, 2013; Nannipieri et al., 2012; Sinsabaugh et al., 2008). These activity indexes of multiple enzymes were related to dissolved organic C (DOC) and extractable nitrogen (EN).

We hypothesized that the contrasting substrate availability between planted soil and litter-amended soil, reflecting the rhizosphere and detritusphere, respectively, would affect the activity of microorganisms and associated enzymes. The effect of the contrasting substrate availabilities on microbial substrate utilization was predicted to decline with depth due to the lower amount and quality of substrates in the subsoil (> 40 cm depth) compared to the topsoil. Furthermore, we hypothesized that lower N contents in the maize-planted soil, due to plant N uptake, would lead to stronger competition between microbes compared to the fallow control. This, in turn, would increase proteases, because of an inversely proportional relationship to low substrate availability (Olander and Vitousek, 2000; Sims and Wander, 2002; Stursova et al., 2006). To our knowledge this is the first

study using a broad range of activity indicators to elucidate the tight interactions between microbial activity and contrasting substrate input down the soil profile.

Table II.3/9 Enzyme indexes

Enzyme indexes	Description	References
1) Specific enzyme activity	Potential activity to microbial biomass	Trasar-Cepeda et al., 2008; Stone et al., 2014
2) Catalytic efficiency	Catalytic properties of same enzyme (V_{max}/K_m)	Moscatelli et al., 2012
3) V_{max} ratio of C- to N- and C- to P-cycling enzymes	Relative activities of C- vs. N- and C- vs. P-acquiring enzymes	Sinsabaugh et al., 2008
4) Proportions of C- to N- and C- to P-cycling enzymes	Proportional enzyme activities of C- vs. N- and C- vs. P-acquiring enzymes	Hill et al., 2014; Moorhead et al., 2013
5) Vector length	Relative C vs. nutrient aquisition	Moorhead et al., 2013, Moorhead et al., 2016
6) Vector angle	Relative P vs. N limitation	Moorhead et al., 2013, Moorhead et al., 2016

3.2 Material and Methods

3.2.1 Study site

The experimental arable field was located in the north-west of Göttingen, Lower-Saxony, Germany (51°33´N, 9°53´E; 158 m NN). The area has a temperate climate with a long-term mean annual precipitation of 645 mm and mean air temperature of 8.7 °C. The dominant soil types are Luvisols occasionally with stagnic properties (Table I.2/1; Kramer et al., 2012; Pausch et al., 2013).

In April 2012 the field was tilled with a chisel plough to a depth of 12 cm and maize was sown at a density of 12 grains m^{-2}. Nitrogen fertilizers (ammonium nitrate urea solution: 110 kg N ha^{-1} and diammonium phosphate: 110 kg N ha^{-1}) were applied to all treatments, shortly before and after sowing the maize. The corn was not irrigated during plant growth.

In September 2012 corncobs were harvested and maize plants were cut at a height of 10 cm above soil surface. The maize above-ground biomass was hackled to a particle size of 1 cm^2 and air-dried to gain litter. In April 2013 the herb layer developed during spring was removed by Glyphosate (4 l ha^{-1}). Three weeks later the soil was tilled to a depth of 12 cm, maize sown at a density of 9 grains m^{-2} and fertilized similarly to 2012. In September 2013 maize plants were harvested and removed from the experimental field site.

3.2.2 Treatments

In May 2012 a total of 12 experimental plots (size 5 x 5 m) were conducted and arranged in two adjacent rows separated by a 5 m buffer stripe within and 2 m buffer stripes between rows. Three treatments, each replicated four times, were established differing in resource quality: plant (maize as crop), litter (application of maize litter) and fallow. Maize was removed from the eight plots within the first three weeks after seeding to set up the litter and fallow treatments. For the litter-treated soil four plots received 0.8 kg m^{-2} dry maize litter (equivalent to 0.35 kg C m^{-2}, C-content = 44%) approximating the above-ground biomass of maize in June. Litter was grubbed into the first 10 cm of soil on

June the 6th 2013. This coincided with the start of the crop growth period to ensure the same conditions for the herbivore and detritivore communities. To accomplish comparable environmental conditions between plots, the litter-amended and the fallow control plots were shaded with blinds (mechanical shading; AGROFLOR Kunststoff GmbH, Wolfurt, Austria). The shading level represented the mean leaf area index of plants during the vegetation period. In addition, plots were regularly weeded to prevent plant carbon input by herbs.

3.2.3 Soil sampling and preparation

We sampled in each plot soil from 0–50 cm in 10 cm increments, and from 60–70 cm depth, of each plot in July 2013. Each plot was sampled in one position, using a Riverside auger (inner diameter 5 cm, Eijkelkamp, Giesbeek, The Netherlands). The soil samples were frozen at -18°C until analysis. Prior to analysis the soil samples were thawed at 4 °C. After thawing the soil samples were sieved (< 2 mm) and fine roots and other plant debris were carefully removed with tweezers. The soil was then pre-incubated at 22 °C for 72 h. Soil sub-samples from each plot and depth were dried at 105 °C for 24 h to determine the moisture content. Moisture contents ranged from 14% for planted to 18% for fallow soil. Prior to incubation the moisture content was adjusted to 60% of the water holding capacity (WHC).

3.2.4 Substrate-induced respiration

The substrate-induced respiration (SIR) method is generally used to measure microbial biomass by amendment with easily available C. We determined the CO_2 efflux following the addition of glucose and mineral salts (Anderson and Domsch, 1985; Anderson and Joergensen, 1997). Production of N_2O was also measured as an indicator of N sources for nitrification and denitrification. In total, 25 g samples of soil were incubated in flasks for 4 hours after addition of the substrate. The SIR substrate mixture contained glucose (10 mg g^{-1}) and mineral salts (1.9 mg g^{-1} $(NH_4)_2SO_4$; 2.25 mg g^{-1} K_2HPO_4; and 3.8 mg g^{-1} $MgSO_4.7H_2O$) (Blagodatsky et al., 2000). Glucose is an important components of root

exudates (Derrien et al., 2004; Whipps and Lynch, 1983). The amount of mineral salts was selected so that the added substrate did not change soil pH (change < 0.1) (Blagodatskaya et al., 2007). Gas samples (15 ml) were taken hourly and the CO_2 as well as N_2O concentrations were analysed by gas chromatography (GC 6000 VEGA series 2, Carlo Erba instruments, UK). The slopes of measured hourly CO_2 and N_2O concentrations were corrected by the specific gas flux (according to the gas constant, air pressure and temperature) and multiplied by the headspace volume (1098 cm^3) to obtain the individual flux rates for each soil sample. Microbial biomass C (C_{mic}) was determined using the individual flux rate (Anderson and Joergensen, 1997) and calculated according to the equation by Anderson and Domsch (1978, 2010):

$$C_{mic}\ (\mu g\ g^{-1}\ soil) = (\mu l\ CO_2\ g^{-1}\ soil\ h^{-1}) \times 40.04 \quad (1)$$

3.2.5 Dissolved organic carbon and extractable nitrogen

Moist soil (7.5 g) was extracted with 30 ml of 0.05 M K_2SO_4 for 1 h by overhead shaking (40 rpm) (Bruulsema and Duxbury, 1996). The soil suspension was centrifuged for 10 min at 2500 x g. The supernatant was then filtered through Rotilabo-rondfilters (type 15A, Carl Roth GmbH & Co.KG). The organic C and N content of the K_2SO_4 extracts were measured using a multi N/C analyzer (multi N/C analyzer 2100S, Analytic Jena).

3.2.6 Enzyme assays

We used 4-methylumbelliferone-β-D-cellobioside, 4-methylumbelliferone-β-D-glucoside, 4-methylumbelliferone-phosphate, 4-methylumbelliferone-7-β-D-xyloside, L-leucine-7amino-4-methylcoumarin hydrochloride and L-tyrosine-7amino-4-methylcoumarin to determine the enzyme activities of β-cellobiohydrolase (exo-1,4-β-glucanase, EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.2), β-xylosidase (EC 3.2.2.27) and leucine-/tyrosine-aminopeptidase (EC 3.4.11.1), respectively. β-glucosidase (BG), β-cellobiohydrolase (CE) and β-xylosidase (BX) represented enzymes in the

C-cycle, whereas leucine- (LE) and tyrosine- (TY) aminopeptidases represented N-cycling enzymes. Acid phosphatase (AP) is responsible for substrate utilization in the P-cycle.

Fluorogenic substrates enable direct quantitative comparison of the activity of enzymes responsible for various functions (Marx et al., 2002; Nannipieri et al., 2012). This is because the fluorogenic compounds (MUF or AMC) are enzymatically released in amounts equimolar to the number of bonds broken (corresponding to enzyme function). Enzyme activity is therefore expressed in the same units for various different enzymes, based on calibration by MUF or AMC. This standard analysis of enzyme kinetics is based on the assumption that the binding of substrate to one enzyme binding site does not affect the affinity or activity of an adjacent site. That is, neither substrate nor product acts as an allosteric modulator to alter the enzyme velocity.

Half a gram of soil was added to 50 ml sterile water in autoclaved jars and dispersed by an ultrasonic disaggregator (50 J s^{-1} for 120 s; De Cesare et al., 2000). Aliquots of 50 µl were withdrawn while stirring the suspension and dispensed into 96-well microplates (Brand pureGrade, black). Fifty microliter of buffer was added (0.1 M MES buffer, pH 6.1 for carbohydrases and phosphatase, 0.05 M TRIZMA buffer, pH 7.8 for leucine-/tyrosine-aminopeptidase) (Marx et al., 2005, 2001). Finally, 100 µl of substrate solution was added at a series of concentrations (20, 40, 60, 80, 100, 200, 400 µmol substrate g^{-1} soil). Plates were kept at 22 °C, agitated and measured fluorometrically (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation using an automated fluorometric plate reader (Wallac 1420, Perkin Elmer, Turku, Finland). Fluorescence was converted into an amount of MUB (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin) by reference to the fluorescence of standard solutions, which had been prepared in subsamples of the various soil suspensions. Each field replicate was measured as an analytical triplicate.

The kinetic parameters V_{max} and K_m were estimated using a non-linear regression model (Michaelis-Menten kinetics) (Marx et al., 2001). V_{max} is the decomposition rate at saturating substrate concentrations; K_m reflects the enzyme´s affinity for the substrate. The K_m corresponds to the weighted sum of rate constants for the dissociation of the enzyme-substrate complex divided by the rate constant for its formation (Koshland, 2002).

V_{max} and C_{mic} were used to determine the specific enzyme activity (Table II.3/9; Index 1) (Nannipieri et al., 2012; Stone et al., 2014). Furthermore, we determined the catalytic efficiency (Table II.3/9; Index 2) for each treatment and enzyme (Gianfreda et al., 1995; Koshland, 2002; Moscatelli et al., 2012). It reflects the total enzyme catalytic process combining enzyme-substrate complex dissociation and the rate of enzyme-substrate complex formation (Cornish-Bowden, 1995; Koshland, 2002). These parameters were selected to compare the catalytic properties of each enzyme with different substrate input (litter, rhizodeposits) (Cervelli et al., 1973; Esti et al., 2011).

We integrated the activities of enzymes involved in the same process as indicators of organic matter degradation and nutrient transformation. It is assumed that the sum of major C-acquiring enzyme activities is a better indicator of total C-acquisition than BG alone (Bell et al., 2014; Nannipieri et al., 2012). Thus, enzyme activity ratios (Table II.3/9; Index 3 and Index 4) were used as a tool for examining relative allocation to energy versus nutrient acquisition (Stone et al., 2014).

We translated the enzyme activity proportions (TAC/(TAC+TAP)) and (TAC/(TAC+TAN)) (Sinsabaugh, 2008) into vector lengths and directions (angles) that provide clear metrics of relative C limitation vs. nutrient limitation (Moorhead et al., 2016, 2013). The angle quantifies the relative P vs. N limitation (Supplementary Figure 1b; Moorhead et al., 2016). Enzyme activity toward P acquisition is reflected by the steepness of the vector angle. With increasing enzyme production toward C acquisition relative to N and P, the vector length increases (Supplementary Figure II.3/20). The increasing vector length is interpreted as a relative increase in C limitation, and increasing vector angle as a relative increase in P vs. N limitation (Moorhead et al., 2013).

Vector length was determined as the square root of the sum of the squared values of x and y. Relative C- vs. P-acquiring enzyme activities were represented by x and the relative C- vs. N-acquiring activities by y (Moorhead et al., 2016).

$$\text{Vector length} = \text{SQRT}\,(x^2+y^2) \qquad (2)$$

The vector angle was calculated as the arctangent of the line extending from the plot origin to point (x, y) (Moorhead et al., 2016):

Angle = DEGREES (ATAN2 (x, y)) (3)

3.2.7 Statistics

The means of four field replicates with standard errors are presented in tables and figures. The Shapiro-Wilk test was applied to test for normality. We used Pearson correlation coefficients to interpret the degree of linear relationships. Multiple t-tests were applied to characterize the effects of contrasting substrate input (litter, rhizodeposits) and soil depths (each layer was tested separately). When significant effects were identified, a multiple post-hoc comparison using the Holm-Sidak test ($P<0.05$) was performed. The kinetic parameters were fitted by minimizing the least-square sum using GraphPad Version 6 software (Prism, USA). The three analytical replicates of enzyme activity curves were used for each of the four replicated soil samples at each depth. Parameter optimization was restricted to the applied model equation as indicated by maximum values of r^2. Outliers were identified by the ROUT method, based on the False Discovery Rate (FDR), where Q was specified as the maximum desired FDR (Motulsky and Brown, 2006).

3.3 Results

3.3.1 Microbial biomass C and N_2O production

Microbial biomass C (C_{mic}), determined by the SIR method, declined sharply with depth (Figure II.3/15 a, b) and was higher in planted soil than in litter-treated ($P<0.01$) and fallow soils ($P<0.05$) in the upper 10 cm (Figure II.3/15 a, b). C_{mic} was strongly correlated to SOC content for litter-treated ($r^2=0.98$, $P<0.05$) and fallow soil ($r^2=0.98$, $P<0.05$), whereas C_{mic} was not significantly correlated to SOC content in planted soil ($r^2=0.31$) (not shown). Total organic C and N content at each depth were similar between the treatments (Figure 2). Furthermore, SIR-derived microbial biomass was closely correlated to N_2O production, irrespective of substrate quality (planted, $r^2=0.96$, $P<0.001$; litter, $r^2=0.92$, $P<0.01$; fallow, $r^2=0.89$, $P<0.05$) (not shown). However, when comparing the specific enzyme activities (Table II.3/9; Index 1) of leucine aminopeptidase (LE) and tyrosine aminopeptidase (TY) with N_2O, the relationships between either LE or TY and N_2O production shifted to a negative correlation (r between -0.21 and -0.62) with depth.

The highest N_2O production was measured in the top 10 cm with similar intensities for all treatments (Figure II.3/15 c). N_2O emission in planted soil dropped by 67% from 0–10 to 10–20 cm depth. N_2O production at 10–20 cm in planted soil was much lower (58%) than that of the litter-amended and fallow soil at corresponding depth.

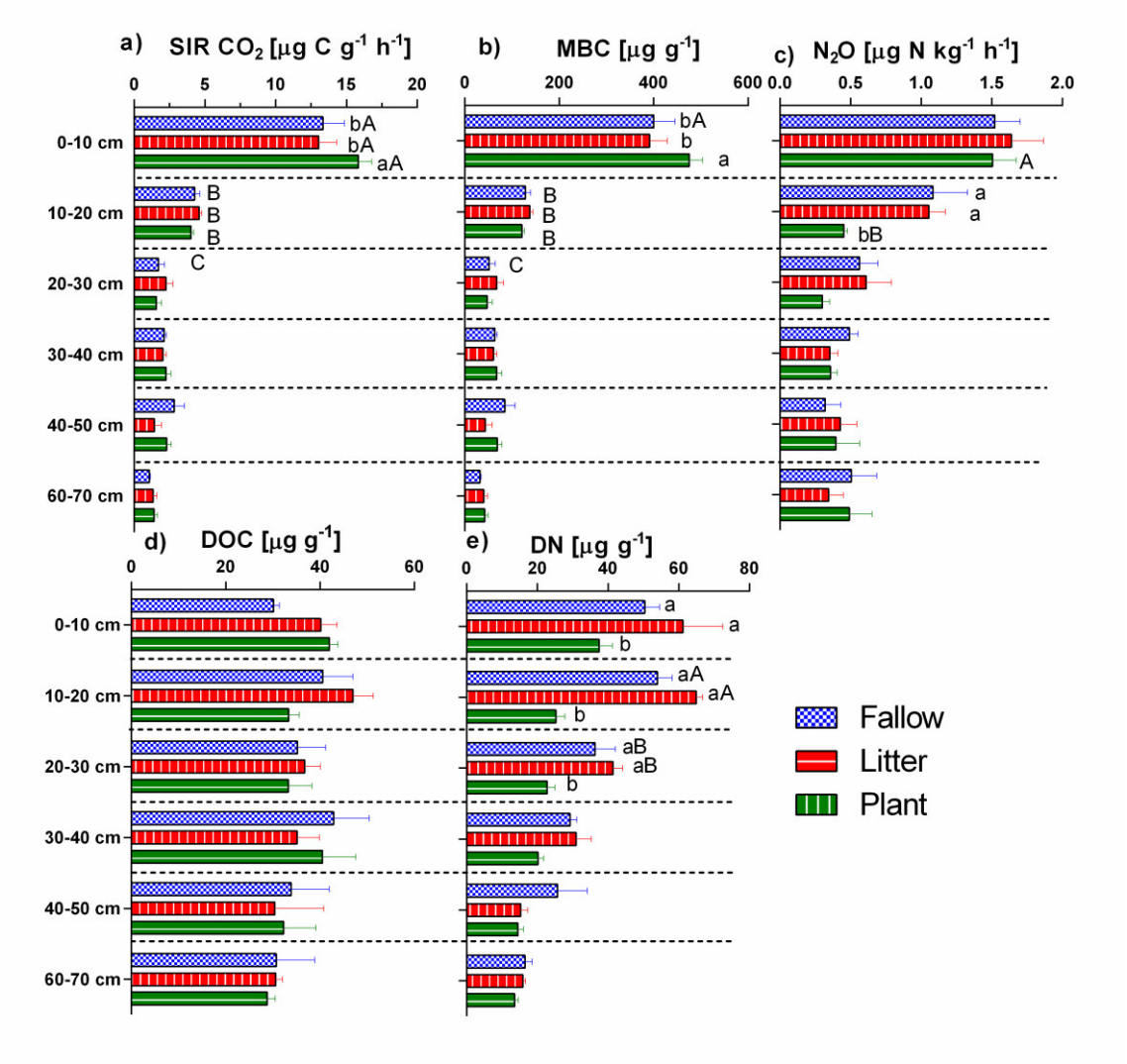


Figure II.3/15 Microbial respiration CO_2, b) microbial biomass, c) N_2O d) dissolved organic C (DOC) and e) dissolved N contents (DN) with depth for bare fallow (Fallow), litter-amended (Litter) and rooted (Plant) soil. Significant differences between the treatments are indicated by lower case letters. Capital letters are used to show significant differences with depth ($P<0.05$).

3.3.2 Dissolved organic carbon and extractable nitrogen

Dissolved organic C (DOC) content was significantly ($P<0.05$) higher in the surface layer of planted compared to fallow soil, indicating the importance of labile C in the rhizosphere (Figure II.3/15 c). Extractable nitrogen (EN) content was reduced in planted soil compared to litter-amended soil by 39, 61, and 45% at 0–10, 10–20, and 20–30 cm depths,

respectively. EN content declined from 10–20 to 20–30 cm depth for litter-amended and fallow soil by 37 and 33%, respectively (Figure 1d).

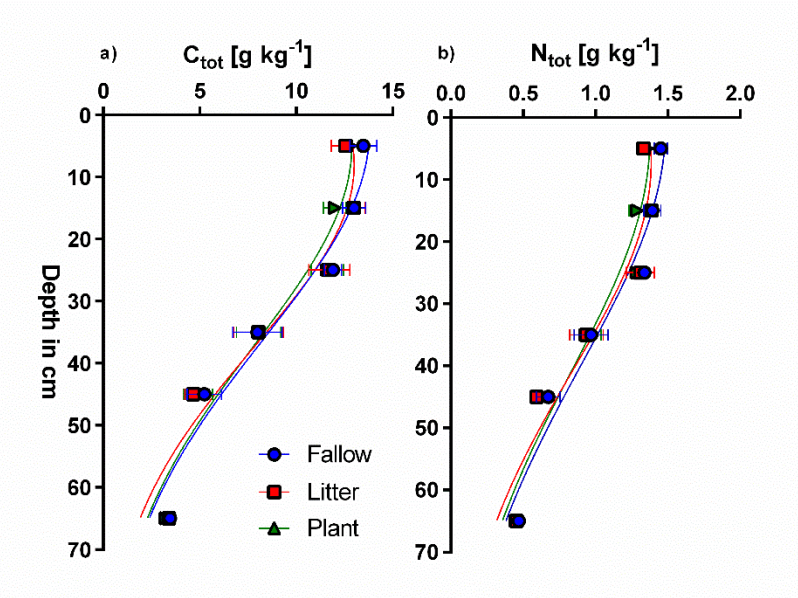


Figure II.3/16 Total carbon (C_{tot}) and nitrogen (N_{tot}) contents with depth for bare fallow (Fallow), litter-amended (Litter) and rooted (Plant) soil.

3.3.3 Soil enzymes

3.3.3.1 Enzyme indexes

It is assumed that the sum of major C-acquiring enzyme activities is a better indicator of total C-acquisition than BG alone. Therefore, we compared proportional enzyme activities (Table II.3/9; Index 4) between the treatments and with depth. When we reduce the information on TAC by using only BG as C-acquiring enzyme (V_{max}(BG)/[V_{max}(BG)+V_{max} (AP)]) and compare it with the proportions of all measured C-acquiring enzymes (TAC/(TAC+TAP)), lower values were obtained when only BG was used as a representative for C-cycling hydrolases in the surface layer of litter-amended and bare fallow soil (Figure II.3/17 a, b). This was constant with specific enzyme activities of CE and BX, which were respectively 88 and 69% lower for planted than for litter-treated soil at 0–10 cm depth, reflecting strong cellulolytic decomposition of plant litter (Supplementary Table II.3/10). In deeper soil layer this effect disappeared.

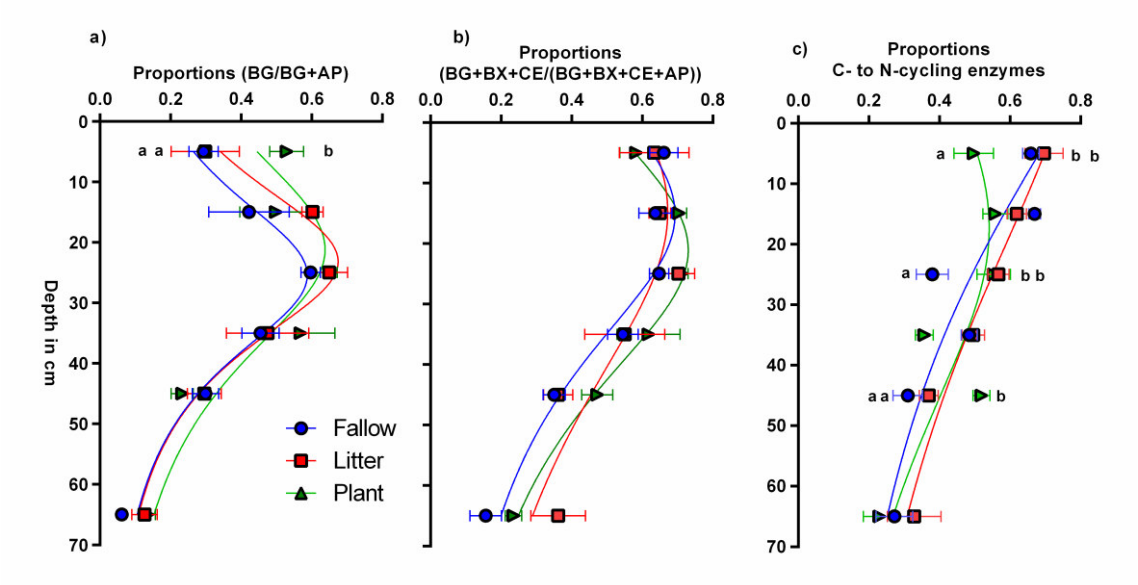


Figure II.3/17 Proportions of C- to P- cycling enzymes with depth for fallow (F), litter-amended (L) and rooted soil (P). a) Proportions of β-glucosidase (BG) to acid phosphatase (AP); b) Proportions of β-glucosidase (BG), β-xylosidase (BX), β-cellobiohydrolase (CE) to acid phosphatase (AP), and c) Proportions of BG, BX, AP to leucine- (LE) and tyrosine- (TY) aminopeptidase. Significant differences between the treatments are indicated by lower case letters (P<0.05).

Potential (V_{max}) and specific (V_s) enzyme activities of LE in the upper 10 cm were higher for planted than for litter-amended and fallow soil (Supplementary Table II.3/10), indicating higher production of proteolytic enzymes. This was confirmed by the sum of N-degrading enzyme activities in the upper 20 cm, which was 41 (0–10 cm) and 43% (10–20 cm) higher in planted soil than in litter-amended soil (Figure II.3/18). The higher proteolytic activity in planted soil was corroborated by lower proportional enzyme activities (TAC/(TAC+TAN)) and lower V_{max} ratio of C- to N-cycling enzymes (TAC/TAN; Figure 4). In the upper 20 cm, a higher activity ratio (P<0.05) of C- to N-cycling enzymes was determined for litter-treated than for planted soil (Figure II.3/18). Vector length (Equation 2) and angle (Equation 3) did not show evidence for N limitation in planted soil, but rather C limitation (30–40 cm) (Supplementary Figure II.3/20).

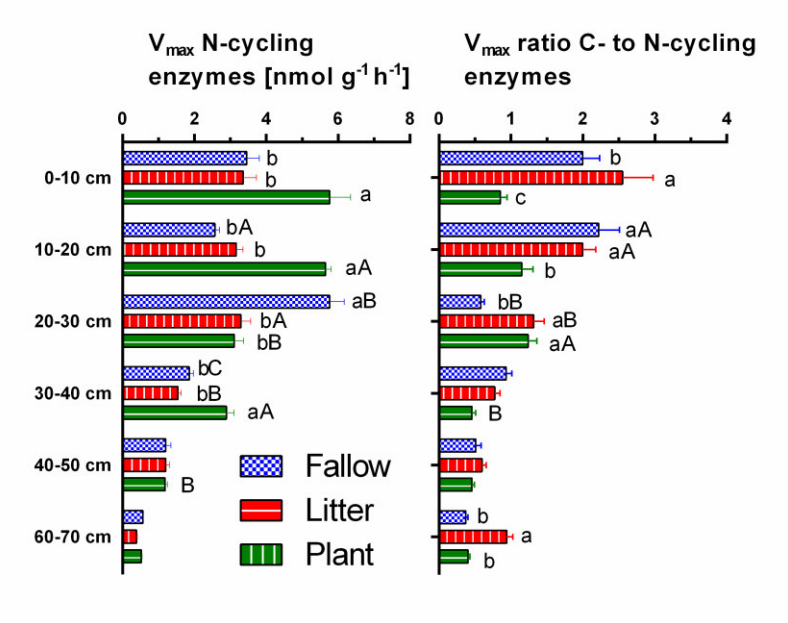


Figure II.3/18 Sum of potential activity rates (V_{max}) of N-cycling enzymes (left); V_{max} ratio between C-cycling (β-glucosidase (BG), β-xylosidase (BX), β-cellobiohydrolase (CE) and N-cycling enzymes (leucine- (LE) and tyrosine- (TY) aminopeptidase) with depth for bare fallow (Fallow), litter-amended (Litter) and rooted (Plant) soil. Significant differences between the treatments are indicated by lower case letters. Capital letters were used to show significant differences with depth ($P<0.05$).

3.3.3.2 Catalytic efficiency

At each depth, the sum of specific enzyme activities, the sum of catalytic efficiencies, and the proportional enzyme activities (TAC/(TAC+TAN)) were computed and tested for correlation to EN and DOC content (Supplementary Table II.3/12). The sum of catalytic efficiencies was better correlated to EN and DOC across all treatments and depths than the sum of specific enzyme activities. Furthermore, the sums of catalytic efficiencies were strongly correlated with the EN for all soils throughout the profile (Supplementary Table II.3/12), with the strongest relationship for planted soil (e.g. $r^2=0.97$, $P<0.01$). The mean catalytic efficiency of enzymes decreased 2- to 20-fold from top- (< 40 cm) to subsoil (> 40 cm), reflecting the lower substrate quality with increasing depth (Figure II.3/19). The catalytic efficiency of TY was higher in planted than in litter-amended topsoil. This indicated highly efficient action of specific aminopeptidases with strong affinity to the substrate in the upper 40 cm of planted soil.

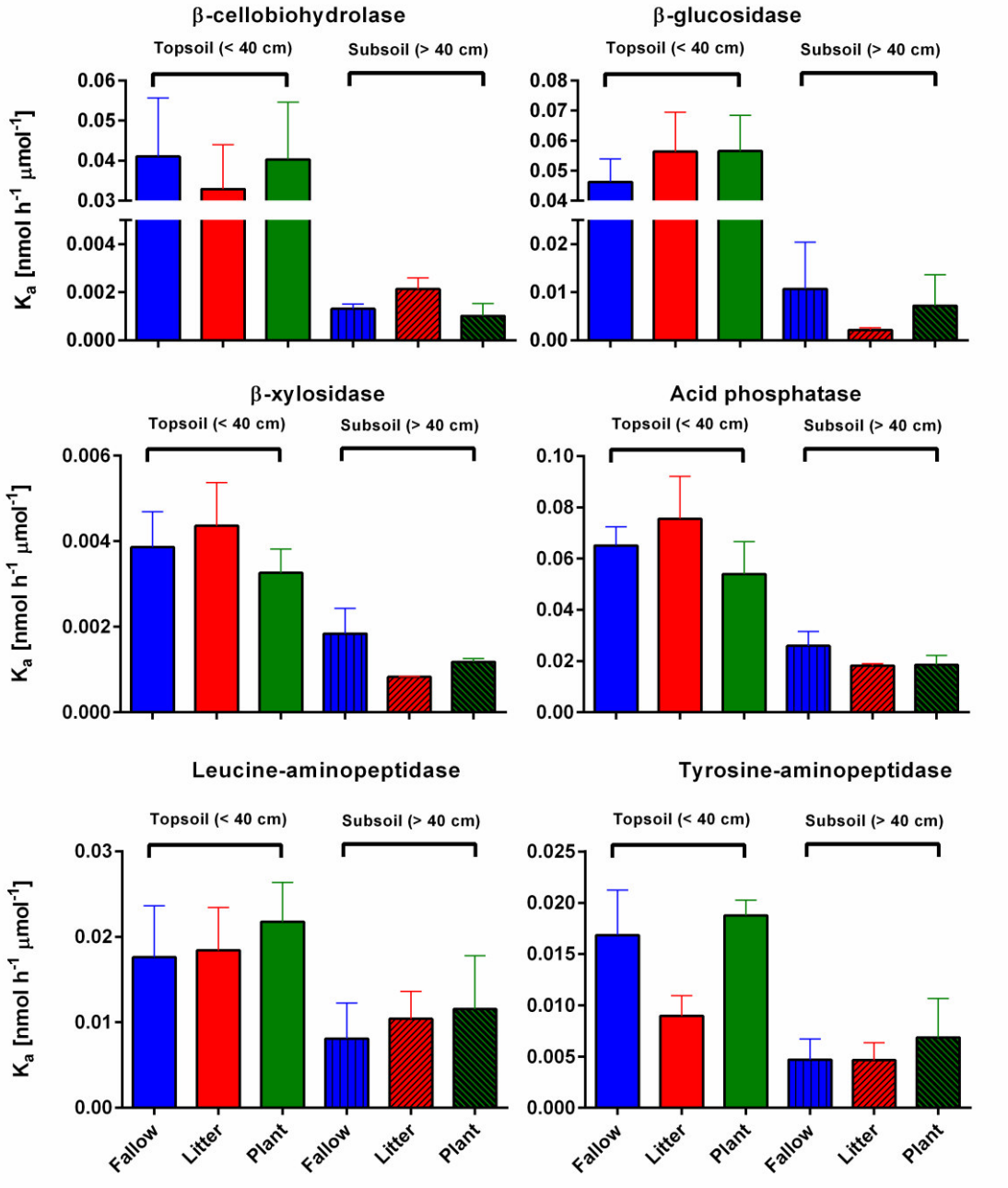


Figure II.3./19 Mean catalytic efficiency (K_a) of C-, N- and P-cycling enzymes for top- (< 40 cm) and subsoil layer (> 40 cm) in bare fallow (Fallow), litter-amended (Litter), and rooted (Plant) soil.

3.4 Discussion

The contrasting substrates (rhizodeposits vs. litter) affected soil microbial activity indicators only in the upper 40 cm, reflecting strong dependence of microbial activities on C and N sources (Šnajdr et al., 2008).

3.4.1 Enzyme indexes

This research provides insights into distinct profiles of soil enzyme indexes as influenced by the rhizosphere and the detritusphere. We compared the applicability of several indexes proposed as indicators of microbial nutrient limitation (Allison and Vitousek, 2005; Moorhead et al., 2016; Sinsabaugh and Follstad Shah, 2012). Proportional activities of C- versus N-cycling enzymes (Table II.3/9; Index 4) and the relative V_{max} of these enzymes (Table II.3/9; Index 3) showed similar patterns with depth. Rhizodeposition may have decreased the relative Vmax and the proportional activity of C- to N-cycling enzymes compared to litter-amended and fallow topsoil, reflecting increased microbial N acquisition in planted soil, due to mining of additional N from SOM (Kuzyakov, 2002; Luo et al., 2006). This suggested that enzyme production was induced by resource limitation.

Specific enzyme activities (V_s) of acid phosphatase increased almost 3-fold from 0–10 to 10–20 cm in planted and bare fallow soil. Higher phosphatase activities in soil enhance the mineralization of organic phosphates when P is limited (Olander and Vitousek, 2000).

Vector length as a measure of C limitation, and vector angle as a measure of P vs. N limitation, did not show any pattern between the treatments in the upper 30 cm (Supplementary Figure II.3/20). However, lower proportions calculated as $V_{max}(BG)/[V_{max}(BG)+V_{max}(AP)]$ relative to TAC/(TAC+TAP), demonstrated that the use of one single enzyme biased the assessment of substrate utilization. When three C-cycling enzymes were considered, the activities of BX and CE counterbalanced the low activities of BG in the detritusphere. Therefore, artificial enzyme indexes, which do not consider

the great redundancy and complex interactions in the suite of soil enzymes, fail to adequately reflect the biological background and mechanisms.

Under natural conditions, enzymes interact with each other in order to utilize the complex substrate structures. The activities of cellulolytic, proteolytic and chitinolytic enzymes are usually assigned to the C- and N-cycles, respectively. A single enzyme, such as BG, for example, is responsible for terminal steps in the decomposition of both cellulose and bacterial/fungal peptidoglycan (Beier and Bertilsson, 2014; Humann and Lenz, 2009; Park and Uehara, 2007), and hence participates in both C and N elemental cycles. Thus, the interpretation of multiple enzyme indexes requires a certain degree of caution.

3.4.2 Top- vs. subsoil

The decrease of microbial biomass C down the soil profile is connected with decreasing availability and quality of organics (Blume et al., 2002; Fierer et al., 2003a, 2003b; Trumbore, 2000). Roots provide easily available C to the microbial community (Nguyen, 2003), which mobilizes nutrients from sources unavailable to plants (Kuzyakov and Xu, 2013). At the same field site more than 50% of the roots were distributed in the upper 10 cm of the Ap horizon and the weighted average root biomass C declined with depth, from 104 kg C ha^{-1} at the 0−10 cm depth to 15 kg C ha^{-1} at the 40−50 cm (Pausch et al., 2013). About 20% of the C assimilated by maize is transferred to below-ground pools at this arable field site (Pausch et al., 2015). The C transferred below-ground by the roots was immediately utilized by microbes in the upper 10 cm, as recently shown by increased specific growth rates (Loeppmann et al., 2015). The effect of diminishing substrate availability with depth on EN and N_2O production was significant only in the upper 30 cm, which reflected that the arable topsoil under maize cultivation is a hot spot for microbial decomposition. Consequently, substrate quality plays an important role in controlling the vertical distribution of enzymes.

In deeper soil layers, the amount and quality of substrate were reduced, which was reflected in the differences between top- (0–40 cm) and subsoil (> 40 cm) for most of the measured indicators of microbial activity, especially in the presence of plants. For example, all potential enzyme activities declined from top- to subsoil (Supplementary Table

II.3/10), as frequently shown before (Gelsomino and Azzellino, 2011; Snajdr et al., 2008; Steinweg et al., 2013). However, the catalytic efficiency down the soil profile was not considered in most of these studies. The catalytic efficiency of enzymes (K_a) describes the specific rate of catalytic reaction, considering the enzyme affinity to the substrate (K_m). The K_a decreased by 2- to 20-fold from top- (< 40 cm) to subsoil, irrespective of the substrate input (Figure II.3/19). The variation of K_m implied that enzyme-specific efficiencies of substrate utilization are strongly dependent on the affinity to the substrate. Nevertheless, the decrease of K_a from top- to subsoil indicated that the driving forces were substrate quantity and quality.

3.4.3 Rhizosphere vs. detritusphere

Microbial biomass C and N_2O production in the upper 20 cm showed significant effects of substrate input (rhizodeposits in the rhizosphere and maize-litter in the detritusphere). Particularly, the decrease of N_2O production in planted soil from 0−10 to 10−20 cm may be defined as greater N limitation, which reflected maize as a sink for N. This was in line with lower EN contents in planted soil compared to litter-amended and bare fallow soil (Figure II.3/15 d). Correspondingly, a weak relationship (r^2=0.31) between C_{mic} and SOC was determined in planted soil, which may be explained by co-limitation of nutrients (e.g. N) in the rhizosphere. However, the strong correlations between Cmic and N_2O production, indicated that N sources for nitrification or denitrification were not the limiting factor for N_2O production. Eventually, O_2 limitation occurred during SIR, which was proportional to the size of the microbial biomass, and mainly controlled the N_2O efflux from the soil. Since N_2O production is mediated by both biotic and abiotic processes and by oxygen availability, the link between soil organic matter degradation and N_2O production is not always straight forward (Blagodatskaya et al., 2014). Moreover, the N_2O reflected the total mineralized N, which strongly varies depending on the substrates used by microorganisms (Zhu et al., 2013).

In the presence of plants, EN, N_2O, V_{max} ratio and proportional activity of C- to N-cycling enzymes were lower than in litter-amended soil at 0–10 and 10–20 cm depths. However,

the catalytic efficiency of tyrosine aminopeptidase strongly increased in planted compared to litter-amended topsoil. This suggested strong effects of N limitation on the decomposer community in the presence of plants (Vitousek and Howarth, 1991). N limitation induced a shift in the catalytic properties of proteolytic enzymes (leucine- and tyrosine-aminopeptidases) which was in accordance with previous studies (German et al., 2011; Rejsek et al. 2008; Sims and Wander, 2002). This reflected higher investment in N-releasing enzyme production in planted soil than in litter-amended and fallow soil (Phillips et al., 2011; Stursova et al., 2006). It also confirmed the production of proteolytic enzymes with high substrate affinity (revealed by low K_m) by competitive microorganisms (Supplementary Table II.3/11) and reutilization of microbial residues for maintenance when nutrients are limited (Bradford, 2013).

Cellulolytic specific enzyme activity was up to 10-fold higher in the litter-amended than in the planted surface layer (Supplementary Table II.3/11). Maize litter may stimulate the decomposition of lignocellulosic materials by fungal communities and their enzymes (Kramer et al., 2012; Moll et al., 2015).

The C:N or the lignin:N ratio of plants is often used as a measure of litter quality and a predictor of decomposition rate, but the role of N in the regulation of litter decomposition is too complex to be characterized by measures of total N concentration (Sinsabaugh et al., 1993; Tian and Shi, 2014). Instead, we determined the activity ratio between C- and N-cycling enzymes (Figure II.3/18 b), which was lower for planted than for litter-treated soil throughout the soil profile. This can be explained by relatively greater access to readily utilized labile root C sources, because N-limitation is defined with reference to relative N vs. C availability, suggesting better nutrient supply for microbes in the detritusphere (Šnajdr et al., 2011). Similarly, the idea of a "better" N supply for microbes in the detritusphere is based on C:N enzyme ratios and remains a relative concept. The recalcitrant substrates (e.g. lignin and tannin) in the detritusphere may affect organic N mineralization (Valenzuela-Solano and Crohn, 2006).

3.5 Conclusions

The availability of C and nutrients in soil and especially in the rhizosphere and detritusphere strongly affected the microbial biomass and the catalytic efficiency (K_a) of hydrolytic enzymes with depth-dependent contrasting patterns. Dissolved N is decisive for enzyme activities, and decreases with depth. In particular, under root-induced N limitation, proteolytic enzymes had increased activity and affinity to substrate, which reflected the energy investment of microorganisms for nutrient acquisition. Enzymes´ catalytic efficiency decreased 2- to 20-fold from top- (< 40 cm depth) to subsoil. The contrasting input and quality of substrates in rhizosphere and detritusphere influenced microbial decomposition only in the topsoil (0–40 cm), whereas in the subsoil (> 40 cm depth) the effects of contrasting substrate input disappeared. Proportions of multiple enzyme activities as well as catalytic efficiencies reflected both stoichiometric and C-quality effects on decomposer communities.

Acknowledgements

We thank Susann Enzmann, Heike Strutz and Viola Schade for their intensive help during the field and laboratory work. This study was supported by the German Research Foundation (DFG) within the Research Unit “Carbon Flow in Belowground Food Webs assessed by Isotope Tracers”.

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Supplementary

Table II.3/10 Potential enzyme activities (V_{max}±SEM) and specific enzyme activities with depth for fallow, litter-amended and rooted soil.

Depth	Vmax		Specific enzyme activity		Vmax		Specific enzyme activity		Vmax		Specific enzyme activity	
cm	nmol h^{-1} g^{-1}		nmol h^{-1} μg^{-1} MBC		nmol h^{-1} g^{-1}		nmol h^{-1} μg^{-1} MBC		nmol h^{-1} g^{-1}		nmol h^{-1} μg^{-1} MBC	
	Fallow	*SEM*	*Fallow*	*SEM*	*Litter*	*SEM*	*Litter*	*SEM*	*Rooted*	*SEM*	*Rooted*	*SEM*
Beta-cellobiohydrolase												
0–10	5.13	0.44	0.0128	0.0018	5.94	1.05	0.0152	0.0031	0.88	0.04	0.0019	0.0001
10–20	2.37	0.38	0.0185	0.0034	0.79	0.07	0.0058	0.0005	2.69	0.45	0.0222	0.0038
20–30	0.52	0.03	0.0102	0.0027	0.83	0.09	0.0123	0.0028	0.63	0.04	0.0135	0.0033
30–40	0.34	0.03	0.0053	0.0006	0.27	0.04	0.0044	0.0009	0.17	0.02	0.0026	0.0005
40–50	0.05	0.00	0.0006	0.0002	0.08	0.00	0.0018	0.0006	0.05	0.00	0.0008	0.0001
60–70	0.07	0.01	0.0022	0.0003	0.14	0.02	0.0035	0.0009	0.04	0.00	0.0009	0.0002
Beta-glucosidase												
0–10	1.45	0.12	0.0036	0.0005	1.73	0.22	0.0044	0.0007	3.71	0.21	0.0078	0.0006
10–20	2.97	0.56	0.0232	0.0048	5.20	0.47	0.0377	0.0038	3.48	0.74	0.0287	0.0063
20–30	2.72	0.12	0.0527	0.0135	3.38	0.31	0.0498	0.0111	2.98	0.19	0.0634	0.0155
30–40	1.28	0.11	0.0201	0.0023	0.83	0.07	0.0136	0.0019	1.07	0.10	0.0158	0.0030
40–50	0.51	0.04	0.0060	0.0016	0.56	0.04	0.0131	0.0046	0.43	0.02	0.0063	0.0010
60–70	0.07	0.01	0.0021	0.0002	0.08	0.01	0.0021	0.0004	0.13	0.01	0.0032	0.0006
Leucine												
0–10	2.51	0.33	0.0063	0.0011	2.43	0.35	0.0062	0.0011	4.78	0.57	0.0100	0.0013
10–20	1.26	0.08	0.0098	0.0011	1.88	0.17	0.0136	0.0013	4.05	0.15	0.0335	0.0019
20–30	3.67	0.22	0.0711	0.0185	2.45	0.19	0.0362	0.0078	1.34	0.15	0.0285	0.0075
30–40	0.76	0.09	0.0120	0.0017	0.75	0.09	0.0123	0.0020	2.14	0.21	0.0316	0.0059
40–50	0.78	0.14	0.0093	0.0029	0.58	0.08	0.0136	0.0051	0.68	0.07	0.0099	0.0017
60–70	0.33	0.01	0.0102	0.0007	0.21	0.01	0.0051	0.0011	0.29	0.01	0.0068	0.0011
Acid phosphatase												
0–10	3.84	0.26	0.0096	0.0013	5.95	0.65	0.0152	0.0022	4.15	0.43	0.0087	0.0010
10–20	3.41	0.41	0.0267	0.0039	3.63	0.37	0.0263	0.0029	2.77	0.21	0.0229	0.0020
20–30	1.90	0.14	0.0368	0.0097	1.74	0.10	0.0256	0.0054	1.64	0.14	0.0348	0.0087
30–40	1.47	0.14	0.0230	0.0028	1.13	0.15	0.0186	0.0033	0.77	0.06	0.0114	0.0021
40–50	1.28	0.09	0.0152	0.0041	1.29	0.08	0.0299	0.0105	1.42	0.09	0.0206	0.0033
60–70	0.74	0.09	0.0228	0.0030	0.70	0.09	0.0173	0.0041	0.89	0.13	0.0209	0.0046
Tyrosine												
0–10	0.94	0.08	0.0024	0.0003	0.92	0.11	0.0024	0.0004	0.98	0.08	0.0021	0.0002
10–20	1.32	0.10	0.0103	0.0012	1.29	0.08	0.0093	0.0007	1.60	0.05	0.0132	0.0007
20–30	2.10	0.35	0.0406	0.0123	0.84	0.20	0.0124	0.0039	1.76	0.21	0.0375	0.0099

Table II.3/10 Potential enzyme activities (V_{max}±SEM) and specific enzyme activities with depth for fallow, litter-amended and rooted soil.

Depth cm	Vmax nmol h^{-1} g^{-1}		Specific enzyme activity nmol h^{-1} μg^{-1} MBC		Vmax nmol h^{-1} g^{-1}		Specific enzyme activity nmol h^{-1} μg^{-1} MBC		Vmax nmol h^{-1} g^{-1}		Specific enzyme activity nmol h^{-1} μg^{-1} MBC	
	Fallow	*SEM*	*Fallow*	*SEM*	*Litter*	*SEM*	*Litter*	*SEM*	*Rooted*	*SEM*	*Rooted*	*SEM*
30–40	1.09	0.07	0.0172	0.0017	0.80	0.04	0.0131	0.0016	0.75	0.04	0.0112	0.0019
40–50	0.41	0.03	0.0049	0.0013	0.62	0.06	0.0144	0.0052	0.50	0.03	0.0072	0.0011
60–70	0.23	0.01	0.0072	0.0006	0.19	0.01	0.0046	0.0010	0.24	0.01	0.0056	0.0009
Beta-xylosidase												
0–10	0.32	0.03	0.0008	0.0001	0.89	0.09	0.0023	0.0003	0.31	0.02	0.0006	0.0001
10–20	0.38	0.03	0.0030	0.0003	0.31	0.02	0.0023	0.0002	0.34	0.02	0.0028	0.0002
20–30	0.11	0.00	0.0021	0.0005	0.11	0.01	0.0016	0.0003	0.23	0.02	0.0049	0.0012
30–40	0.12	0.01	0.0018	0.0002	0.10	0.01	0.0016	0.0002	0.08	0.00	0.0012	0.0002
40–50	0.05	0.00	0.0006	0.0002	0.08	0.00	0.0018	0.0006	0.05	0.00	0.0008	0.0001
60–70	0.07	0.01	0.0022	0.0003	0.14	0.02	0.0035	0.0009	0.04	0.00	0.0009	0.0002

Table II.3/11 Half-saturation constant (K_m±SEM) for fallow, litter-amended and rooted soil.

Depth cm	Km µmol		Km µmol		Km µmol	
	Fallow	*SEM*	*Litter*	*SEM*	*Rooted*	*SEM*
Beta-cellobiohydrolase						
0–10	70.2	13.9	114.9	38.0	15.1	3.2
10–20	40.7	18.6	15.3	5.7	38.2	18.7
20–30	25.6	6.1	46.7	11.0	28.5	6.7
30–40	27.4	9.1	27.2	15.4	16.9	9.5
40–50	48.2	17.2	46.4	14.2	35.6	10.6
60–70	47.2	16.6	54.8	16.3	78.7	28.4
Beta-glucosidase						
0–10	48.9	10.6	31.0	13.1	43.2	6.2
10–20	45.0	23.4	65.1	13.6	67.3	33.3
20–30	55.4	6.3	48.4	12.0	49.6	8.4
30–40	31.8	8.0	42.0	8.1	37.6	11.8
40–50	24.8	8.1	37.7	8.7	31.7	5.5
60–70	67.3	14.6	129.3	21.8	182.0	27.2
Leucine						
0–10	326.0	52.3	162.3	24.6	149.9	33.9
10–20	38.5	7.5	103.4	19.8	232.1	14.7
20–30	166.3	20.3	76.2	15.1	119.2	31.0
30–40	94.7	27.0	89.6	25.4	80.7	19.6

Table II.3/11 Half-saturation constant (Km±SEM) for fallow, litter-amended and rooted soil.

cm	Km µmol g^{-1}		Km µmol g^{-1}		Km µmol g^{-1}	
	Fallow	*SEM*	*Litter*	*SEM*	*Rooted*	*SEM*
Leucine						
40–50	198.8	58.4	81.1	24.5	129.5	23.0
60–70	27.1	3.6	15.2	3.8	16.1	3.4
Acid phosphatase						
0–10	61.9	13.8	54.9	10.3	48.2	7.6
10–20	40.8	6.6	38.4	8.5	68.2	12.5
20–30	28.6	7.1	26.4	5.4	26.9	8.2
30–40	30.3	9.5	33.7	14.3	27.6	7.9
40–50	40.7	9.5	68.1	11.4	95.4	15.3
60–70	36.3	13.4	39.9	15.1	39.9	17.9
Tyrosine						
0–10	110.2	22.3	170.9	40.2	68.6	14.8
10–20	130.9	22.5	113.2	15.7	78.0	6.4
20–30	87.5	29.8	143.4	60.6	84.6	21.5
30–40	44.1	8.7	59.9	8.9	38.9	7.6
40–50	154.1	21.2	210.3	39.9	161.1	17.8
60–70	34.7	7.7	29.2	8.5	22.3	2.9
Beta-xylosidase						
0–10	61.4	14.1	199.5	37.5	63.6	9.9
10–20	149.5	22.8	93.8	13.4	106.6	17.0
20–30	20.6	4.2	15.4	4.7	91.5	19.4
30–40	49.5	13.1	39.7	10.6	32.4	6.7
40–50	22.0	8.1	94.1	13.4	49.2	7.6
60–70	57.3	19.6	170.8	42.7	31.0	11.9

Table II.3/12 Pearson correlation coefficients between a) the sum of measured specific enzyme acitivities, b) the sum of measured catalytic efficiencies of enzymes and c) proportions of C- to N-cycling enzymes (explained in the text); both to either dissolved organic nitrogen (DN) or dissolved organic carbon contents (DOC) for fallow, litter-amended and rooted soil. Significant differences between treatments are indicated by asterisks (P<0.01).

a) Specfic enzyme activities		**DN**			**DOC**	
	Fallow	*Litter*	*Rooted*	*Fallow*	*Litter*	*Rooted*
Fallow	0.45			-0.02		
Litter		0.69			0.53	
Plant			0.56			0.22
b) Catalytic efficiencies		**DN**			**DOC**	
	Fallow	*Litter*	*Rooted*	*Fallow*	*Litter*	*Rooted*
Fallow	0.94*			0.22		
Litter		0.94*			0.91	
Plant			0.97*			0.80
c) Proportions C- to N-cycling enzymes		**DN**			**DOC**	
	Fallow	*Litter*	*Rooted*	*Fallow*	*Litter*	*Rooted*
Fallow	0.93			0.30		
Litter		0.95			0.84	
Plant			0.46			0.19

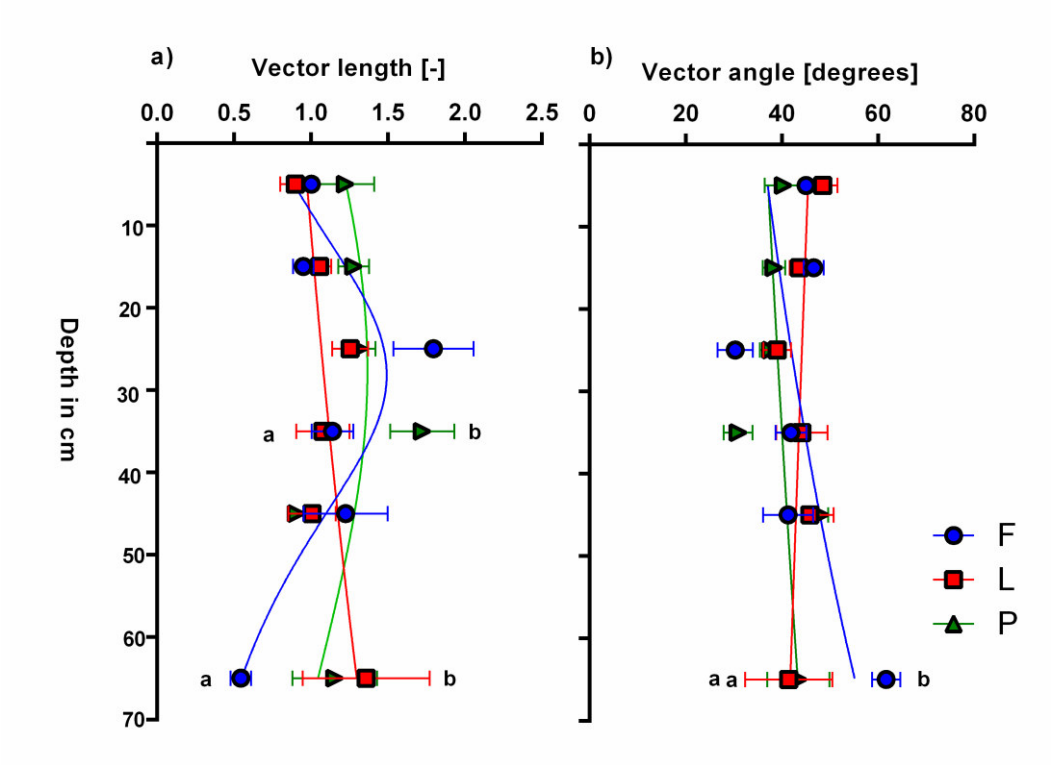

Figure II.3/20 Vector length and b) vector angle for fallow (F), litter-amended (L) and rooted soil (P) with depth. Calculations and interpretation for vector length and vector angle are described in the text. Significant differences between the treatments are indicated by lower case letters (P<0.05).

4. Towards to physiological status of soil microorganisms determined by RNA:dsDNA ratio

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In preparation for submission to *Ecological Indicators*

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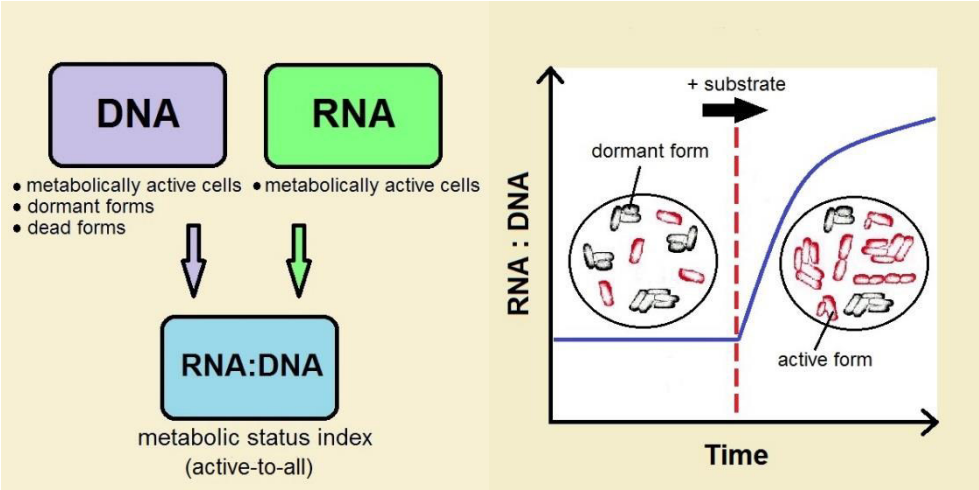
Graphical abstract



Abstract

Despite soil microorganisms spend most of their lifetime at dormant or resting states, they are quickly activated by substrate input and easily switch to growth. As both the dsDNA- and RNA-contents increase during microbial growth, the RNA:dsDNA ratio should be a useful predictor, whether the response of a microbial community to environmental changes results from an increase in population (by dsDNA) or in activity (by RNA). This prediction of the RNA:dsDNA ratio can be accomplished by the comparison of microbial respiration approaches with and without addition of easily available substrates. We exhibited the RNA:dsDNA ratios in four contrasting soil types during substrate-induced growth. After glucose addition, a strong increase of dsDNA and RNA contents were determined in most of the soil types during 72 h of incubation. Furthermore, we identified distinct temporal soil-specific RNA:dsDNA patterns. The dsDNA- and RNA-contents yielded 26–174 and 0.3–30 µg g^{-1} soil, respectively. The soil texture was strongly associated with the reduction of RNA recovery, by means of an exponential decrease of RNA-content with increasing clay content. The lower RNA recovery in virgin and arable Chernozem (>30%) compared to soil types with lower clay contents (<17% for Retisol, Luvisol and Calcisol) suggests, that the underestimation of RNA yields in soils, exceeding 30% clay content, biased the RNA:dsDNA ratio, and subsequently the physiological state of the microbial community is not adequately represented.

Keywords: dsDNA yield, RNA yield, nucleic acids, RNase, particle size distribution, glucose amendment, microbial growth

4.1 Introduction

Bulk soil is regarded as an oligotrophic environment, as it is generally poor in labile organic compounds (Van Elsas and van Overbeek, 1993). This low amount of readily-available organic carbon (C) precludes slow bacterial growth and low activity. Many soil organisms, therefore, have very low rates of metabolic activity and frequently spend most of their lifetime in dormant or resting phases, especially in soils of low C and N contents (Sparling et al., 1981; Van Elsas and van Overbeek, 1993). The input of readily assimilated C substrates (e.g. sugars) either shifts microbial population from dormancy to activity, thereby strongly accelerates microbial metabolism, and may induce microbial growth, which leads to increasing DNA- and RNA- contents. Growing microbial cells are actively dividing, whereas active cells are measurably metabolizing, but are not necessarily dividing (Blazewicz et al. 2013; Jones and Lennon, 2010).

DNA and RNA molecules perform the storage of genetic information and the translation of this genetic information for protein synthesis, correspondingly. The DNA extracted from soil in relatively large amounts represents organisms at any physiological state – dead, dormant or active (Levy-Booth et al., 2007; Blagodatskaya and Kuzyakov, 2013). In contrast to DNA, the RNA content in dormant cells is extremely low, while it increases dramatically after microbial activation. Since the amount of RNA per cell is proportional to metabolic activity of microorganisms (Mills et al., 2004; Molin and Givskov, 1999; Penannen et al., 2004), the RNA-based approaches provide information on the metabolically active pools of microbial community. More than a hundred studies are available using rRNA to identify currently active microorganisms in batch studies but also in the marine and terrestrial environment (Blazewicz et al., 2013; Hunt et al., 2013; Jones and Lennon, 2010, Wu et al., 2011). The RNA:dsDNA ratio, therefore, is a promising indicator of the metabolic status of bacterial (Dell'Anno et al., 1998; Kerkhof and Ward, 1993; Muttray and Mohn, 1999) and of microbial communities as a whole (Hahn et al., 1990; Tsai et al., 1991).

Determination of DNA content in soil is well established (Marstorp and Witter, 1999; Blagodatskaya et al., 2003) and is possible by application of commercially available kits

(Fornasier et al., 2014). Quantitative extraction of microbial DNA from soil (Marstorp et al., 2000) can be used as a measure of total microbial biomass (Joergensen and Emmerling, 2006; Renella et al., 2006). Most successful quantitative DNA extraction is based on cells destruction with FastPrep system accompanying by highly sensitive PicoGreen staining enabling strong dilution of DNA samples and therefore minimizing the biases due to humic acids (Blagodatskaya et al., 2003). Such extraction is more precise and differs from DNA determination by NanoDrop, which is more suitable for community structure studies. The positive linear correlation between dsDNA content and total microbial biomass was already confirmed by the number of studies (Blagodatskaya et al., 2003; Anderson and Martens, 2013).

Relatively stable conversion factor from units dsDNA to units microbial C in a narrow range of 5.0 (Anderson and Martens, 2013), 5.4 (Blagodatskaya et al., 2003) and 5.6 (Lloyd-Jones and Hunter, 2001) has been frequently revealed. A review of various studies also showed an averaged conversion factor from dsDNA into microbial biomass of 6, which indicates that approximately 13% of microbial C stems from DNA (Joergensen and Emmerling, 2006).

Commonly, the RNA is more difficult to extract from soil than DNA and quantitative extraction of soil RNA comprises a number of challenges. The RNA pool of a microbial cell is mainly composed of rRNA (82–90%) (Neidhardt, 1987). The RNA recovery from soil still remains very low and rarely exceeds 10% (Duarte et al., 1998). The RNA yields extracted from soil range from tens of nanograms to several micrograms per gram of soil (Borneman et al., 1997; Bürgmann et al., 2003; Mettel et al., 2010; Moran et al., 1993; Sessitsch et al., 2002; Wang et al., 2008; 2009). Such a wide range of RNA yield may be caused by interaction of several factors, such as activity state of soil microorganisms, contamination of RNA sample by humic substances or the loss of RNA during purification (Wang, 2012). Furthermore, the strong losses of RNA during isolation may be caused by an RNase activity and by adsorption to the soil clay fraction. The RNA:dsDNA ratio is not only affected by biotic factors, such as the substrate quality, but also strongly decrease with higher clay-contents, caused by immobilization mechanisms of nutrients and organo-mineral associations (Vogel et al., 2014). Although, both DNA and RNA could be adsorbed by soil particles (Goring and Bartholomew, 1952), the adsorption of single-

strand RNA molecules can be especially strong in soils with clay and clay-loam texture (Tournier et al., 2015). The main restrictions of modern RNA isolation methods refer to soils with high clay content (Novinscak and Filion, 2011). Despite low recovery, the relative changes in RNA content within same soil type can shed light on shifts in physiological state of soil microorganisms (Bakken and Frostegard, 2006; Blagodatskaya and Kuzyakov, 2013). Since not all active microorganisms are growing, but all the growing microorganisms are active (Blazewicz et al., 2013), a differentiation between microbial growth and activity in soils remains a great challenge in order to identify the underlying mechanisms of soil microbial communities functioning.

We hypothesized that 1) the growth of microorganisms is strongly dependent on the C and N status of the respective soil type and 2) strong growth of microorganisms subsequently indicates high microbial activity.

To test these hypotheses we determined the RNA:dsDNA ratio along a climatic gradient with five zonal soil types (Figure I.2./3) varying in pH, soil organic carbon content (C_{org}), soil nitrogen content (N_{tot}) and particle size distribution. Virgin and arable Chernozems were characterized by the highest C_{org}, N_{tot}, microbial biomass (C_{mic}) and soil C:N ratios. Retisol and Luvisol had almost similar soil properties, and Calcisol was lowest in C_{org}, N_{tot} and microbial biomass. Thus, these five soils represent the reduced enrichment gradient of C and N from Chernozems over Retisol and Luvisol to Calcisol. Based on these data, we expected that the highest DNA- and RNA-contents occur in the C and N rich Chernozem, and the lowest in Calcisol. We also switched physiological state of soil microorganisms by addition of glucose in order to reveal shifts of the RNA:dsDNA ratio during microbial growth. These changes of the RNA:dsDNA ratio are linked to the shift in physiological state of microorganisms and we aimed to prove whether this ratio provide reliable prediction on microbial activity and growth in these contrasting soil types.

4.2 Material and Methods

4.2.1 Soils and sampling sites

The dynamics of RNA:dsDNA ratio were tested in top 10 cm-layers of five soils located in European part of Russia: Gleyic Retisol, Luvisol, virgin and arable Chernozem and Haplic Calcisol (IUSS Working Group WRB, 2015). Terrestrial Biomes, precipitation and temperature for these soils are displayed as map (Figure I.2/3).

Retisol was sampled at the bottom (accumulative) part of the slope in Tver region (56°46' N, 36° 3' O). The territory of Tver region is characterized by the areal extent of the various formations of Valdai glaciation: lacustrine, alluvial, fluvioglacial and lacustrine-glacial deposits (Dorofeev, 1992).

The Luvisol was sampled at the top (autonomous) part of the slope at the right bank of the Oka River near the town Pushchino in Moscow region (54°49' N, 37°35' O). The right bank of the river Oka belongs to Zaoksky physiogeographic province, which occupies the northern spurs of the Central Russian Upland, on the border of taiga forest and steppe natural zones (Annenskaya et al., 1997).

Chernozem was sampled in Russian Federal Nature Preserve "Kamennaya Step" located in Talovsky District in Voronezh region, at the watershed of rivers Bitug and Khoper (51°02´ N, 40°72´ O). The territory of the "Kamennaya Step"' is a slightly rolling plain with sloping beams and unformed steppe depressions (Cheverdin, 2013).

Calcisol was sampled in the Astrakhan region. Astrakhan region is located in the south-east of the East European Plain within the Caspian lowland (47°93´ N, 46°11´ O). This region is characterized by a temperate semi-arid climate with large annual and diurnal amplitudes in summer air temperature, low precipitation and high potential evapotranspiration (Pankova et al., 2014).

4.2.2 Soil dsDNA extraction procedure

DNA extraction was performed according to the manufacturer's protocol with 0.5 g of fresh moist soil treated by the FastDNA® SPIN kit for Soil (MP Biomedicals, Germany). Bead beating and a silica matrix were used to isolate DNA from soil. Before extraction, soil samples were placed into a freezer overnight to ensure higher DNA yields. Soils were added to lysing matrix tubes containing silica and glass spheres of different diameters, were treated with sodium phosphate buffer (Na_2HPO_4; pH 8.0, 0.12 M) and MT buffer (1% sodium dodecyl sulfate – SDS, 0.5% Teepol, and PVP40 with EDTA and Tris) were subjected to bead beating in the FastPrep® instrument and processed by protein precipitation solution (150 µL of 3 M CH_3COOK and 4% glacial acetic acid). DNA was bound to a DNA binding matrix (1 mL of glassmilk diluted 1:5 with 6 M guanidine isothiocyanate), washed by a salt ethanol wash solution (SEWS – ultra-pure 100% ethanol and 0.1 M sodium acetate) and finally, eluted in DNase-free water (DES). After extraction, purified DNA samples were immediately measured according to the dsDNA quantification procedure (see below).

4.2.3 Soil RNA extraction procedure

RNA extraction was performed according to the manufacturer's protocol with 0.5 g of fresh moist soil by the FastRNA® Pro Soil Direct kit (MP Biomedicals, Germany). Soil samples were placed to lysing matrix tubes containing silica and glass spheres of different diameters, treated with RNApro™ Soil Lysis Solution provided RNase inhibition, subjected to bead beating in the FastPrep® instrument. Phenol:Chloroform (1:1) solution was added; the upper aqueous phase were taken and processed by Inhibitor Removal Solution and cold 100% isopropanol. After mixing, the solution was incubated for 30 minutes at –20° C, centrifuged, and the pellet was washed by cold 70% ethanol (with DEPC-H_2O). RNAMATRIX Binding Solution and RNAMATRIX Slurry were used to bind RNA molecules. The binding matrix with caught RNA was washed by RNAMATRIX Wash Solution and pure RNA extract was eluted by DEPC-H_2O. Purified RNA samples were immediately measured according to the RNA quantification procedure.

4.2.4 Soil dsDNA and RNA quantification

The quantity of dsDNA obtained in the extract was determined by diluting the extract 150-fold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Aliquots of 0.1 ml were transferred to 96-well microplates (Brand pureGrade, black). For staining the dsDNA a 200-fold dilution of the dsDNA fluorescence dye PicoGreen® (Molecular Probes, Life Technologies, Germany) was prepared in plastic containers. The dye (0.1 mL) was added to the wells with diluted DNA extract (final 300-fold dilution of the extracts) and left to react at 23 °C protected from light for 2 min. Fluorescence intensity was measured with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland) of excitation 485 nm, emission 525 nm and measurement time 1.0 s. The dsDNA of bacteriophage lambda was used as a standard; samples for the standard curve were prepared in TE-buffer in the same way as the experimental samples (Blagodatskaya et al., 2014).

The quantity of RNA obtained in the extract was determined by making a 5-fold dilution of the extract in RNase-free TE buffer (with DEPC-treated water). Aliquots of 0.1 ml were then transferred to 96-well microplates. For staining the RNA a 200-fold dilution of the fluorescence dye RiboGreen® (Molecular Probes, Life Technologies, Germany) in RNase-free TE buffer was prepared in plastic containers. The dye (0.1 mL) was added to the wells with diluted RNA extract (final 10-fold dilution of the extracts) and left to react at 23 °C protected from light for 2 min. Fluorescence intensity was measured with an automated fluorometric plate-reader of excitation 485 nm, emission 525 nm and measurement time 1.0 s. The ribosomal RNA (16S and 23S rRNA from *E. coli*) was used as a standard; samples for the standard curve were prepared in RNase-free TE-buffer in the same way as the experimental samples.

4.2.5 Soil dsDNA and RNA extraction efficiency

To verify DNA and RNA recovery we performed a pre-experiment adding certain amount of DNA and RNA standards to the variants with and without soils in probe. The DNA recovery ranged from 94% to almost 100% for all samples tested. An efficiency of RNA extraction was verified by adding 3 µg of rRNA standard: 1) directly to the soil sample,

2) to the control probe without soil, 3) to the untreated soil already as suspension with the RNApro^{tm} Soil Lysis Solution (inhibitor of RNase) from the kit and, finally, 4) to the threefold autoclaved soil suspension with attached inhibitor solution.

In contrast to DNA extraction, we encountered a problem with RNA stability which was reflected in low percentage of recovery. RNA standard added directly to the soil sample was completely decomposed, e.g. no change in RNA content was detected. This effect was expected when taking into account the activity of air and soil RNases. Transferred RNA standard to the control probe without any soil recovered in average 5.5%. The recovery of the RNA standards added to the untreated soil suspensions with RNase inhibitor varied in a small range between 4 and 6% and averaged at 5.5%. The highest yields (10% of added RNA standard) were revealed for those probes where RNA was added to the suspension of autoclaved soil with RNase inhibitor. Since our experiment was carried out with non-autoclaved samples, we used RNA recovery index of 5.5% for recalculation of total soil RNA yield. Despite low recovery is common for most of RNA studies, the reproducibility of the RNA extraction was high and the data variability was low (5–10%).

4.2.6 Estimation of microbial biomass and basic characteristics of soils

Microbial biomass-C was analyzed by chloroform fumigation-extraction (CFE) (Brookes et al., 1985; Jenkinson and Powlson, 1976; Vance et al., 1987). We extracted the unfumigated soil samples (5 g) with 20 ml of 0.05 M K_2SO_4 and agitated the samples for 1 h with an overhead shaker (40 rev min^{-1}). The same amount of soils was fumigated with ethanol-free chloroform and then extracted in the same way. The fumigation was done in desiccators at 20°C for 24 h (Friedel and Scheller, 2002; Joergensen and Mueller, 1996). After 5 min centrifugation of the soil suspension at 2500 × g, the supernatant was filtered through Rotilabo-rondfilters (type 15A, Carl Roth GmbH & Co.KG). The centrifugation of soil suspension was applied to shorten the filtration time (Rousk and Jones, 2010). The organic C-content of the K_2SO_4 extracts was measured using a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena, Germany). Microbial biomass C and microbial biomass N were calculated by dividing the microbial C flush (EC), i.e. the difference between

extracted C from fumigated and non-fumigated soil samples, with a k_{EC} factor of 0.45 (Joergensen and Mueller, 1996; Wu et al., 1990).

The soil moisture content was determined gravimetrically by drying the soil samples for 24 h at 105 °C (Black, 1965). C_{org} was analyzed in sieved (1 mm) and dried samples which had been pounded with a mortar to a fine powder prior to removing inorganic carbon by HCl-treatment (Nelson and Sommers, 1982). C_{org} and N_{tot} contents were determined using a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena, Germany).

4.2.7 Particle size distribution analysis

Particle size distribution analysis was performed with a Laser-Particle-Sizer «Analysette 22 comfort» (FRITSCH, Germany), equipped with a low-power (2 mW) Helium-Neon laser with a wavelength of 632.8 nm as the light source. The device has active beam length of 2.4 mm, and it operates in the range 0.01 to 1250 µm, combining out of two measurements with focal lengths of 9 and 474 mm in the same suspension. The suspension is pumped through a sample cell placed in the convergent laser beam and the forward scattered light falls on the 31 photosensitive sensor rings. The sample obscuration was adjusted to an optimal value of 45%. The reference refractive index for standard deionized water was 1.33. Before determination, the samples were introduced into the ultrasonic bath. Particle size distribution was obtained by fitting full Mie scattering functions for spheres (Kerker, 1969). The Mie theory approach was selected instead of the Fraunhofer one, because it provides a better estimation of particle size in the clay fraction (deBoer et al., 1987).

4.2.8 Statistics

The means of three replicates with standard errors are presented in tables and figures. A Shapiro-Wilk test was applied to test for Gaussian distribution. We used the Pearson correlation coefficients to interpret the degree of linear relationships. Significant differences in time between the soil types were assessed by repeated measurements ANOVA includ-

ing a Holmes-Sidak post-hoc correction. A multiple t-test was performed to test for significant ($P<0.05$) differences of basic parameter using GraphPad Version 6 software (Prism, USA).

4.3 Results

4.3.1 Basic soil parameters, dsDNA, RNA contents and RNA:dsDNA ratio

Similar decreasing pattern was determined for C_{org}, N_{tot}, and C_{mic} from virgin and arable Chernozems over Retisol and Luvisol to the Calcisol (Figure II.4/21). C_{org}, N_{tot}, C_{mic} and clay content varied significantly ($P<0.05$) between the soil types (except for C_{org}, N_{tot} in Retisol and Luvisol).

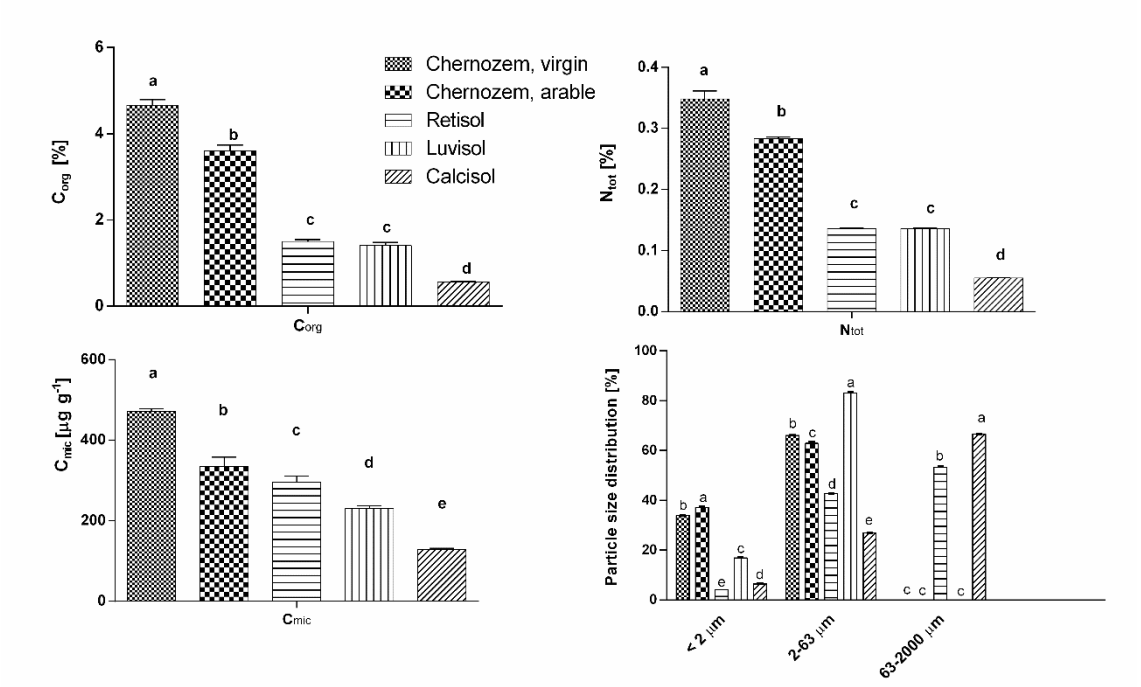


Figure II.4/21 Basic parameter, such as total organic C (C_{org}), total nitrogen (N_{tot}) and microbial biomass content (C_{mic}) as well as the soil clay content for the four different soil types. Significant ($P<0.05$) differences between the soil types were given by lower-case letters.

For non-activated soil samples, the total dsDNA contents ranged from 26-107 µg g^{-1} soil (Figure II.4/22). By the addition of glucose, dsDNA content increased by 64, 94, 154,

147 and 149% for virgin and arable Chernozem, Retisol, Luvisol and Calcisol, respectively during 72 h, reflecting the growth of microbial cells. During the first 24 hours after glucose addition, the dsDNA content increased ($P<0.05$) by 21, 67 and 51% for Luvisol, Retisol and Calcisol, respectively (Figure II.4/22). In case of Chernozems, the DNA contents increased by 56-83% during the first 24 hours, and it leveled off thereafter.

The dsDNA content of non-activated soils was characterized by strong positive linear correlation with C_{org} ($R^2=0.97$, $P<0.0001$), C_{mic} ($R^2=0.85$, $P<0.0001$) and N_{tot} ($R^2=0.84$, $P<0.0001$) (not shown). The conversion factor from dsDNA content to C_{mic} was 4.87 ($R^2 = 0.98$).

For non-activated soils, the total RNA contents ranged from 0.3 to 4.2 µg g^{-1} soil (Figure II.4/22). By the addition of glucose, the RNA contents increased by the factor of 2.8, 1.5, 1.8, 1.2 and 90 fold for virgin and arable Chernozem, Retisol, Luvisol and Calcisol, respectively, reflecting strongly active microbial cells. In contrast to dsDNA, the lowest RNA contents were determined in C-rich Chernozems for both non-activated and activated soils, despite dsDNA-derived C_{mic} and C_{org} were the highest.

In all soils the dsDNA and the RNA increased stronger during 0-72 h compared to 0-24 h or 24-72, except the RNA extracted from Luvisol, which was highest at the end of the experiment (Figure II.4/22). The lowest RNA:dsDNA ratios ($P<0.05$) were revealed in virgin and arable Chernozem soils compared to all other soil types and increased by 131 and 28%, respectively during 72 h of incubation (Figure II.4/22). In Retisol the RNA:dsDNA ratio increased by 132% during the first 24 h and then decreased 52% to the end of incubation. The RNA:dsDNA ratio of Calcisol increased by 53-fold during the first 24 h and decreased by 31% to the end of incubation. In summary, we distinguished three distinct patterns of the RNA:dsDNA responses. 1) In Calcisol, the increase in RNA was much faster than dsDNA (0-72 h and 0-24 h). 2) In Luvisol, the RNA and dsDNA increased simultaneously (0-72 h and 24-72 h), and it was reflected by a comparatively constant RNA:dsDNA ratio, ranging from -19 to 10% during incubation. Finally, 3) in Retisol, the initial growth of RNA retarded after 24 hours, while the dsDNA content progressively increased during incubation.

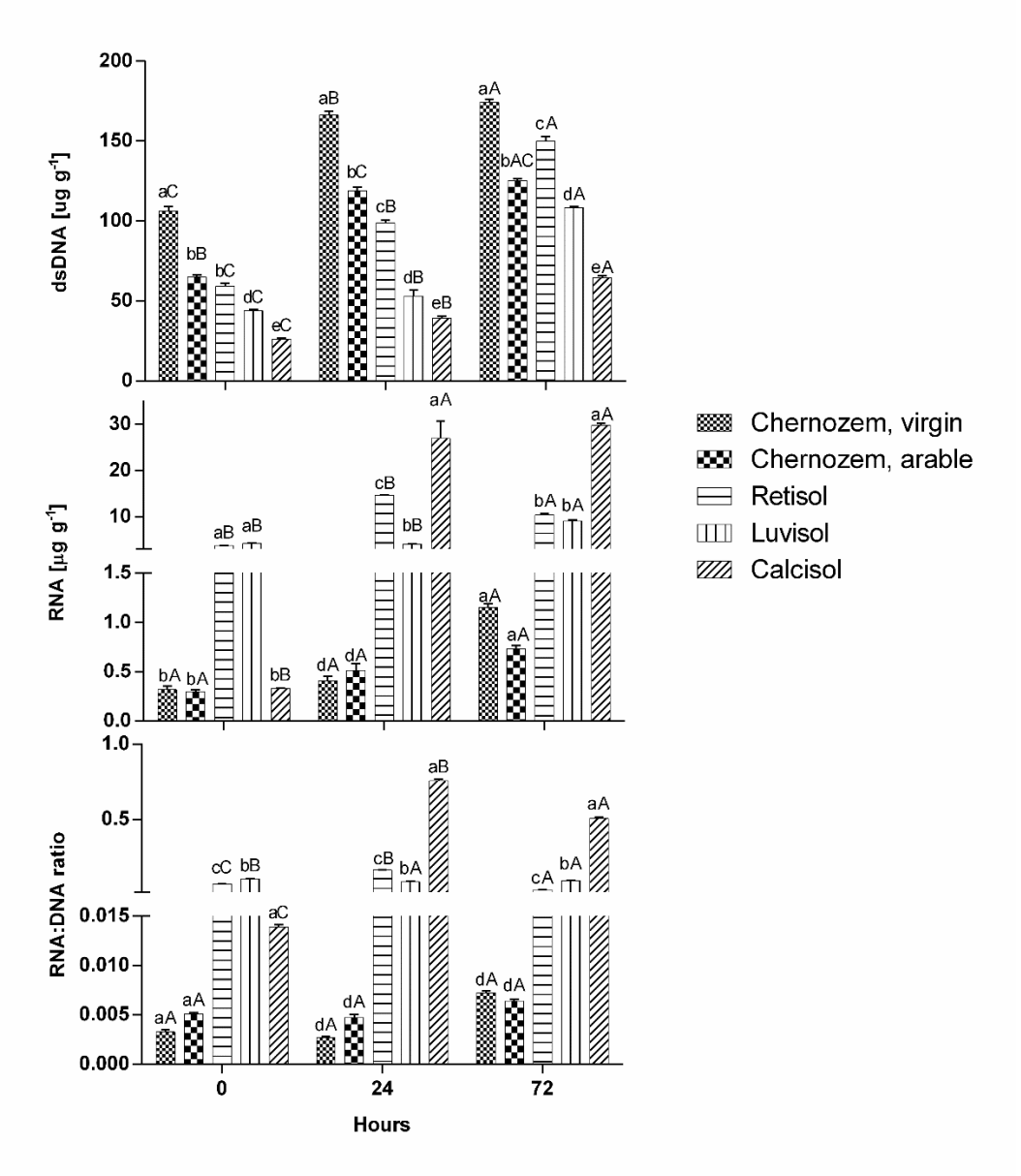


Figure II.4/22 Dynamic of total soil dsDNA and RNA contents as well as RNA:dsDNA ratio in non-acivated soils (0 hours) and after glucose amendment (24 and 72 hours). Significant differences in time between the soil types were assessed by repeated measurements ANOVA including a Holmes-Sidak post-hoc correction. Capital letters indicate significant ($P<0.05$) differences in time of the same soil type. Significant ($P<0.05$) differences between the soil types were given by lower-case letters.

4.3.2 RNA content as affected by particle size distributions

All soil types showed significantly ($P<0.05$) different particle size distributions. Highest clay contents were demonstrated by Chernozems, intermediate for Retisol and Luvisol and lowest for Calcisol (Figure II.4/21). The regression analysis between extractable RNA and soil clay contents exhibited an exponential decay relation (Figure II.2/23 a), which reflected low RNA yields with high clay contents. This negative exponential effect held also true for the RNA:dsDNA ratio at both sampling points (24 h and 72 h) (Figure II.4/23 b).

Table II.4/13 Particle size distribution determined by the Laser-Particle-Sizer (Analysette 22 comfort).

Soil	<2µm	2–63 µm	63–2000 µm
Chernozem virgin	33.92	66.08	0.00
Chernozem arable	37.10	62.90	0.00
Luvisol	16.89	83.11	0.00
Retisol	4.12	42.61	53.27
Calcisol	6.48	26.93	66.60

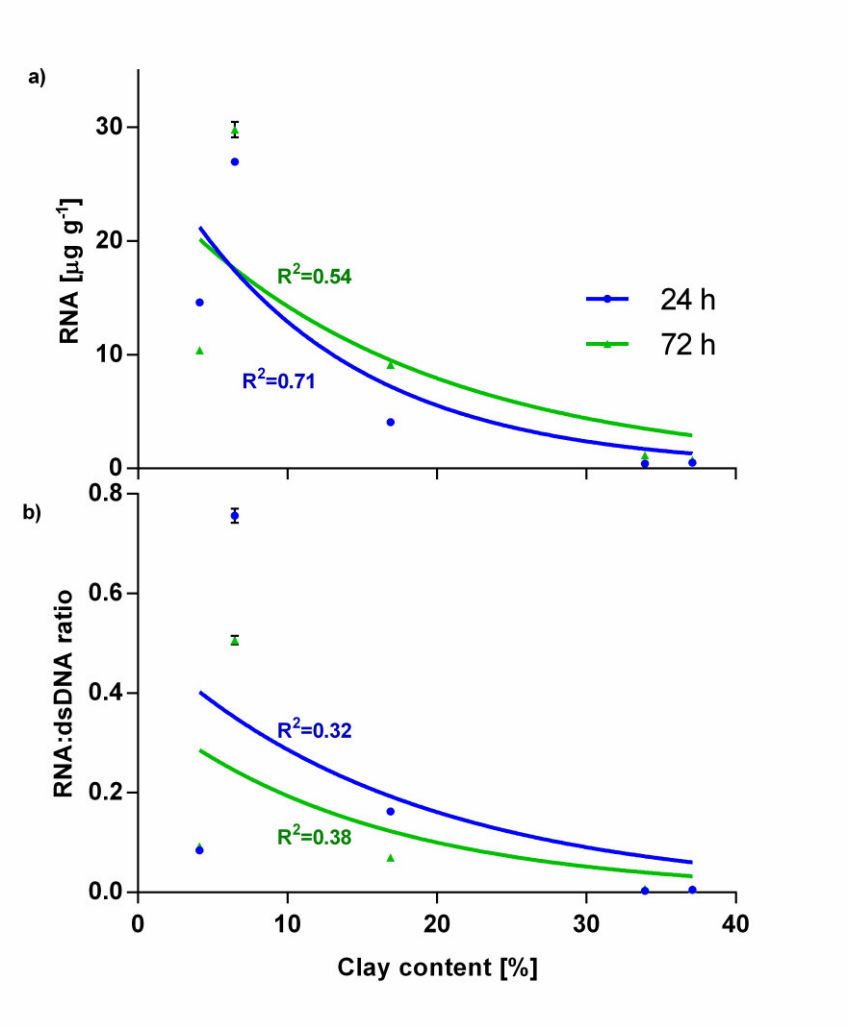


Figure II.4/23 Negative effect of soil clay fraction on RNA yields recovery: a) The RNA contents (±SEM) and b) the RNA:dsDNA ratios (±SEM) after 24 and 72 hours incubation with glucose were considered.

4.4 Discussion

4.4.1 RNA:dsDNA ratio

As cellular DNA concentration does not vary strongly due to environmental changes (Muttray et al., 2001), and because of its considerably strong correlation to microbial biomass in the wide range of soil types (Anderson and Martens, 2013), we considered the quantitative evaluation of dsDNA as a stable indicator for microbial biomass. The dsDNA increased in all soils after glucose addition. Calcisol, with the lowest C- and N-contents, demonstrated a completely different pattern of metabolic activity and growth behavior of microorganisms compared to Retisol and Luvisol with intermediate C- and N-contents. The Calcisol demonstrated a strong increase in RNA content, reflecting strong microbial activity, whereas Retisol and Luvisol showed a slight increase in RNA during 72 h after activation. However, the microbial biomass doubled in the latter two soil types, reflecting strong microbial growth. In contrast, the microbial biomass was lowest in nutrient-poor sandy Calcisol, reflecting activation of microbial population rather than strong microbial growth. Remarkably, a decrease in the active microbial pool, which was often linked to decreasing rRNA contents in nutrient-limiting environments (Davis et al., 1986; Tolker-Nielsen et al., 1997). Different factors affect the relationship between microbial activity and RNA in environmental samples, such as cell physiology (Licht et al., 1999), cell life history and cell life strategy (Lepp and Schmidt, 1998), enzymatic substrate utilization, rRNA synthesis and degradation rates (Gausing, 1977). For S*ynechococcus* and *Prochlorococcus* strains analyzed in pure culture studies a three-phase relationship between growth and rRNA concentration was suggested: (1) at low growth rates, rRNA concentration remains constant, (2) at intermediate growth rates, rRNA concentration increases linearly with growth rate and (3) at higher growth rates, rRNA content decreases as growth rate increases (Blazewicz et al., 2013; Worden and Binder, 2003). The 16S rRNA of marine isolates elucidated different relationship to metabolic activity during non-steady-state growth (Kerhof and Kemp, 1999). In the heterogeneous soil environment this three-phase model may not be such straight forward processes. Here, Retisol and Luvisol

showed “stable” RNA:dsDNA ratios (± 10%) during 72 h hours of incubation with intermediate N contents. When microbial growth was low in Chernozems (5-6%) at the end of incubation, the microbial activity still increased up to 190%, indicating the domination of non-growth activity. However, in the beginning of the experiment (0-24), the increase of dsDNA was stronger than the increase of RNA, which contradicted our hypotheses on microbial activity.

The RNA:dsDNA ratios were 10-times lower than the ratios obtained for pure cultures of various bacteria (Kerkhor and Ward, 1993). The discrepancy can be explained by the dominance of fungi (up to 90% for considered Luvisol) over bacteria in microbial biomass for all studied soils (Ananyeva et al., 2006; Semenov et al., 2013), which have lower nutrient requirements and lower metabolic activity than bacteria, and contain much less RNA (Bardgett et al., 1996; Cross et al., 2005; van der Wal et al., 2006). The RNA:dsDNA ratio increased during incubation for most of the measured soil types, especially for Calcisol (36-fold). This indicated strong non-growth associated microbial activity, reflected by a slow growing but large fraction of active microbial biomass (Loeppmann et al., 2016a). Only for Luvisol, the RNA:dsDNA ratio slightly decreased by 12% during 72 h of incubation. This is in consistence to batch culture studies, which reported a decrease of RNA:dsDNA ratio of activated microorganisms with time (Muttray et al. 2001).

4.4.2 Effect of clay particles distribution on RNA recovery

RNA yield in nutrient-poor sandy Calcisol exceeded for hundredfold the RNA yields in rich clayey Chernozems after glucose amendment. The numbers of both bacterial and archaeal active cells determined by RNA-FISH method in Chernozem were 10-times higher than in Calcisol (Semenov et al., 2016). From methodological perspective, RNA-FISH provides direct intracellular detection of RNA and, therefore, avoiding its contact with RNases or soil environment. Indeed, external RNases may have declined total RNA recovery for all samples during RNA extraction procedure. Nevertheless, the RNA contents, with about 4 $\mu g\ g^{-1}$ soil (non-activated) extracted from Retisol and Luvisol, corresponded fairly well to yields extracted from soil by Tournier and co-workers (2015). By

contrast, the RNA contents extracted from the Chernozems were up to 30-times lower independently of soil activation, reflecting possible adhesion of RNA to clay particles, known to reduce RNA recovery in soils (Wang et al., 2012). Since the RNA content in the range of studied soils varied within the two orders of magnitude, this high difference in RNA yield could be the result of RNA underestimation in some of studied soils due to various interfering factors (Ehlers et al., 2010). The strong correlation between RNA and the clay content of virgin and arable Chernozem during 72 h of incubation clearly depicted the challenge for RNA extraction on clayey soils. The correlation between RNA:dsDNA ratios and clay contents corresponded well to the study of Tournier et al. (2015).

Another consequence of such small quantities of isolated RNA is that we are still unable to properly apply molecular biological RNA-based approaches (transcripts sequencing or quantification by qPCR, etc.) on clayey soils, such as the studied Chernozems. Thus, development of RNA extraction methodologies to provide sufficient RNA yields in high-clay soils are still required.

4.5 Conclusions

The procedure of isolation and quantification of total soil DNA and RNA yields and determination of RNA:dsDNA ratios was demonstrated in order to elucidate the metabolic status of soil microbial communities in contrasting soil types. The identification of several microbial growth pattern in terms of RNA:dsDNA responses due to the addition of glucose enabled insights into the physiological state of the soil microbial community. In general, soil RNA yields increased stronger than soil DNA yields after glucose addition. Consequently, the RNA:dsDNA ratio was mostly governed by the dynamics and the behavior of RNA. Especially, the RNA:dsDNA ratio extracted from nutrient-poor sandy Calcisol increased strongly by 36-fold after glucose amendment, indicating rather highly active microbes than fast microbial growth. For Retisol and Luvisol with intermediate C and N contents, the RNA:dsDNA ratio remained comparatively constant after soil activation. The RNA yield was strongly affected by the clay content (> 30% in Chernozems) of the soils, which was indicated by the lower RNA recovery in virgin and arable Chernozem compared to soil types with lower clay contents. This suggests, that the underestimation of RNA yields in clayey soils biased the RNA:dsDNA ratio, and subsequently the physiological state of the microbial community is not adequately represented in soils with clay contents exceeding 30%.

Acknowledgments

We would like to thank Prof. Eugeniy Milanovskiy and Anna Yudina for supplying with particle size distribution analysis and interpretation of obtained data. This research was supported by DAAD programm «Forschnungsstipendien für Doktoranden und Nachwushswissenschaftler» 2013–2014 and Russian Science Foundation, Project No 14-14-00625.

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5. Effects of Acanthamoeba grazing on carbon flux and enzyme activities in rhizosphere and detritusphere

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In preparation for *Plant and Soil*

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Abstract

Predation by soil fauna on microorganisms controls microbial activity and, hence, the decomposition of organics, a process sequence termed the `microbial loop`. These microbial food webs are affected by the complexity and accessibility of carbon (C) in two major C inputs rhizodeposits and litter. Thus, the amount and quality of substrates entering the soil control microbial processes in the rhizosphere and detritusphere. We labelled root exudates (^{14}C, rhizo-C) in corn (*Zea mays*)-planted microcosms and duplicated all treatments with an added model protist (*Acanthamoebae castellani*). For further identification of C resources fueling microbial-protozoan interactions, *Lolium perenne* root litter was added to the system. To uncover the complexity of interactions between the C sources, microorganisms and Acanthamoeba grazing, we used enzyme kinetics (β-glucosidase, acid phosphatase, β-xylosidase, leucine-aminopeptidase and N-acetyl-β-glucosaminidase) as an indicator for microbial activity and determined the microbial community structure by PLFA analyses.

Acanthamoeba grazing strongly increased microbial uptake of rhizo-C (^{14}C) in planted soil with and without root litter addition, reflecting preferred utilization of easily available substrates. The presence of Acanthamoeba increased the activity of C-cycling enzymes, especially β-glucosidase raised by 9-fold. In conclusion, C fluxes and enzyme activities were driven by substrate input and quality in the rhizosphere and detritusphere and further stimulated by faunal grazing.

Key words: Protists; Faunal predation; 14C; DNA; Substrate quality; Enzyme affinity

5.1 Introduction

Living plants and plant residues provide carbon (C) to C-limited soil microbial communities (Wardle, 1992), potentially increasing decomposition and nitrogen (N) release from soil organic matter (SOM) (Kumar et al., 2006; Kuzyakov, 2002; Kuzyakov et al., 2007). Protists increase the available N pool in soil through the ingestion and destruction of bacterial cells and excretion of ammonia (Stout, 1980). Since N is a limiting nutrient in the rhizosphere (Kuzyakov and Xu, 2013), increased N availability in presence of protists stimulates plant growth through the so-called `microbial loop` network (Clarholm, 1985). Plant growth and root exudation may lead to higher microbial activity and higher SOM decomposition and N release (Gerhardson and Clarholm, 1986; Kuikman et al., 1990; Bonkowski et al., 2000a; Ekelund et al., 2009).

There are several plant-stimulated hotspots of microbial growth and activity in soil, including the rhizosphere and detritusphere (Bonkowski et al., 2000b; Bonkowski and Clarholm, 2012; Blagodatskaya and Kuzyakov, 2013). In the rhizosphere, the area affected by living plant roots, rhizodeposits are a primary source of C and energy for microbial biomass growth (Gregory, 2006; Neumann and Römheld, 2007; Haichar et al., 2008). Belowground C input by maize amounts to 29±13% of shoot biomass at a growth state of physiological maturity (Amos and Walters, 2006). Because of ongoing root exudation, supplying a large quantity and diversity of easily available substrates (Lynch and Whipps, 1990; Walker et al., 2003; Hinsinger et al., 2005), the rhizosphere is characterized by high microbial abundance and activity (Alphei et al., 1996; Paterson, 2003; Paterson et al., 2007; Haichar et al., 2008).

The detritusphere, a more recalcitrant food source for microorganisms, is characterized by dead plant residues (e.g. root litter) that typically contain large amounts of cellulose, hemicelluloses and lignin (Kandeler, 1999; Marschner et al., 2012; Nannipieri et al., 2012). Microorganisms decompose root debris by extracellular depolymerization, hydrolysis and oxidation. Recent studies proposed the amount and quality of plant-derived substrates entering the soil drive microbial substrate utilization in the rhizosphere and detritusphere, which is largely mediated by extracellular enzymes (Wallenstein et al., 2011;

Loeppmann et al., 2016). For example, chitinase (N-acetyl-glucosaminidase) is involved in both C- and N- cycles in soils (Beier and Bertilsson, 2013). Moreover, chitin contains about 6% N (e.g. peptidoglycan, bacterial cell walls) and is a major source of organic N (Ekenler and Tabatabai, 2002; Kelly et al., 2011).

Little is known about the tight biotic interactions between enzymatic substrate utilization and microbial activity (Blagodatskaya and Kuzyakov 2013). In particular, the feedback of Acanthamoeba-affected functions into the microbial loop remained unclear in rhizosphere and detritusphere (Ekelund et al., 2009; Bonkowski and Clarholm, 2012).

Increased root biomass and rhizodeposition frequently result in higher prey density (Bonkowski and Brandt, 2002; Phillips et al., 2007; Bonkowski, 2004). The numbers of bacterial-feeding protists increase up to 30-fold in the rhizosphere compared with bulk soil (Griffiths, 1990; Zwart and Brussaard, 1991), significantly enhancing microbial turnover (Coleman et al., 1984; Gerhardson and Clarholm, 1986; Alphei et al., 1996) and the proportion of active bacteria (Rosenberg et al., 2009). Consequently, soil respiration and specific respiration increase in the presence of micro-fauna (Kuikmann et al., 1990; Scheu et al., 1996).

To investigate C flux and changes in enzyme production in the rhizosphere and detritusphere during Acanthamoeba grazing, microcosms with living corn plants (*Zea mays*), or with *Lolium perenne* root litter, or with both were established and all treatments were duplicated with an added model protist (*Acanthamoebae castellani*). Maize plants were pulse labelled with $^{14}CO_2$. ^{14}C activity in soil, CO_2, and extractable organic C were determined. The dsDNA and PLFA contents as well as enzyme activities were analysed. Our hypotheses were that 1) rhizo-C (maize-derived ^{14}C) and enzyme activities increase with Acanthamoeba grazing in comparison to non-grazing, 2) enzyme activities show a stronger increase in the rhizosphere (available C) than in the detritusphere (more stabile C) and 3) simultaneous presence of available and recalcitrant plant C sources in soil leads to maximum enzyme activities.

5.2 Material and methods

5.2.1 Soil sampling

Soil was taken from the uppermost 20 cm of a loamy Luvisol with partly stagnic properties at an arable field site near Göttingen (Holtensen), Germany. The soil is carbonate-free and has the following characteristics: pH ($CaCl_2$) 6.0, C_{org} 11.7 mg g^{-1}, N_{tot} 1.2 mg g^{-1}, C:N 9.8, NO_3 0.08 mg g^{-1} (Kramer et al., 2012). The soil was stored at 6 °C for 10 days and then sieved for homogenization. Soil was autoclaved (3-times) to eliminate living microorganisms as well as spores (Tuominen et al., 1994). All further preparation, transfer of soil into the microcosms and subsequent inoculation were performed under sterile conditions to avoid contamination by airborne cysts of protist and fungi.

5.2.2 Experimental setup

We implemented a full factorial design with the following treatments with autoclaved soil with a re-inoculated bacterial community: a control, soil (Bulk), a rhizosphere treatment with a corn seedling with sufficient root density to fill the microcosm (Rhizo), a detritusphere treatment with ground *Lolium perenne* root litter homogenously mixed with the soil (Detritus), and a treatment which combined both the root litter and plant (Rhizo + Detritus). Finally, each treatment was duplicated with the addition of a model protist, *Acanthamoeba castellani*. The 8 treatments were replicated 6 times. However, from the rhizosphere treatment, only 4 replicates were selected for $^{14}CO_2$ pulse labelling and further analysis.

Perennial ryegrass (*Lolium perenne*) root powder (1.7 g) was homogeneously mixed with 550 g of dry weight of soil and transferred into each microcosm (Detritus, Rhizo + Detritus), and the entire microcosms were again autoclaved. The microcosms consisted of transparent glass jars with a volume of 870 ml (height of 14.5 cm and diameter 9.5 cm) (Parisienne de Verreries, Orly, France) (Figure 1). The lid was perforated four times: one central hole for plant growth (16 mm diameter), two holes near the rim (6 mm diameter) for gas inflow and outflow, and a small hole (2 mm diameter) with syringe and filter for

watering. A detailed picture of the experimental set up is presented in Figure II.5/24. Air from the root compartment of the microcosms was pumped through a NaOH trap (5 ml, 1 M) in a glass vial by multi-channel peristaltic pumps (40 rpm) (Watson-Marlow 205S, Watson-Marlow GmbH, Rommerskirchen, Germany). To achieve CO_2 free air inflow into the root compartment, each microcosm was fitted with a syringe (5 ml, AMEFA GmbH, Limburg, Germany) filled with sterile cotton wool and soda lime. The microcosms were then covered with aluminum foil to prevent growth of photosynthetic - chemolithoautotrophic organisms in the soil.

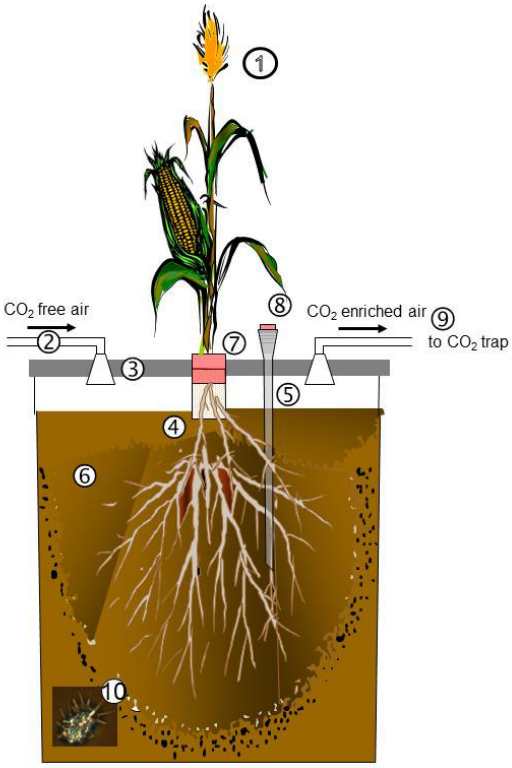

Figure II.5/24 Microcosm setup: (1) shoots of maize, (2) inflow of CO_2-free air, (3) airtight lid of the microcosm, (4) roots of maize, (5) hypodermic needles, (6) soil mixed with root litter, autoclaved and re-inoculated with bacterial community, (7) lid perforated for plant growth and sealed airtight with silicone, (8) sterile hydrophobic cotton, (9) outflow of CO_2-enriched air from soil respiration, (10) amoeba (*A. castellanii*) (modified after Koller et al., 2013) .

5.2.3 Inoculation

To isolate a protozoa-free bacterial community, 5 g of fresh soil were suspended in 20 ml of Neff's modified Amoebae Saline (NMAS; Page, 1976) and moderately shaken (200 rotations per minute) for 20 minutes. In order to filter out protists and fungi, the suspension was pressed through the 5 µm and then 1.2 µm Isopore filters (Millipore, Schwalbach, Germany; Bonkowski and Brandt, 2002). In order to check for fungal or protistic contaminants, a subsample of each filtrate was cultured for one week in sterile nutrient broth (NB; Merck, Darmstadt, Germany) with NMAS at 1:9 v/v (NB-NMAS; Page, 1976). In the treatments without Acanthamoeba, the inoculum consisted of 5 ml of the soil extracted bacterial community was diluted in 45 ml water. In the treatments containing Acanthamoeba, the inoculum consisted of 5 ml bacteria extracted, 43.58 ml water and 1.42 ml suspension of amoebae (*Acanthamoeba castellanii*) in NMAS (Rosenberg et al., 2009). Treatments with amoebae therefore received 200 amoebae per gram soil. However, the density was anticipated to increase up to carrying capacity as the soil community had time to develop before establishment of the plants. The inoculum was dispensed with a 10 ml pipette onto the soil surface.

5.2.4 Plant growth conditions and incubation procedure

Surface-sterilized seeds of *Zea mays* were germinated in Petri dishes with NMAS agar (1%) at room temperature for 3 days. Germinated seedlings were grown at aseptically conditions for 14 days. The microcosms (with and without plants) were incubated in a climate chamber at 18:22 °C night:day temperature, 70% humidity, 16 h photoperiod and 460±80 µmol m^{-2} s^{-1} light photon flux density. During the first five days, the NaOH in the traps was changed daily due to high C mineralisation from the soil. After the first five days when the C flux was more stable, the traps were changed every few days.

Plants were subsequently transferred on top of the root compartment of the microcosms. The tubes with grown plants were then placed aseptically on top of the root compartment of the microcosms. Microcosms were randomized to locations and soil moisture was checked gravimetrically and kept at 20% moisture content by adding sterile distilled water

through the syringe containing a sterile filter (pore size, 0.2 μm; Sartorius) into the root compartment.

5.2.5 Plant ^{14}C pulse labelling

The ^{14}C labelling of plants was done after 35 days of plant growth. All plants were placed in an acrylic glass chamber and labelled simultaneously in a $^{14}CO_2$ atmosphere. The chamber and the labelling technique are described in detail elsewhere (Kuzyakov et al., 1999; Kuzyakov and Siniakina, 2001; Werth and Kuzyakov, 2008). Briefly, the chamber was connected by tubing to a flask containing 10 ml of $Na_2{}^{14}CO_3$ solution with an activity of 4.5 MBq. By adding 5 ml of 5 M H_2SO_4 solution $^{14}CO_2$ was released into the labelling chamber. During the labelling procedure the assimilation of $^{14}CO_2$ by plants was measured by gas sampling. The remaining unassimilated $^{14}CO_2$ was trapped by pumping the chamber air through 50 ml of 1 M NaOH solution for 3 h. 66% of the activity was net assimilated by the plants. After pulse labelling, the $^{14}CO_2$ from the microcosms was trapped in 15 ml of 1M NaOH solution until harvest with the NaOH trap changed after 3, 15, 42, and 66 h after pulse labelling.

5.2.6 Sampling

Harvest of the plants was done 3 days after ^{14}C pulse labelling. Above-ground biomass was divided into shoot and crown roots. To collect the fine roots, soil was sieved (< 5 mm) and fine roots and other plant debris were carefully removed with tweezers and washed. The washing water was kept for further analysis. As root density was high, all soil in the planted microcosms was taken to be rhizosphere soil. Soil was separated into 4 portions: 1) a soil sample was dried at 105 °C (24 h) to determine the soil moisture contents, 2) a subsample was flash frozen by liquid N_2 and stored at -20 °C for determination of enzyme and dsDNA analysis, 3) a part of fresh soil (stored at 5 °C) was used to analyse the abundance of Acanthamoeba 4) another soil sample was stored at -20 °C for the phospholipid fatty acid analysis.

5.2.7 Sample analyses

No significant differences were detected in pH, C_t, or N_t contents of the soils.

5.2.7.1 Carbon mineralization, microbial biomass, and analysis of soil, root and plant material

The total CO_2 trapped in NaOH was measured with a C analyser (Shimadzu).

Soil microbial biomass C (MBC) was determined by the chloroform fumigation extraction (CFE) procedure described by Brookes et al. (1985) and Vance et al. (1987). Briefly, 7.5 g of fresh soil was extracted with 30 ml 0.05 M K_2SO_4 solution (Bruulsema and Duxbury, 1996) by overhead shaking (40 rev min^{-1}) for 30 min. Another 7.5 g of soil was first fumigated with ethanol-free chloroform for 24 h at room temperature and then extracted in the same way. The soil suspension of the fumigated and the non-fumigated samples was centrifuged for 10 min at 2500 g. Afterwards, the supernatant was filtered through Rotilabo-rondfilters (type 15A, Carl Roth GmbH & Co.KG).

Activity of ^{14}C in MBC, extractable organic C (EOC) and NaOH solutions was measured by liquid scintillation counting (LS 6500 Multi-Purpose Scintillation Counter, Beckman, USA) using scintillation cocktail Rotiszint Eco Plus (Carl Roth, Germany) which was mixed with a sample in a ratio of 1:2.5. The ^{14}C counting efficiency was at least 70% and the measurement error did not exceed 4%. The ^{14}C activity found in each compartment was determined as described in Werth and Kuzyakov (2008). Rhizo-C-derived MBC, EOC and CO_2 was calculated based on ^{14}C activity in the plant shoots according to Kuzyakov et al. (1999) and Werth and Kuzyakov (2008). The ^{14}C activity of all solid samples (shoots, crown and fine roots and soil samples) was determined by liquid scintillation counting after dry combustion of samples at 800 °C for 4 min (Oxysolve C-400) and trapping CO_2 into scintillation cocktail.

5.2.7.2 dsDNA extraction and quantification procedure

The extraction of total soil DNA was done by the FastDNA® SPIN kit for soil (MP Biomedicals, Germany). Briefly, 0.5 g soil was added to lysing tubes, treated with lysis buffer, subjected to bead beating in the FastPrep® instrument and treated with protein

precipitation solution. DNA was bound to a silica matrix, washed, and eluted in DNase-free water.

A 150-fold dilution of the extract was prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) for fluorometric dsDNA determination. Aliquots of 0.1 ml were transferred to 96-well microplates (Brand pureGrade, black). A 200-fold dilution of the dsDNA fluorescence stain PicoGreen® (Molecular Probes, Life Technologies, Germany) was prepared in sterile plastic tubes. This dye (0.1 ml) was added to each well (final 300-fold dilution) and left to react at 22 °C for 2 min, protected from light. Fluorescence intensity was measured (measurement time 1.0 s) with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland) at excitation and emission wavelengths of 485 and 525 nm for excitation and emission, respectively. The dsDNA yield was determined using dsDNA of *Bacteriophage lambda* as a standard (Molecular Probes, Life Technologies, Germany). Samples for the standard curve were prepared in TE-buffer in the same way as the experimental samples (Blagodatskaya et al., 2014).

Microbial biomass was calculated as (Anderson and Martens, 2013):

$$\text{Microbial biomass C } (\mu g\ g^{-1}\ \text{soil}) = 5.02 \times \text{dsDNA } (\mu g\ g^{-1}\ \text{soil}) \quad (1)$$

5.2.7.3 Extraction of phospholipid fatty acids

Total lipids were extracted from the 6 g of moist soil with a one-phase mixture of chloroform, methanol and 0.15 M citric acid (1:2:0.8 v/v/v) (Frostegard and Baath, 1996). 19:0-phospholipid (100 µl, 1µg μl^{-1}) was used as a first internal standard and was added into the soil samples before extraction. Purification of the phospholipid fraction was done on a silica column, with elution of neutral-, glyco- and phospholipids by chloroform, acetone and methanol, respectively. Phospholipid fatty acids were saponified with 0.5 ml 0.5 M NaOH in dry MeOH for 10 min at 100 °C. The free FAs were methylated with 0.75 ml BF_3 in methanol (10%, 1.3 M, Fluka) for 15 min at 80 °C. Fatty acid methyl esters (FAMEs) were extracted three times with 1 ml hexane by liquid-liquid extraction and combined hexane aliquots were dried under N_2. For final analysis FAMEs were re-dissolved in 185 µl toluene with the addition of 15 µl of a second internal standard (IS2) (13:0 FAME at 1 mg ml^{-1}). Final quantification of FAME content was performed by gas

chromatography with a Hewlett Packard 5890 gas chromatograph coupled to a mass-selective detector 5971A. Chromatography parameters were as follows: 15 m HP-1 methylpolysiloxane column coupled with a 30 m HP-5 (5% Phenyl)-methylpolysiloxane column both with an internal diameter of 0.25 mm and a film thickness of 0.25 µm; He flow rate of 2 ml min^{-1}; injection volume of 1 µl; temperature programme: 80 ºC ramped to 164 ºC at 10 ºC min^{-1}, then to 230 ºC at 0.7 ºC min^{-1}, and finally to 300 ºC at 10 ºC min^{-1}. Quantification of PLFAs was based on 29 external standards (Gunina et al., 2014).

5.2.7.4 Enzyme kinetics

We used 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-phosphate, 4-methylumbelliferyl-7-β-D-xylopyranoside, L-leucine-7-amino-4-methylcoumarin hydrochloride and 4-methylumbelliferyl N-acetyl-β-D-glucosaminide to determine the enzyme activities of β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.2), β-xylosidase (EC 3.2.2.27), leucine aminopeptidase (LAP) (EC 3.4.11.1) and N-acetyl-β-glucosaminidase (chitinase) EC 3.2.1.52), respectively. Half a gram of wet soil was mixed with 50 ml of sterile water and dispersed by an ultrasonic disaggregator (50 J s^{-1} for 120 s) (De Cesare et al., 2000). Aliquots of 50 µl were withdrawn and dispensed into 96-well microplates (Brand pureGrade, black) while stirring the suspension. The substrates were dissolved in 300 µl dimethyl sulfoxide (DMSO) and then diluted with 80 ml of 0.1 M, pH 6.1 MES (for carbohydrases and phosphatase) or by 0.05 M, pH 7.8 TRIZMA (for leucine-/tyrosine-aminopeptidase) to obtain 1 mM of working solution (Marx et al., 2001; 2005). A concentration series of 20, 40, 60, 80, 100, 200, 400 µmol substrate g $soil^{-1}$ was then prepared and 100 µl of the respective solutions was added to the wells.

Fluorescence was measured (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation at 22 °C with an automated plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland). Fluorescence was converted to an amount of MUB (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin), by comparison to standard solutions of MUB and AMC prepared in separate sub-samples of the various soil suspensions. The substrate-dependent rate of reaction (v) mediated by hydrolytic enzymes, followed Michaelis-Menten kinetics (Marx et al., 2001; 2005; Nannipieri et al., 2012).

$$v = (V_{max} \times [S]) / (K_m + [S]) \qquad (2)$$

Initial reaction rate (v) was plotted against substrate concentration (S). Using experimental data, the calculation allows characterization of each enzyme-substrate reaction by 2 kinetic parameters: 1) V_{max}, the maximum rate of enzyme catalysis that theoretically is attained when the enzyme has been saturated by an infinite concentration of substrate, and 2) K_m, the Michaelis constant, which is numerically equal to the concentration of substrate for the half-maximum rate (Cornish-Bowden, 1995; Koshland, 2002; Marx et al., 2005). V_{max} represents decomposition rates at saturating substrate concentrations while K_m reflects the enzyme affinity to the substrate Gianfreda et al., 1995; Koshland, 2002; Moscatelli et al., 2012). The parameters of the equation were fitted by minimizing the least-square sum by using GraphPad Version 6 software (Prism, USA). The 3 analytical replicates of enzyme activity curves were used for each soil replicate. Parameter optimization was restricted to the applied model equation as indicated by maximum values of statistic criteria: r^2, the fraction of total variation explained by the model defined as ratio of model weighted sum of squares to total weighted sum of squares. Outliers were identified by the ROUT method, based on the False Discovery Rate (FDR), where Q was specified to define the maximum desired FDR (Motulsky and Brown, 2006).

5.2.7.5 Enumeration of protozoa

Total numbers of amoebae were estimated as described in Koller et al. (2013). Briefly, 5 g fresh weight of soil was suspended in 20 mL sterile NMAS (Page, 1976) and gently shaken on a vertical shaker for 20 min. A dilution series with nutrient broth (Merck) and NMAS at 1:9 v/v were prepared in 96-well microtiter plates (VWR, Darmstadt, Germany) with four replicates each. The microtiter plates were incubated at 15 °C in darkness, and the wells were inspected for the presence of amoebae using an inverted microscope at 9100 and 9200 magnification (Nikon, Eclipse TE 2000-E, Tokyo, Japan) after 3, 6, 11, 19 and 26 days.

5.2.8 Statistics

All data were expressed as means ± standard errors (SEM). A Shapiro-Wilk test was performed to test for Gaussian distribution. Effects of soil treatments were assessed by two-way ANOVA. Therefore the method described in detail by Glantz and Slinker (1990) was

applied. This method converts the ANOVA problem to a multiple regression problem and then displays the results as ANOVA (Fox, 2008). Since the data was unbalanced, analysis of unweighted means was applied (Fisher and van Belle, 1993). When significant effects were identified, a multiple post-hoc comparison using the Holm-Sidak test ($P<0.05$) was performed.

5.3 Results

5.3.1 Protozoan abundances and dsDNA-derived microbial biomass

At harvest, Acanthamoeba were detected in all microcosms to which they had been added. Treatments showed no differences (P<0.05) in protozoan numbers (Supplementary Figure II.5/25). Overall, the microbial biomass was strongly increased by additional substrate input, whereas Acanthamoeba grazing slightly reduced the abundance of bacteria in all treatments (Figure II.5/26).

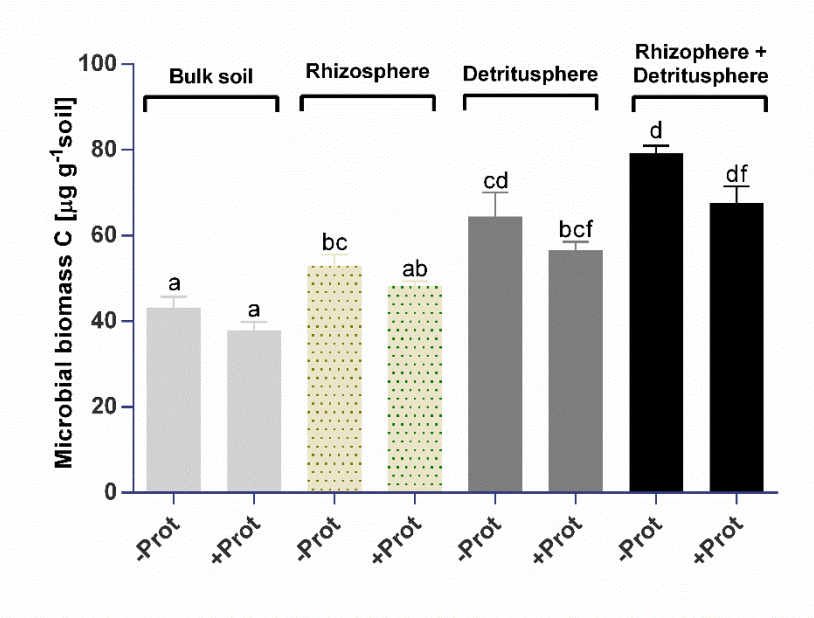

Figure II.5/25 Microbial biomass calculated from dsDNA content in absence (-Prot) or presence of Acanthamoeba (+Prot) for bulk soil, rhizosphere (Rhizo), detritusphere (Detritus) and combined-substrate input (Rhizo + Detritus). Significant differences (P<0.05) between the treatments are indicated by lower-case letters.

5.3.2 CO_2 production and rhizo-C

Grazing had no significant effect on CO_2 production in all treatments (Figure II.5/26). CO_2 production was lower ($P<0.05$) in root litter-treated than in rhizosphere soils, irrespective of grazing (Figure II.5/26).

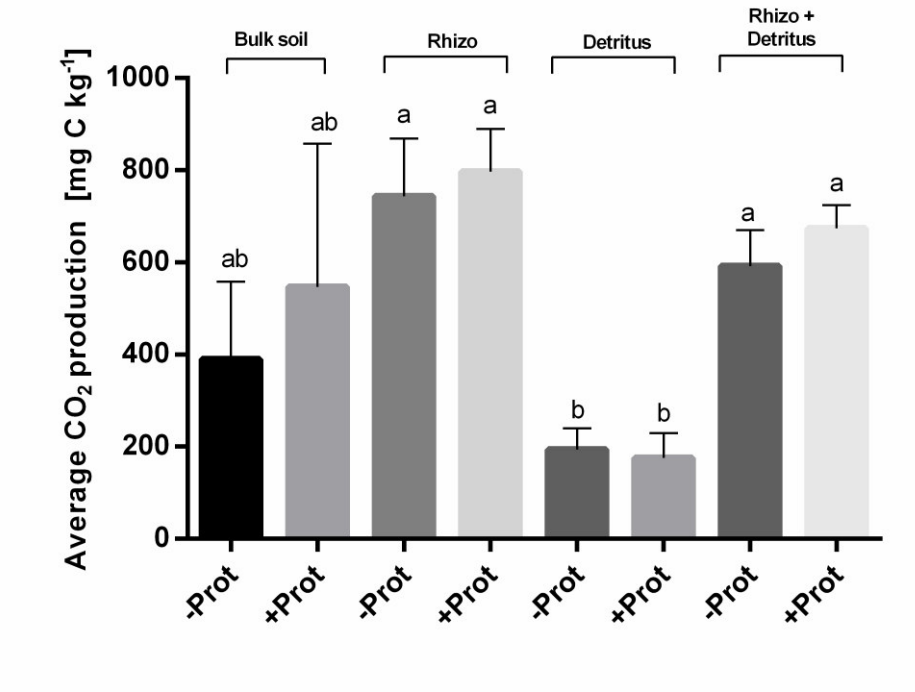

Figure II.5/26 Average CO_2 production (±SEM) after ^{14}C pulse labelling in the absence (-Prot) and the presence of Acanthamoeba (+Prot) for bulk soil, rhizosphere (Rhizo), detritusphere (Detritus) and combined-substrate input (Rhizo + Detritus).

The recovered ^{14}C input was partitioned to shoots (70.7%), roots (13.4%), crown roots (8.4%), CO_2 (5.7%), soil (1.5%), microbial biomass (0.3%). Maize-derived ^{14}C (rhizo-C) in CO_2 decreased by 24% for both non-grazed and grazed combined substrates (Rhizo + Detritus) compared to rooted (Rhizo) soil, reflecting decreased mineralization of fresh C sources (Figure II.5/27 a). Grazing slightly increased mineralization of rhizo-C ($^{14}CO_2$ release) by 28% and 32% for rooted soil and combined-substrate input relative to the absence of Acanthamoeba but was not significant.

Highest incorporation of rhizo-C into microbial biomass was exhibited in rooted (Rhizo) soil in the presence of Acanthamoeba. Grazing increased ($P<0.05$) the microbial uptake of maize-derived ^{14}C in both rooted (by 143%) and combined substrate (by 215%), reflecting preferred incorporation of easily available substrates into microbial biomass (Figure II.5/27 b). Higher rhizo-C in EOC (by 34%; $P<0.05$) was determined for rooted (Rhizo) soil compared to combined-substrate input (Rhizo + Detritus) irrespective of Acanthamoeba grazing (Figure II.5/27 c).

Acanthamoeba grazing raised microbial incorporation of rhizo-C (^{14}C in MBC). Highest microbial uptake and mineralization of rhizo-C occurred in the rhizosphere (Rhizo) with Acanthamoeba grazing.

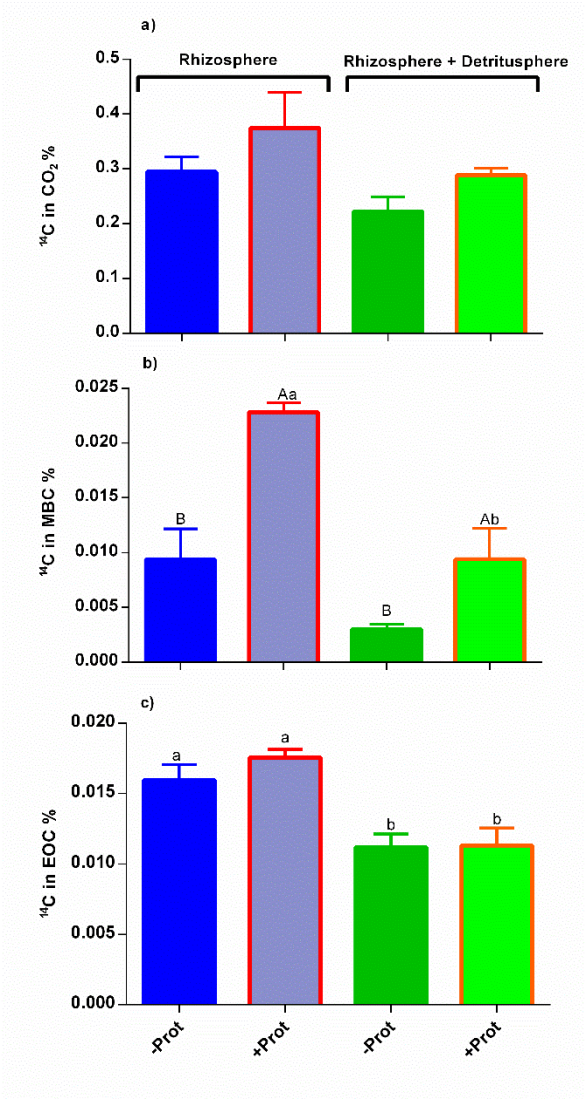

Figure II.5/27 Maize-derived ^{14}C (rhizo-C) in a) CO_2, b) microbial biomass, c) extractable organic carbon (EOC) (±SEM) in absence (-Prot) or presence of Acanthamoeba (+Prot). Significant differences ($P<0.05$) between absence and presence of Acanthamoeba are indicated by capital letters. Lower-case letters indicate significant differences ($P<0.05$) between rhizosphere (Rhizo) and combined-substrate input (Rhizo + Detritus).

5.3.3 Enzyme kinetics parameters

The potential enzyme activities (V_{max}) of chitinase and leucine-AP were affected ($P<0.05$) by rhizodeposition (Figure II.5/28 b, d). Both rates increased in the presence of living plants, whereas the enzyme activities for the root litter-amended soil were similar to bulk

soil. For β-glucosidase, the activity rates were higher for all treatments ($P < 0.05$) compared to the bulk soil (Figure II.5/28 c), showing strong substrate effects.

Acanthamoeba grazing increased ($P<0.05$) the potential enzyme activities of chitinase as well as β-glucosidase and decreased activity of leucine-AP for all treatments (except leucine-AP for combined-substrate input) (Figure II.5/28 b, c, d), reflecting strong grazing effects.

The K_m of chitinase was unaffected by substrate input, whereas the presence of Acanthamoeba raised ($P<0.05$) the half saturation constant (K_m) in all treatments (Figure II.5/28 5b). The strongest increase of K_m of chitinase (by 144%) was determined for the combined-substrate input (Rhizo + Detritus) with grazing, indicating the lowest enzyme affinity to the substrate. Root litter-amended (Detritus) soils showed reduced K_m for β-glucosidase (Figure 5c), and therefore, higher affinity to the substrate. During Acanthamoeba grazing in rooted (Rhizo) soil, only the K_m of β-glucosidase increased (by 227%).

Both, the substrate input and the Acanthamoeba grazing demonstrated strong effects on microorganisms and their associated extracellular enzymes. Furthermore, the C flux into the microbial community was affected by substrate quality and the presence of Acanthamoeba.

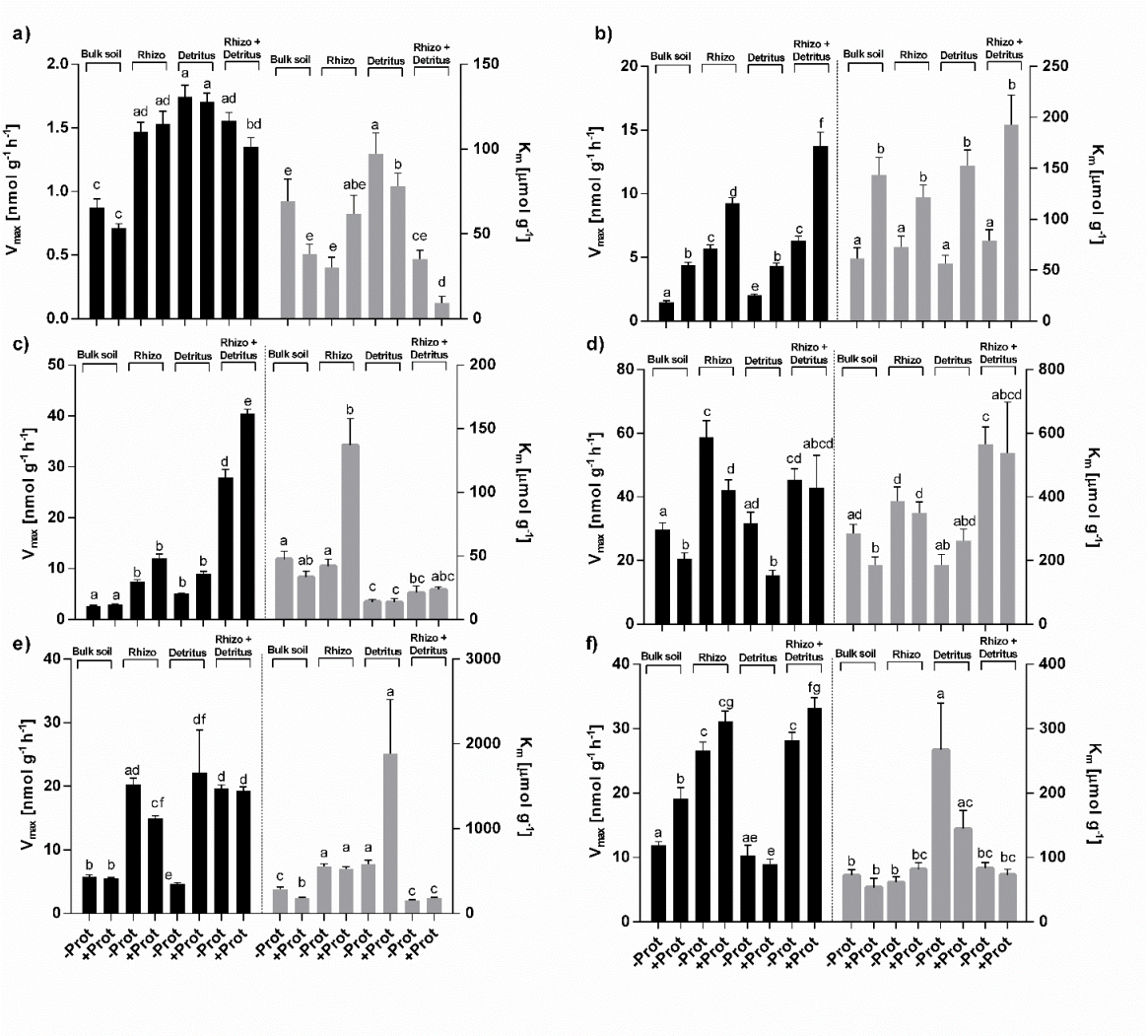

Figure II.5/28 Enzyme kinetics parameters of a) β-xylosidase b) chitinase, c) β-glucosidase and d) leucine-aminopeptidase e) tyrosine-aminopeptidase f) acid phosphatase. Potential enzyme activity (V_{max}) on the left axis and half saturation constant (K_m) on the right axis (±SEM). Both are shown in the absence (-Prot) or presence of Acanthamoeba (+Prot) Lower-case letters indicated significant differences ($P<0.05$) between bulk soil, rhizosphere (Rhizo), detritusphere (Detritus) and combined-substrate input (Rhizo+Detritus).

5.3.4 Phospholipid fatty acids

Higher content of Gram- bacterial biomarkers was found in the presence of rhizodeposition compared to Gram+, irrespective of Acanthamoeba predation. However, Acanthamoeba grazing did not affect the microbial community structure. Higher PLFA contents were determined for planted (Rhizo; Rhizo + Detritus) compared to unplanted treatment (Detritus) irrespective of the presence of Acanthamoeba (Figure II.5/29).

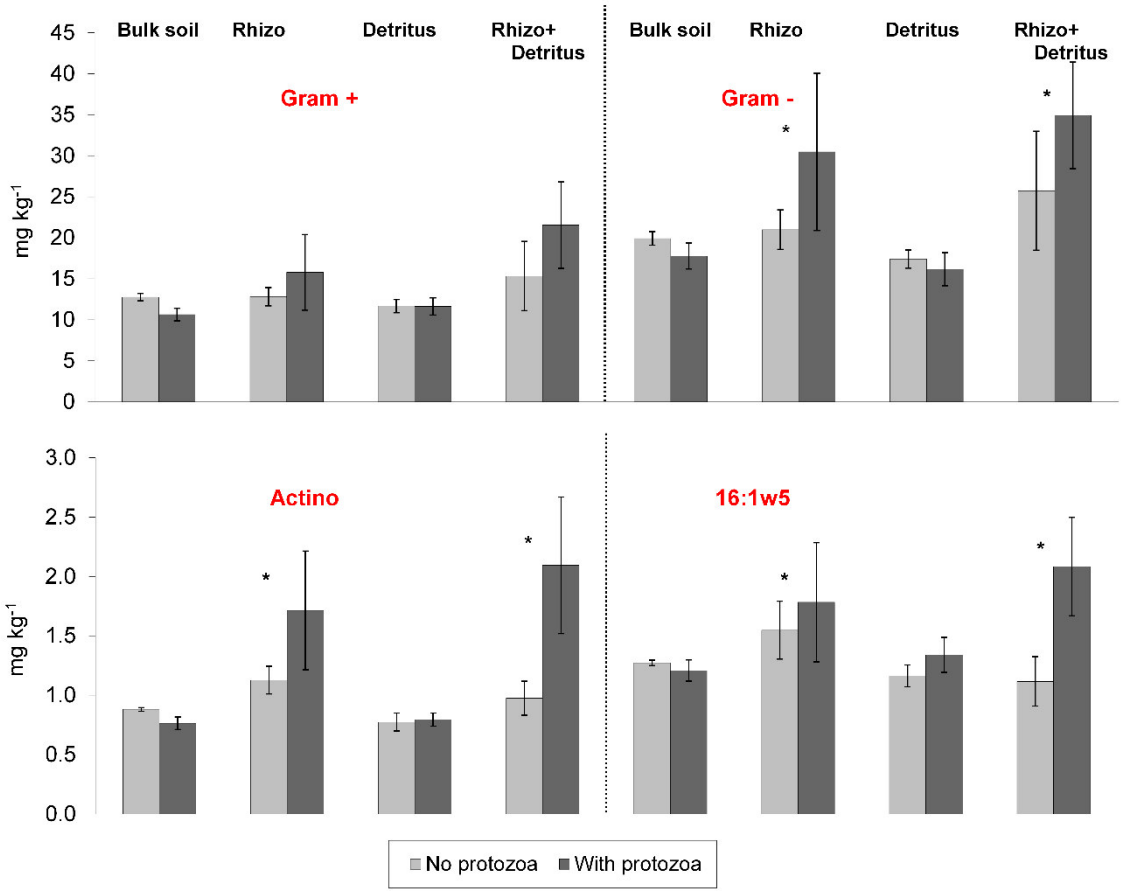

Figure II.5/29 Content of G+, G-, actinobacterial and 16:1w5 biomarkers (mg kg-1 soil) (±SEM) in absence or presence of protozoa in bulk soil, rhizosphere (Rhizo), detritusphere (Detritus) and combined-substrate treatment (Rhizo+Detritus). Stars reflect significant differences between planted (Rhizo; Rhizo + Detritus) and root litter-amended soil (Detritus) (P<0.05).

5.4 Discussion

5.4.1 Grazing effects

Despite MBC was stable, microbes increased incorporation of rhizo-C (^{14}C) in the presence of Acanthamoeba. Microbes enhanced their activity for the utilization of easily available substrates in the rhizosphere (Kuzyakov et al., 2002; Loeppmann et al., 2016a) and may have shifted from dormant to active state (Lennon and Jones, 2011; Blagodatskaya and Kuzyakov et al., 2013; Parry et al., 2014). The increased microbial activity with Acanthamoeba predation, as shown by increased potential enzyme activities, was in line with the higher incorporation of rhizo-C for all treatments.

Total microbial respiration in presence of Acanthamoeba was comparable irrespective of the substrate quality. Pure culture studies revealed that the respiration increased in the presence of the same amoeba (Levrat et al., 1989, 1992) as used in our study (*A. castellanii*). Many studies have demonstrated an increase in CO_2 evolution in the presence of Acanthamoeba in soil (Singh, 1964; Coleman et al., 1977; Kuikman et al., 1990). This is generally regarded as an increase in C mineralization resulting from enhanced microbial activity.

Under substrate limitation, microbes invest energy and nutrients (N and P) in enzyme production to ensure their supply of resources (Schimel and Weintraub, 2003; Kelly et al., 2011; Schimel and Schaeffer, 2012). There are life strategies of microorganisms (Fierer et al., 2007) which are more competitive in the rhizosphere through the consumption of available substrate, such as r-selected microorganisms (Blagodatskaya et al. 2009; 2014; Loeppmann et al., 2016b). These microorganisms may further benefit from a higher availability of nutrients (e.g. N and P) caused by Acanthamoeba grazing on the slow-growing bacteria (Sherr et al., 1992). This is in line with the higher abundance of Gram- compared to Gram+ bacteria in the rhizosphere with protozoan predation as shown by PLFAs. It has been frequently proposed that proteobacteria (r-strategists e.g. bacteria from the *Pseudomonadaceae* or *Burkholderiaceae* family) are the dominant microbes in

the rhizosphere, with the ability to utilize a broad range of root-derived C (Phillipot et al., 2013).

Root exudate-consuming decomposers tend to mine for other N sources to support their fast growth because of the high C to N ratio of root exudates (Nguyen, 2003; Kelly et al., 2011). This is supported by increased activities of chitinase during Acanthamoeba predation, suggesting higher microbial allocation to C-cycling enzyme production (Allison et al., 2011), since N is less limited. When Acanthamoeba were present, much lower affinity of chitinase to the substrate was revealed, irrespective of the treatment. That suggested changes in the relative dominance of organisms with different life strategies (Fierer et al., 2007; Loeppmann et al., 2016b) which are differentiated by the substrate affinity of their enzyme systems (Killham and Prosser, 2015). Leucine-AP and tyrosine-AP clearly demonstrated decreased potential enzyme activities with Acanthamoeba grazing (except Detritus), because there was no need to increase proteolytic enzyme production, due to the higher N availability. Acanthamoeba excretion of ammonia provided an additional substrate pool for microbes and plants.

Beside the C- and N-cycling enzymes, the acid phosphatases play a crucial role in soils, since they catalyze the hydrolysis of monophosphoesters (Nannipieri et al., 2012). Living plants are able to produce high amounts of extracellular acid phosphatase and compete with microbes for P acquisition. The transformation of organic phosphates into mineral P forms is strongly linked to higher acid phosphatase activities (Olander and Vitousek, 2000; Gahoonia et al., 2001). Acanthamoeba predation induced higher P demand in presence of plants as shown by an increase in acid phosphatase activities, which might indicate that amoebae are additional constitutive producers of phosphatases. This corresponds with the results of Gould et al. (1979) who concluded that solely bacteria and the combination of bacteria and amoeba stimulate phosphatase activity in the rhizosphere to support their nutrient demand.

5.4.2 Substrate effects

The dsDNA contents increased with the addition of substrate. This reflected stronger microbial growth in rhizosphere and detrituspehre compared to the bulk soil, resulting in higher microbial biomass.

Microorganisms´ uptake of rhizo-C (^{14}C) in the rhizosphere (Rhizo) compared to the combined-substrate input (Rhizo + Detritus), which indicated a high fraction of active (growing) microorganisms because only the active cells drive the biochemical cycles (Blagodatskaya and Kuzyakov, 2013; Parry et al., 2013). Microbial cells that maintain a potentially activity status (Bodegom, 2007), are ready for energy and nutrient uptake when labile substrates enter the soil through root exudation. This suggests that microbial activity is highly dependent on root activity.

It can be concluded that root litter addition hampered microbial uptake and mineralization as well as allocation of maize-derived ^{14}C to EOC, despite higher substrate amount compared to rhizosphere (Rhizo). Roots are known to be major producers of both β-glucosidase and phosphatase enzymes (Conn and Dighton, 2000).

Especially leucine-AP was significantly higher in the presence of living plants, irrespective of root litter addition, suggesting higher N demand in the presence of plants. These regulatory processes ensure that enzymes are produced only when substrate is available and the end-products of the enzymatic reaction are scarce (Allison et al., 2010, Allison et al., 2011).

All tested enzymes showed increased activities relative to the non-grazed bulk, except for the root litter-treated soil. This reflects that the decomposition of this recalcitrant substrate, such as root litter, reduced the V_{max} of enzymes. This is explained by highly recalcitrant root litter, rich in secondary cell walls that contain lignin and covalent bridges between heteroxylans and lignin (Amin et al., 2014). The degradation processes and the microbial succession resulted in a similar substrate utilization pattern as for soil organic matter decomposition (e.g. as in bulk soil). Because all non-lignified polysaccharides in the litter were already consumed, the decomposition of the remaining material was tied to the oxidative breakdown of lignin and humic condensates by slow-growing decomposers (Joseleau et al., 1994; Allison et al., 2007). Our results confirm a relatively larger

proportion of K- versus r-selected microorganisms on decaying litter (Blagodatskaya et al., 2007; Amin et al., 2014; Loeppmann et al., 2016b). For root litter-treated soil, higher enzyme affinity to substrate was found for β-glucosidase, leucine-aminopeptidase and acid phosphatase relative to the combined-substrate input (Rhizo + Detritus), implying more efficient substrate utilization. This was consistent with lower CO_2 production in root litter-amended soil, indicating lower microbial turnover and lower sequestration of nutrients in soil (Schimel and Schaeffer, 2012).

5.5 Conclusions

Acanthamoeba grazing strongly affected C fluxes and enzyme activities depending on contrasting substrate quality. Microbial activity in planted soil increased with predation, as shown by the increased microbial incorporation of rhizo-C (^{14}C) and activities of C-cycling enzymes, suggesting higher microbial- and enzymatic turnover times under Acanthamoeba grazing in the rhizosphere. The additional N from protozoan excretions supplied microbes and plants with nutrients and accelerated C-, N-, and P-cycling in the rhizosphere. Thus, the proteolytic enzyme production decreased. Accordingly, Acanthamoeba grazing was accompanied by a shift in enzymatic systems towards higher chitinase activities with lower binding affinities, implying an increased degradation of bacterial residues. Root litter hampered C mineralization by microbes in the rhizosphere irrespective of Acanthamoeba grazing, due to the utilization of highly recalcitrant substrates.

In conclusion, enzyme systems, which are essential factors in microbial decomposition mechanisms in soil, implied differential susceptibility of microbes on Acanthamoeba grazing. Consequently, substrate availability and the stimulation by micro-fauna is a crucial driver for microbial decomposition in the rhizosphere and detritusphere.

Acknowledgments

Many thanks to Susann Enzmann, Heike Strutz and Viola Schade for their intensive help in the laboratory. This study was supported by the German Research Foundation (DFG) within the Research Unit “Carbon Flow in Belowground Food Webs assessed by Isotope Tracers”. The authors disclose any potential conflict of interest.

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Supplementary

Table II.5/14 Potential enzyme activity (V_{max}) and half-saturation constant (K_m).

	Chitinase					Tyrosine-AP				
	Vmax	SEM	Km	SEM	Vmax/ Km	Vmax	SEM	Km	SEM	Vmax/ Km
Bulk soil-Prot	1.5	0.1	65	11	0.02	5.8	0.3	286	24	0.02
Bulk soil+Prot	4.4	0.2	144	17	0.03	5.5	0.1	182	9	0.03
Rhizosphere-Prot	5.7	0.3	73	11	0.08	20.3	1.0	552	38	0.04
Rhizosphere+Prot	9.3	0.4	121	12	0.08	14.9	0.5	526	24	0.03
Detritusphere-Prot	2.0	0.1	56	8	0.04	4.6	0.3	583	48	0.01
Detritusphere+Prot	4.3	0.2	152	16	0.03	22.2	6.6	1883	642	0.01
Rhizosphere+Detri-tusphere-Prot	6.3	0.3	79	11	0.08	19.7	0.5	155	9	0.13
Rhizosphere+Detri-tusphere+Prot	13.7	1.1	193	29	0.07	19.3	0.7	178	12	0.11

	Beta-glucosidase					Beta-xylosidase				
	Vmax	SEM	Km	SEM	Vmax/ Km	Vmax	SEM	Km	SEM	Vmax/ Km
Bulk soil-Prot	2.7	0.1	47	6	0.1	0.9	0.07	69	13	0.01
Bulk soil+Prot	2.9	0.1	33	5	0.1	0.7	0.03	38	6	0.02
Rhizosphere-Prot	7.5	0.3	42	6	0.2	1.5	0.08	30	6	0.05
Rhizosphere+Prot	12.0	0.9	137	21	0.1	1.5	0.10	62	11	0.02
Detritusphere-Prot	5.1	0.1	14	2	0.4	1.7	0.09	97	12	0.02
Detritusphere+Prot	9.1	0.3	14	3	0.7	1.7	0.07	78	8	0.02
Rhizosphere+Detri-tusphere-Prot	27.9	1.6	21	5	1.3	1.6	0.07	35	5	0.04
Rhizosphere+Detri-tusphere+Prot	40.4	0.9	24	2	1.7	1.4	0.07	9	4	0.14

	Acid Phosphatase					Leucine-AP				
	Vmax	SEM	Km	SEM	Vmax/ Km	Vmax	SEM	Km	SEM	Vmax/ Km
Bulk soil-Prot	11.9	0.5	72	8	0.16	29.7	2.1	285	29	0.10
Bulk soil+Prot	8.9	1.2	123	38	0.07	20.6	1.8	186	26	0.11
Rhizosphere-Prot	26.6	1.3	62	9	0.43	58.9	5.1	386	45	0.15
Rhizosphere+Prot	31.2	1.5	81	10	0.38	42.2	3.2	349	36	0.12
Detritusphere-Prot	10.3	1.6	267	73	0.04	31.7	3.5	187	33	0.17
Detritusphere+Prot	8.9	0.8	145	28	0.06	15.4	1.5	261	38	0.06
Rhizosphere+Detri-tusphere-Prot	27.9	1.6	21	5	1.33	45.4	3.5	566	54	0.08
Rhizosphere+Detri-tusphere+Prot	33.3	1.6	73	9	0.46	42.9	10.1	538	160	0.08

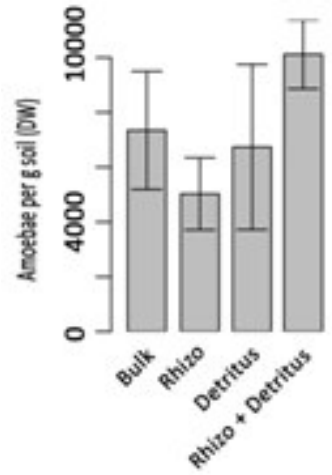

Figure II.5/30 Amoeba abundance (±SEM) in bulk soil, rhizosphere (Rhizo), detritusphere (Detritus) and combined-substrate treatment (Rhizo+Detritus).

6. Effect of root hairs on rhizosphere priming

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Accepted *Soil Biology and Biochemistry* (2016), 10.1016/j.soilbio.2016.05.009

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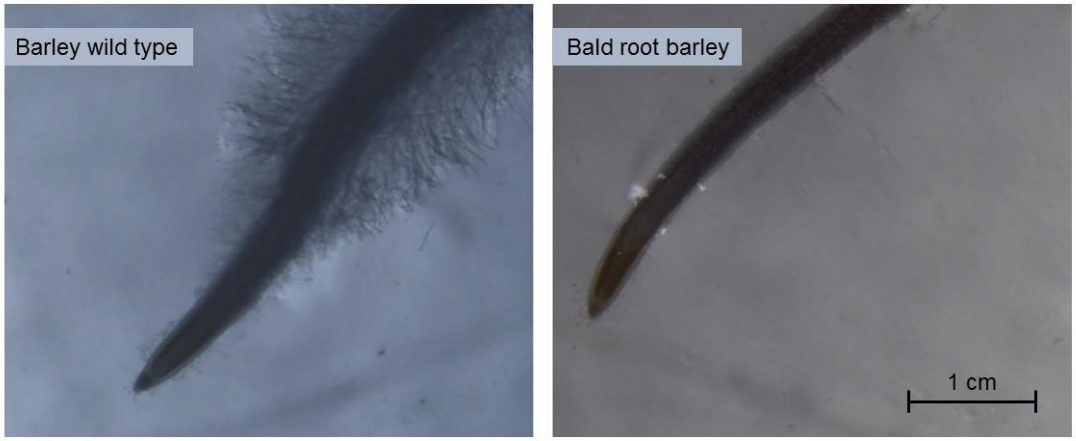
Research highlights

- Root hairs have strong effects on rhizosphere priming of SOM decomposition.
- The presence of root hairs accelerated SOM decomposition by 70%.
- Root hairless barley mutant suppressed SOM decomposition at tillering by 28%.
- At head emergence, SOM decomposition was strongly accelerated by 209%.
- Positive rhizosphere priming was accompanied with higher chitinase and ß-xylosidase activities.

Graphical abstract



Abstract

The influence of plant roots and the associated rhizosphere activities on decomposition of soil organic matter (SOM), the rhizosphere priming effect, has emerged as a crucial mechanism regulating global carbon (C) and nitrogen (N) cycles. However, the role of root morphology in controlling the rhizosphere priming effect remains largely unknown. To investigate the link between root hairs, a critical part of the entire root morphology, and the rhizosphere priming effect, we grew a barley wild type and a barley mutant without root hairs in a greenhouse and continuously labeled them with ^{13}C depleted CO_2. Soil CO_2 efflux was measured during tillering and head emergence stages of plant growth. Based on its $\delta^{13}C$ signature, total CO_2 was partitioned for root-derived and SOM-derived CO_2, and the SOM decomposition primed in the rhizosphere was calculated. Soil microbial biomass C and N, and the activities of six extracellular enzymes (β-cellobiohydrolase, β-glucosidase, acid phosphatase, β-xylosidase, leucin-aminopeptidase, and N-acetyl-β-glucosaminidase) were measured to test the effects of root hairs.

During the early stage of development (tillering), when plants were sufficiently supplied with nutrients, the barley mutant without root hairs used photosynthates more efficiently for plant biomass production. In contrast, high C costs for root hair formation reduced the growth of the barley wild type. At this stage, the wild type with regular root hairs produced a positive rhizosphere priming effect (69% increase), but the mutant without root hairs produced a negative priming effect on SOM decomposition (28% decline). At the head emergence stage, when nutrients were scarce, the inefficiency of nutrient uptake without root hairs reduced the plant biomass production of the mutant. At this stage, both barley types produced positive rhizosphere priming effects (72% and 209% increase for the wild type and the mutant, respectively) and the microbial biomass was higher for both planted soils compared to the unplanted soil. Extracellular enzymes responsible for the decomposition of stable SOM had higher activities in cases of positive priming effects. Concluding, root hairs have strong effects on rhizosphere priming of SOM decomposition.

Key words: Rhizosphere priming; Soil CO_2 efflux; Root morphology; Root hairless Barley mutant; Isotope labeling; Enzyme activities

6.1 Introduction

Soil CO_2 is one of the largest fluxes in the global C cycle, approximately ten-fold greater than CO_2 emissions from fossil fuel combustion (Schlesinger and Andrews, 2000; Amundson, 2001). The majority of this flux results from the decomposition of soil organic matter (SOM) and litter by microbes (Kuzyakov, 2006). In recent years, there is an emerging view that, in addition to temperature and moisture, carbon substrate availability is a key factor controlling SOM turnover (Fontaine et al., 2007; Paterson and Sim, 2013). These changes in the rate of SOM turnover following the input of easily decomposable substrates for microorganisms are termed 'priming effects'.

While decaying leaf and root litter provides some labile substrate for soil microbes, the majority of the labile substrate in soils comes from roots. For example, some studies have reported that SOM decomposition may be 380% greater in soils with roots compared to unplanted soils (positive rhizosphere priming effects; RPE) (Cheng et al., 2014). Accordingly, the magnitude of RPE may control C fluxes at the ecosystem level and influence ecosystem feedbacks to climate (Cheng et al., 2014; Finzi et al., 2015).

The explanation for most of the reported positive RPE is microbial activation, i.e. the stimulation of growth and activity induced by root-derived substrates. Microbes utilize this energy subsidy to produce extracellular enzymes (exoenzymes) that enhance the release of nutrients from SOM (Blagodatskaya and Kuzyakov, 2008). While microbes benefit from the nutrients released through enhanced decomposition, plants may benefit too - suggesting that RPE may be an evolutionary stable strategy (Cheng et al., 2014).

Living roots release numerous available low molecular weight substrates such as sugars, carboxylic acids and amino acids throughout the soil profile and over the course of the growing season (Nguyen, 2003; Jones et al., 2009). These substrates are not homogeneously distributed along the root segments but are rather released in distinct areas, mainly at the root tips (McDougall and Rovira, 1970; Nguyen, 2003; Dennis et al., 2010; Pausch and Kuzyakov, 2011). For this reason, root morphology (e.g. lateral root formation, number of root tips, root hair formation) may largely impact exudation (Nguyen, 2003), and may, hence, be decisive for rhizosphere priming effects. The root morphology, in turn, is

mainly controlled by the nutrient availability in the soil since changes in root architecture can alter the capacity of plants to take up nutrients (López-Bucio et al., 2003). Several strategies have been developed by plants to increase the uptake of limited nutrients from the soil. An efficient strategy to acquire limited nutrients is the production of root hairs, which could differ in numbers, density and length between plant species depending on the kind of nutrients and nutrient availability in the soil (Jungk, 2001). The substantial contribution of root hairs to plant nutrition and accompanied therewith nutrient shortages in the rhizosphere and high energy supply to microbes through exudation, as well as direct and indirect enhancement of enzyme activities (Spohn and Kuzyakov, 2014) may be crucial for rhizosphere priming effects. A barley mutant lacking root hairs completely was discovered by Gahoonia et al. (2001). This mutant enabled us to study the role of root hairs for rhizosphere priming effects.

In the present experiment a barley wild type with root hairs and the root hairless mutant were grown under controlled conditions. Rhizosphere priming effects, i.e. changes in the rate of SOM decomposition, are indicated by an increase or decrease of SOM-derived CO_2 production in planted compared to an unplanted soils. Continuous labeling of shoots with ^{13}C depleted CO_2 allowed to differentiate root-derived CO_2 from SOM-derived CO_2 and finally to calculate RPE as the difference in SOM-derived CO_2 between a planted and unplanted soils. To investigate the influence of plant age, the soil CO_2 efflux was trapped at two growth stages of the plants (tillering and head emergence). Microbial parameters (microbial biomass C and nitrogen (N), enzyme activities) were analyzed to assess changes of microbial activities.

We hypothesize that the rhizosphere priming effect depends on root morphology. More specifically, a better nutrient acquisition of the wild type with root hairs through a higher total root surface area will cause a larger plant biomass production, thus, leading to higher exudation and higher positive RPE. We also expect that the higher the positive priming is the more active are exoenzymes responsible for the decomposition of more stable substrates (organic C and N). Plant age is known to play an important role for the intensity of priming (Fu and Cheng, 2002; Pausch et al., 2013). Due to different growth pattern and nutrient demands, we hypothesize that plant age influences rhizosphere priming on SOM

decomposition differently for the hairless barley mutant and the barley wild type with root hairs.

6.2 Material and Methods

6.2.1 Experimental Setup

Two barley (*Hordeum vulgare* L.) types, a wild type (cv. optic; WT) and a root-hairless mutant called bald root barley (*brb*, Gahoonia et al., 2001), were grown in a greenhouse and were continuously labeled with ^{13}C depleted CO_2 (Cheng and Dijkstra, 2007). The plants were exposed to the tracer from the emergence of the first leaf till the end of the experiment. Briefly, a constant CO_2 concentration of 400±5 ppm and a constant $\delta^{13}C$ value of about -18‰ was maintained inside the greenhouse by regulating the flow of pure ^{13}C depleted CO_2 (99.9% CO_2, $\delta^{13}C$ of -38‰) from a tank and setting CO_2-free air flow rate proportional to the leakage rate (300 L/min) of the greenhouse (Zhu and Cheng, 2012; Pausch et al., 2013). The CO_2-free air was produced from compressed air passed through six soda lime columns (20 cm diameter, 200 cm length) filled with approximately 40 kg soda lime (pellets made of NaOH and $Ca(OH)_2$ mixture) each. The CO_2-free air flow was set at 120 L/min. The CO_2 concentration inside the greenhouse was continuously monitored by an infra-red gas analyzer (Model LI-820, Li-COR, Lincoln, NE, USA) and stabilized at 400±5 ppm by computer controlled CO_2 injection from the tank. A fan was used to ensure a uniform distribution of the CO_2 inside the greenhouse. For the duration of the experiment, the $\delta^{13}C$ value of the greenhouse air was measured every three days during the light period by pumping air through a glass airstone immersed in 50 mL of 0.5 M NaOH solution. The CO_2 trapping efficiency was nearly 100% as checked by an infra-red gas analyzer (Model LI-6262, Li-COR, Lincoln, NE, USA). An aliquot of the sample was precipitated with $SrCl_2$ as $SrCO_3$ using the method described by Harris et al. (1997) and analyzed for $\delta^{13}C$ (relative to PDB standard) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The mean $\delta^{13}C$ value of the CO_2 in the greenhouse air was -18.2±0.3‰.

The two barley types were grown in PVC pots (15 cm diameter, 40 cm height, equipped with an inlet tube at the bottom for aeration and soil CO_2 trapping). A nylon bag filled with ~1500 g sand was placed at the bottom of each PVC pot to improve air circulation.

Each pot was filled with about 7 kg sieved (<2mm) soil. The soil was taken from the plough horizon (top 30 cm) of a sandy loam (Mollisol) from a farm on the campus reserves of the University of California, Santa Cruz. The soil contained 1.18±0.01% organic C and 0.13±0.001% N, had $\delta^{13}C$ and $\delta^{15}N$ values of -26.45±0.07‰ and 7.12±0.02‰, respectively, and a pH value of 5.8. All filled pots were wetted to 20% gravimetric soil moisture content (equivalent of 80% of the water holding capacity) with deionized water.

For each barley type, 10 pots were set up. In addition, 8 unplanted pots (unplanted soil; US) were prepared (in total 28 pots). The seeds were presoaked overnight and 6-8 barley seeds were planted per pot. The inlet tube at the bottom of each pot was connected to an aquarium pump to aerate the pots. This was done 2 times during the dark period to avoid contamination of the growth chamber $\delta^{13}C$ signal with that of soil-derived CO_2 during the assimilation period.

The soil moisture content was measured gravimetrically and adjusted daily to 80% of the water holding capacity. To maintain homogeneous soil moisture and good soil structure, water was added through perforated tubes buried at the center of the pot (inner diameter 0.32 cm, total length 20 cm, buried length 10 cm). The location of the pots in the greenhouse was changed weekly by mixing them randomly to guarantee similar growing conditions for the plants. The day time air temperature inside the greenhouse was maintained at 23°C by two air conditioning units. The night time temperature was kept above 17°C. Artificial lighting (1100W lights, P.L. Light Systems, Beamsville, ON) was used to ensure an adequate light intensity throughout the experiment. The light intensity was kept above 900 W m^{-2}. The photoperiod was set from 4:30AM to 4:30PM. The relative air humidity was kept at 45% by a dehumidifier (Kenmore Elite 70 pint, Sears, Chicago, IL, USA).

6.2.2 Measurements

Soil CO_2 efflux

Soil CO_2 efflux from each pot was measured at two growth stages of barley, 29-30 days after planting (DAP) at tillering (T1) and 64-65 DAP at head emergence (T2), by using a closed-circulation CO_2 trapping system (Cheng et al., 2003; Pausch et al., 2013). Prior to

each CO_2 trapping the pots were sealed with non-toxic silicone rubber (GI-1000, Silicones Inc., NC, USA) added directly to the soil surface.

Soil CO_2 trapping was performed on 4 replicates each of the unplanted soil, and the barley with and without root hairs at T1. At T2, CO_2 was trapped from 6 replicates of planted pots and 4 replicates of unplanted pots. Shortly before CO_2 trapping, the CO_2 inside the pots was removed by circulating the isolated air through a soda lime column (3 cm diameter, 50 cm length) for 40 min. Then CO_2 produced in the sealed pots was trapped for 24 h in 400 ml of 0.5 M NaOH solution. Four blanks were included to correct the total inorganic C content for possible contamination from carbonate in the NaOH stock solution and from sample handling (Cheng et al., 2003; Pausch et al., 2013). An aliquot of each NaOH solution was analyzed for total inorganic carbon using a Shimadzu TOC-5050A Total Organic Carbon Analyzer. Another aliquot was precipitated as $SrCO_3$ (Harris et al., 1997) and analyzed for $\delta^{13}C$ by means of a continuous flow isotope ratio mass spectrometer as described above.

Shoot, root and soil analyses

After each CO_2 sampling the pots were destructively harvested. The shoots were cut at the base. The soil of each pot was pulled out and the roots were separated by hand-picking. Subsamples of about 1 kg soil were stored in a freezer (-18°C) until further analysis. Shoots, root, and soil samples were dried at 60°C for 3 days, weighed, grinded in a ball mill and measured for $\delta^{13}C$ and $\delta^{15}N$ using a Carlo Elba 1108 elemental analyzer interfaced to a Thermo-Finningan Delta Plus XP isotope ratio mass spectrometer at the Isotope Facility of University of California-Santa Cruz.

Dissolved N (DN) which is extractable with K_2SO_4 was determined as described below (2.2.3). Extractable phosphorus (bioavailable inorganic ortho-phosphate) was determined on dried and grinded soil samples by the Bray-Method at the Analytical Laboratory, University of Davis (http://anlab.ucdavis.edu/).

Microbial biomass C and N

Soil microbial biomass C (MBC) and soil microbial biomass N (MBN) were determined on all soil samples by the chloroform fumigation extraction method described by Vance

et al. (1987) with the modification that fumigated and non-fumigated soil samples (7.5 g) were extracted for 1 hour with 30 mL of 0.05 M K_2SO_4 solution. The samples were filtered and the extracts were analyzed for total organic C and N by means of a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena). Total N content of the non-fumigated extracts were used as a measure of available N. The difference between the extracts of fumigated and non-fumigated samples gave the amount of chloroform-labile C and N (hereafter referred to as MBC and MBN). We noted that these values did not correspond to total amount of MBC and MBN as the extraction efficiency was not taken into account. Reported conversion factors k_{ec} (or k_{en} for N) ranged from less than 0.2 to 0.45 among different soils (Wu et al., 1990; Dictor et al., 1998; Bailey et al., 2002). Thus, for the purpose of comparing treatment effects and avoiding biases of conversion factors, the data presented in this study were not corrected by conversion factor.

Enzyme assays

To determine the activities of the enzymes β-cellobiohydrolase (exo-1,4-β-glucanase, EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.2), β-xylosidase (EC 3.2.2.27), leucin-aminopeptidase (LAP) (EC 3.4.11.1), and N-acetyl-β-glucosaminidase (chitinase, EC 3.2.1.52), we used 4-methylumbelliferyl-β-D-cellobioside, 4-methylumbelliferyl-β-D-glucopyranoside, 4-methylumbelliferyl-phosphate, 4-methylumbelliferyl-7-β-D-xylopyroniside, L-leucine-7amino-4-methylcoumarin hydrochloride and 4-methylumbelliferyl N-acetyl-β-D-glucosaminide, respectively. The soil suspension was dispersed by an ultrasonic disaggregator (50 J s^{-1} for 120 s) after addition of half a gram of soil to 50 ml sterile water in autoclaved jars (De Cesare et al., 2000). While stirring the soil suspension 50 µl aliquots were withdrawn and dispensed in 96-well microplates (Brand pureGrade, black). Buffer (80 ml) was added (0.1 M MES buffer, pH 6.1 for carbohydrases and phosphatase, 0.05 M TRIZMA buffer, pH 7.8 for leucine-aminopeptidase) (Marx et al., 2001; 2005; Loeppmann et al., 2016).

We added 100 µl of series concentrations of substrate solutions (20, 40, 60, 80, 100, 200, 400 µmol substrate g^{-1} soil) to the wells and kept the temperature at 21 °C. The microplates were agitated and measured fluorometrically (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation with an automated fluorometric plate-reader (Wallac 1420, Perkin Elmer, Turku, Finland). Fluorescence was converted into an amount of

MUB (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin), according to specific standards, which had been prepared in sub-samples from the various soil suspensions.

6.2.3 Calculations

The contribution of CO_2 derived from SOM decomposition ($C_{SOM\text{-}DERIVED}$, mg C kg^{-1} soil day^{-1}) to total soil respiration was calculated using a linear two-source isotopic mixing model:

$$C_{SOM-DERIVED} = C_{TOTAL} \cdot \frac{\delta^{13}C_{TOTAL} - \delta^{13}C_{ROOT-DERIVED}}{\delta^{13}C_{SOM-DERIVED} - \delta^{13}C_{ROOT-DERIVED}} \quad (1)$$

$$C_{ROOT-DERIVED} = C_{TOTAL} - C_{SOM-DERIVED} \quad (2)$$

where C_{TOTAL} is the total CO_2 efflux of the planted soil (mg C kg^{-1} soil day^{-1}) and $\delta^{13}C_{TOTAL}$ the corresponding $\delta^{13}C$ value (‰). $\delta^{13}C_{SOM\text{-}DERIVED}$ is the $\delta^{13}C$ value of CO_2 from SOM decomposition measured in the unplanted soils (‰). $C_{ROOT\text{-}DERIVED}$ is the root-derived CO_2 in the planted soils (mg C kg^{-1} soil day^{-1}) with $\delta^{13}C_{ROOT\text{-}DERIVED}$ as the corresponding $\delta^{13}C$ value (‰).

To consider isotopic fractionation, we accounted for ^{13}C differences between the isotopic composition of roots and that of root-derived CO_2 (Pausch et al., 2013). The fractionation factor (*f*) was taken from Zhu and Cheng (2011) and was -0.87‰. This fractionation factor was measured for wheat (*Triticum aestivum*) and was chosen since barley and wheat are both belonging to the *Poaceae* family and are both monocotyledons with similar properties.

$\delta^{13}C_{ROOT\text{-}DERIVED}$ was calculated by correcting the $\delta^{13}C$ value of the root ($\delta^{13}C_{ROOT}$) by a fractionation factor (f):

$$\delta^{13}C_{ROOT-DERIVED} = \delta^{13}C_{ROOT} + f \qquad (3)$$

Table II.6/15 End member values (±SEM) used in two-source isotopic mixing models in order to calculate the contribution of SOM-derived CO_2 to total soil CO_2 of the planted treatments.

Sampling time	Treat-ment	Root-derived CO_2 [‰]	SOM-derived CO_2 of the unplanted soil [‰]
	US		-24.20±0.51 (4)
T1	**WT**	-38.65±0.28 (3)	
	brb	-39.65±0.36 (4)	
	US		-24.90±0.22 (4)
T1	**WT**	-38.57±0.22 (6)	
	brb	-39.29±0.21 (4)	

The RPE on SOM decomposition was calculated by subtracting the CO_2 flux of the un-planted soil ($C_{SOM\text{-}DERIVED}$ (US)) from the SOM-derived CO_2 flux of the planted soil ($C_{SOM\text{-}DERIVED}$ (WT, *brb*)).

$$RPE = C_{SOM-DERIVED}(WT, brb) - C_{SOM-DERIVED}(US) \qquad (4)$$

The RPE was related to total root biomass (gDW) as well as expressed as percentage of basal respiration of the unplanted soil.

6.2.4 Statistics

The values presented in the figures and tables are given as means ± standard errors. A one-way analysis of variance (ANOVA) was conducted to test for significant differences in all measured data between the unplanted soil, and barley with and without root hairs by calculating the ANOVA separately for each sampling date. The significance of differences between individual means was obtained by a *post hoc* unequal N HSD test. To test for significant differences within each treatment but between T1 and T2 (phenological effects) a dependent (paired) t-test was used. Moreover, rhizosphere priming values were tested for significant deviation from zero by a t-test. All statistical analyses were performed with the statistical package STATISTICA for Windows (version 7.0; StatSoft Inc., OK, USA).

For enzyme analyses, we used a non-linear regression (Michaelis-Menten kinetics) to estimate the kinetic parameter V_{max} (Marx et al., 2001). Each soil sample was measured as an analytical triplicate. The kinetic parameters were fitted by minimizing the least-square sum using GraphPad Version 6 software (Prism, USA). Parameter optimization was restricted to the applied model equation as indicated by maximum values of statistic criteria: r^2. Outliers were identified by the ROUT method, based on the False Discovery Rate (FDR) (Motulsky and Brown, 2006). A multiple t-test was applied to test for differences in enzyme activities between the unplanted soil, and the barley with and without root hairs and between T1 and T2. Statistical significance was determined using the Holm-Sidak method ($P \leq 0.05$).

6.3 Results

6.3.1 Plant biomass and $\delta^{13}C$ and $\delta^{15}N$ values

The main difference between the two barley types was the production of plant biomass; shoots and roots. While the mutant (*brb*), completely lacking root hairs, produced higher shoot biomass during the tillering stage (T1), both root and shoot biomass was reduced at the head emergence stage (T2) compared to the barley wild type (WT) (Table II.6/17).

At T1, C and N contents of shoots were on average 34.3±0.4% and 6.1±0.1%, respectively. At T2 a higher C content of 38.2±0.4% was measured while the N content decreased to 1.7±0.1% compared to T1. This led to a much higher C/N ratio at T2 compared to T1 (Table II.6/18). Similarly, the C content of roots was higher at T2 (33.6±1.1%) compared to T1 (22.1±2.0%). The N content of roots, however, was similar between the two sampling times. The C/N ratio of roots increased from 13.9±0.6 at T1 to 25.3±1.4 at T2.

The plants were successfully labeled with ^{13}C depleted CO_2 as shown by the $\delta^{13}C$ values of shoots and roots (Table II.6/17). The $\delta^{13}C$ value of shoots was -41.4±0.1‰ at T1 which was lower than at T2 (-39.3±0.3‰). The $\delta^{13}C$ value of roots did not differ between T1 and T2. Interestingly, the $\delta^{15}N$ value of roots increased between T1 and T2 and was about 2.6‰ higher at T2 (Table II.6/17).

Table II.6/16 Plant (shoot and root) biomass, C and N contents, C/N ratios, and $\delta^{13}C$ and $\delta^{15}N$ values (±SEM) 30 (T1) and 65 (T2) days after planting. Significant differences ($P \leq 0.05$) between the treatments are indicated by different lower-case letters. The asterisk indicates significant differences between T1 and T2.

Sampling time		**T1**		**T2**	
Treatment		**WT**	***brb***	**WT**	***brb***
Shoot	**Biomass [g_{DW} pot^{-1}]**	3.03±0.13b	3.70±0.07a	38.87±2.38*	30.70±2.54*
	C content [%]	34.08±0.74	34.51±0.31	38.67±0.21*	37.69±0.69*
	N content [%]	6.15±0.06	6.08±0.09	1.47±0.10*	1.87±0.16*
	C/N	5.54±0.08	5.67±0.05	26.72±2.09*	20.64±1.95*
	$\delta^{13}C$[‰]	-41.45±0.05	-41.40±0.10	-38.75±0.10*	-39.75±0.42*
	$\delta^{15}N$[‰]	5.23±0.28	5.07±0.21	6.13±0.40	5.67±0.50
Root	**Biomass [g_{DW} pot^{-1}]**	1.65±0.04	1.88±0.43	12.30±1.21a*	7.37±0.90b*
	C content [%]	21.13±2.69	23.04±3.18	32.14±0.96*	34.99±1.83*
	N content [%]	1.61±0.35	1.68±0.26	1.27±0.11	1.47±0.15
	C/N	13.89±1.29	13.88±0.46	25.94±1.75*	24.57±2.47*
	$\delta^{13}C$[‰]	-37.84±0.21	-38.78±0.36	-37.70±0.27	-38.46±0.33
	$\delta^{15}N$[‰]	3.61±0.63	2.51±0.27	5.87±0.25*	5.53±0.33*
Shoot/Root		1.91±0.11	2.21±0.35	3.37±0.63	4.25±0.31*

6.3.2 Soil N and P contents, microbial biomass C and enzyme activities

Nutrient uptake by plants led to lower dissolved N (DN) and plant available P in planted soils. The DN content was reduced by ~46% in planted soil compared to the unplanted

soil at tillering and further decreased to 14% at head emergence stage. A small reduction (from 66.79±1.2 to 59.92±1.70 mg P kg^{-1} soil) of available P with time only occurred for the *brb*.

Table II.6/17 Dissolved nitrogen (DN), available phosphorus (Bray-P), and chloroform-labile microbial biomass C and N, at tillering (T1) and head emergence stage (T2) for unplanted soil (US), the barley wild type (WT) and the root-hairless barley mutant (*brb*).

Sampling time	**T1**			**T2**		
Treatment	**US**	**WT**	***brb***	**US**	**WT**	***brb***
K_2SO_4-extractable DN [mg N kg^{-1} soil]	104.62 ±4.68a	57.13± 1.34b	57.74± 3.18b	nd	15.00± 1.71*	15.61± 1.59*
Bray-P [mg P kg^{-1} soil]	66.71± 1.87	62.74± 0.69	66.79± 1.20	68.35± 1.58	60.82± 2.52	59.92± 1.70*
Chloroform-labile MBC [mg C kg^{-1} soil]	67.60± 5.41	62.03± 2.72	66.61± 6.26	81.25± 7.55	101.46 ±14.42 *	92.21± 7.16*
Chloroform-labile MBN [mg N kg^{-1} soil]	8.92±1. 77	6.72±1. 81	8.66±0. 39	10.21± 1.93	13.19± 1.33*	14.49± 1.21*
MBC/ MBN	8.70±2. 18	11.41± 2.71	7.84±1. 13	9.47±3. 05	7.67±0. 56	6.48±0. 63

6.3.3 Microbial biomass C and N and enzyme activities

MBC and MBN (chloroform-labile C and N) was similar between the unplanted soil, and the barley with and without root hairs (Table II.6/18). However, at the head emergence

stage of plant growth (T2) MBC and MBN increased for both barley types compared to the tillering stage (T1).

At tillering stage, the presence of root hairs (WT) decreased the activities of β-glucosidase, β-cellobiohydrolase, and acid phosphatase, while the activity of β-xylosidase and chitinase were higher compared to the unplanted soil (Figure II.6/31). In contrast, the activities of β-glucosidase, β-cellobiohydrolase, and LAP did not differ between *brb* and the unplanted soil, but a higher activity of β-xylosidase and acid phosphatase was measured. At head emergence stage, both barley types induced lower activity rates of β-glucosidase and LAP, while the activities of β-xylosidase and chitinase increased through planting. β-cellobiohydrolase and acid phosphatase activities of the planted soils were similar to that of the unplanted soil at T2.

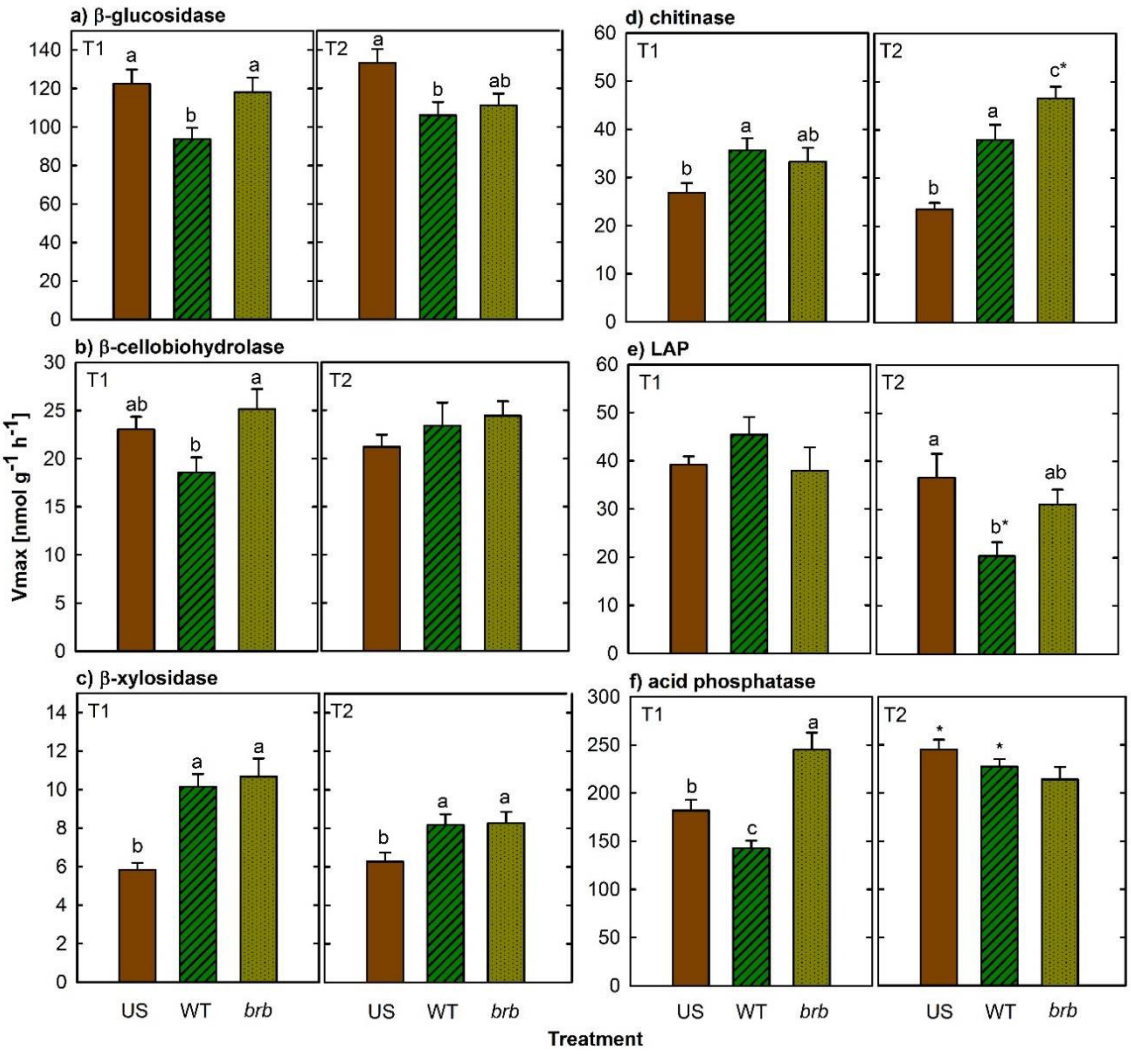


Figure II.6/31 Potential enzyme activities for a) β-glucosidase, b) β-cellobiohydrolase, c) β-xylosidase, d) chitinase, e) leucine-aminopeptidase (LAP), and f) acid phosphatase (±SEM) at tillering (T1) and head emergence stage (T2) for unplanted soil (US), the barley wild type (WT) and the root-hairless barley mutant (*brb*). Bars followed by different lower-case letters indicate significant differences (P≤0.05) between the treatments at one sampling date. Significant differences between T1 and T2 are indicated by an asterisk.

6.3.4 CO_2 efflux partitioning

Total soil CO_2 efflux was influenced by planting and by the presence of root hairs as well as by sampling time. At T2 all planted soils showed higher total soil CO_2 efflux (sum of SOM- and root derived CO_2) compared to the unplanted soils (Figure II.6/32).

SOM-derived CO_2 was higher for WT (29.2±0.6 mg C kg^{-1} soil day^{-1}) compared to *brb* (12.5±1.8 mg C kg^{-1} soil day^{-1}) at T1 (Figure II.6/32, top). Moreover, at T2 both barley types had higher SOM-derived CO_2 (24.3±3.0 mg C kg^{-1} soil day^{-1} for WT and 43.8±9.7 mg C kg^{-1} soil day^{-1} for *brb*) compared to the unplanted soil (14.2±0.3 mg C kg^{-1} soil day^{-1}). However, this was only statistically significant (P>0.05) for the root-hairless mutant. While SOM-derived CO_2 remained relatively constant for the unplanted soil and the WT between T1 and T2, the *brb* showed higher SOM-derived CO_2 at T2.

Root-derived CO_2 consists of CO_2 released from root respiration per se and of CO_2 released through microbial decomposition of rhizodeposits. Root-derived CO_2 positively correlates with root biomass (R^2=0.99, data not shown) (Pausch et al., 2013). At tillering, root-derived CO_2 did not differ between the barley wild type and the mutant. However, root-derived CO_2 increased at head emergence for both barley types with increasing root biomass (Figure II.6/32 (bottom), Table 2). Moreover, the lower root biomass of the *brb* at T2 compared to WT is reflected in a slight, but not significant, lower root-derived CO_2.

On a root dry weight basis, root-derived CO_2 was similar in the two barley types and between sampling dates (Figure II.6/32, inlet). However, there was a trend of less root-derived CO_2 at head emergence (Figure II.6/32, inlet).

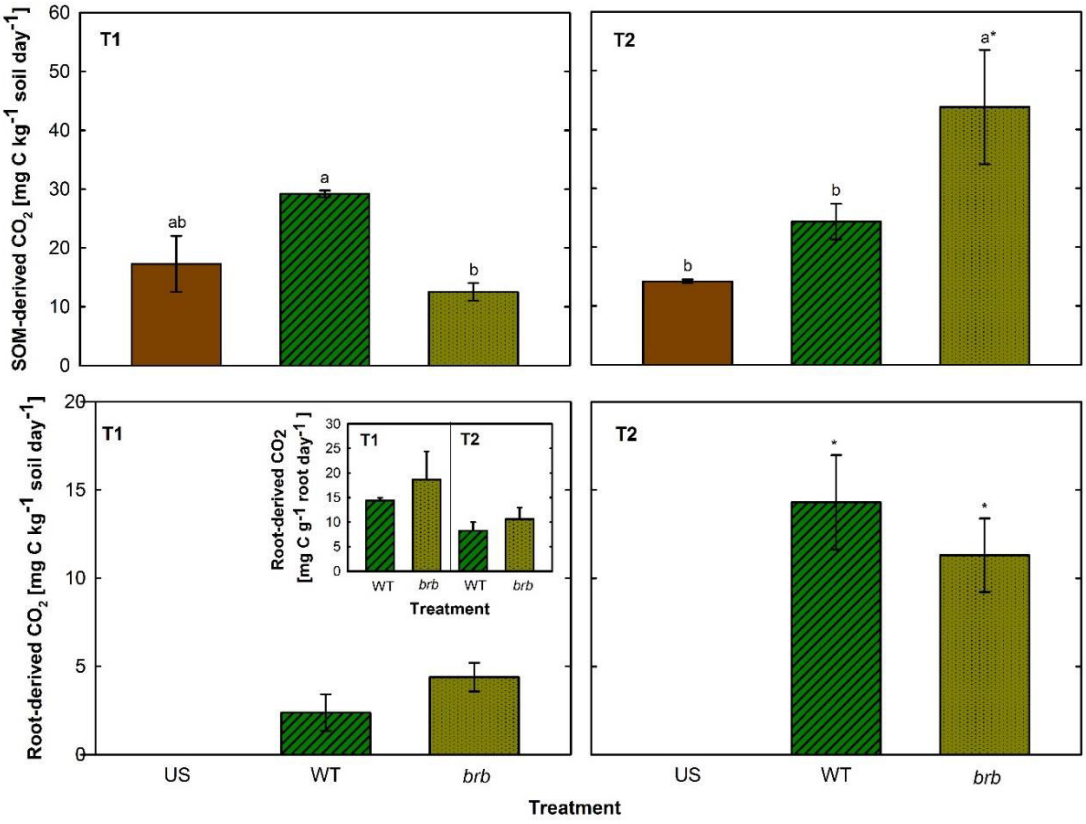


Figure II.6/32 SOM-derived CO_2 (top) and root-derived CO_2 (bottom) at T1 (left) and T2 (right). Bars followed by different lowercase letters indicate significant differences ($P \leq 0.05$) between the treatments at one sampling date. Significant differences between T1 and T2 are indicated by an asterisk. The inlet shows the root-derived CO_2 per g root for both barley types and sampling stages.

6.3.5 Rhizosphere priming effect

During the early stage of plant growth (T1), rhizosphere priming was largely controlled by the presence of root hairs. While SOM decomposition was increased for the wild type by 69% compared to the unplanted soil (P=0.002), it decreased for the root-hairless barley by 28% (P=0.051; Figure II.6/33, right y-axis). At the head emergence stage (T2), both barley types showed positive priming effects with even higher intensity under the mutant. Rhizosphere priming was enhanced for the wild type compared to the unplanted soil by 72% (P=0.020). The highest positive priming effect was measured for the barley mutant lacking root hairs, amounting to 209% of the unplanted soil (P=0.055).

To account for root properties effects, a specific RPE was calculated by relating total primed C to root biomass (Fig. II.6/33, left y-axis). The specific RPE was highest for the wild type at the early stage of plant growth (47.9±1.1 mg C g^{-1} root day^{-1}). A negative priming effect for the *brb* was measured at T1 with -22.2±8.5 mg C g^{-1} root day^{-1}). At the head emergence stage (T2) the WT showed with 5.7±2.0 mg C g^{-1} root day^{-1}, a lower specific RPE than during tillering (T1). In contrast, the barley mutant shifted from negative priming at T1 to a positive RPE at T2 (28.2±9.7 mg C g^{-1} root day^{-1}).

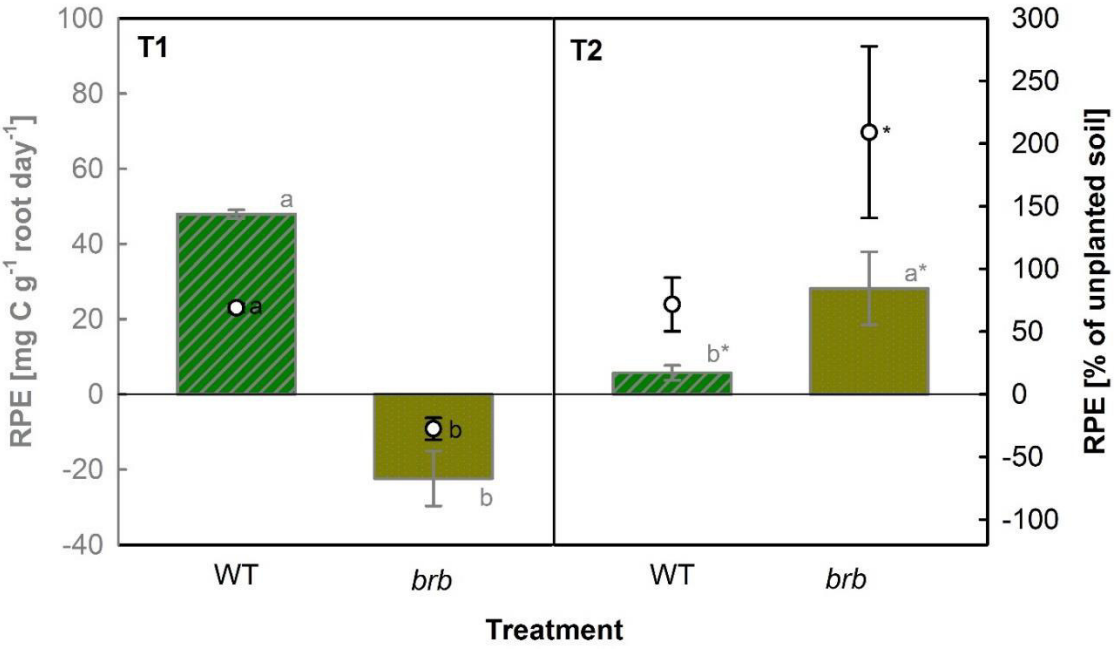


Figure II.6/33 Rhizosphere priming effects per root dry weight (±SEM) (left axis, bars) and as % of the unplanted soil (±SEM) (right axis, dots) at T1 and T2 in treatments with a barley wild type (WT) and a root hairless barley mutant (*brb*). Bars followed by different lower-case letters indicate significant differences (P≤0.05) between the treatments. Significant differences between T1 and T2 are indicated by asteriks.

6.4 Discussion

During the tillering stage of plant growth, SOM decomposition was enhanced (positive priming) in soils with the barley wild type by 69% compared to the basal respiration of the unplanted soil. The data are within the range of priming results published for wheat, another monocotyledon plant from the *Poaceae* family. Wheat showed positive priming effects ranging from 42% of the unplanted soil (28 day old wheat) (Cheng and Johnson, 1998; Cheng et al., 2014) to 75% for 30 days old wheat (Pausch et al., 2013). Positive priming effects could be explained by the 'microbial activation hypothesis' (Kuzyakov, 2002; Cheng and Kuzyakov, 2005), which assumes that the activity and growth of microorganisms is enhanced through metabolizing labile substrates (e.g. root exudates), further leading to an accelerated SOM turnover. In the presence of labile plant C microbes start decomposing SOM to acquire N ('Microbial nitrogen mining' Crain et al., 2007).

Interestingly, the barley mutant lacking root hairs showed a complete opposed effect on SOM turnover. SOM decomposition was reduced by 28% compared to the unplanted soil; hence, the *brb* induced negative priming at the tillering stage. As both barley types produced same amounts of roots at the tillering stage, our result point to root morphology (here the presence or absence of root hairs) as a main determinant for RPE. Negative priming effects were observed in short-term experiments (Cheng et al., 2014) and were explained either by 1) 'Preferential substrate utilization' (PSU), i.e., microorganisms, not limited in N, can switch from the decomposition of SOM to the decomposition of easily available rhizodeposits or by 2) 'Microbial competition hypotheses'. The latter suggests that microbes and plants compete for nutrients and thus, microbial growth decreases, thereby, depressing SOM decomposition (Kuzyakov, 2002; Cheng and Kuzyakov, 2005). At tillering, when mineral nutrients were still abundant, the activity of extracellular enzymes measured in soils with the root-hairless mutant did not differ or even increased (ß-xylosidase, acid phosphatase) compared to the unplanted soil. This may point to PSU.

The present study shows that already at the tillering stage, root morphology plays a major role for rhizosphere priming effects. The extension of the rhizosphere by root hairs accelerated SOM decomposition.

At the head emergence stage, both barely types induced positive rhizosphere priming effects. When referred to the unplanted soil, the wild type primed 72%, while the *brb* increased SOM-decomposition by 209% compared to the unplanted soil. In a recent study Mwafulirwa et al. (2016) investigated barley genotypes and reported negative priming at day 19 after planting while all genotypes induced positive priming after 27 days of growth when nutrients are becoming scarce. Overall, a higher root biomass per pot (10 times higher for WT between T1 and T2, and 4 times higher for *brb*) at increasing N limitation (about 7 times lower at T2 compared to the US at T1) triggers the positive priming effects at T2 in the present study. Since the dissolved N is highly reduced at T2, microorganisms start mining for N to meet their N demand and thus, decompose SOM more intensively. This effect is clearly indicated by the $\delta^{15}N$ values of the plants. $\delta^{15}N$ values increase with SOM stabilization (Kramer et al., 2003), hence, more stabilized SOM pools are likely enriched in ^{15}N. For both barley types the $\delta^{15}N$ values of roots were higher at the head emergence stage indicating a higher N gain from more stabilized SOM sources and hence positive rhizosphere priming effects.

Plant phenology plays a major role for the magnitude of rhizosphere priming effects. The specific rhizosphere priming (RPE per root dry weight) was reduced for the barley wild type when comparing between the tillering and head emergence growth stages. A reduction of RPE at later growth stages has also been reported for wheat after flowering (Cheng et al., 2003). Young plants translocate higher proportions of assimilated C belowground than older plants (reviewed by Nguyen, 2003). This is indicated by the lower (yet not significant) root-derived CO_2 per root dry weight of the tillering stage compared to the head emergence stage. Root exudates are mainly derived from recent assimilates (Bertin et al., 2003; Pausch et al., 2011). Accordingly, the photosynthetic activity is intimately coupled with RPE, with time lags between assimilation of C and occurrence of priming effects as short as 48 h in the case of young annual crops (Cheng et al., 2014; Kuzyakov and Cheng 2001, 2004). At the head emergence stage, barley had a lower photosynthetic activity per leaf area than during tillering as indicated by higher $\delta^{13}C$ value of the shoots at T2 than at T1. The higher $\delta^{13}C$ of the shoots indicates a reduced intercellular partial pressure of CO_2 as a result of either 1) lower stomatal conductance at a constant photosynthetic capacity or 2) increased photosynthetic capacity at a constant stomatal conductance (Farquhar et al., 1989; Scheidegger et al., 2000). Simultaneously, the C/N ratio of

roots was about twice as high at the head emergence stage compared to the tillering and C/N ratios of shoots were even 4 times higher when comparing the two sampling dates. Thus, the large N demand of the plant is likely to induce the positive priming effects measured at T2.

At the head emergence stage, when nutrients are becoming scarce, the barley mutant without root hairs suffered from the inefficiency in nutrient uptake as indicated by a lower shoot and root biomass compared to the wild type. However, in contrast to the specific RPE of the wild type, which was reduced between the sampling dates most likely because of reduced allocation of assimilates belowground (Nguyen, 2003), the root hairless mutant showed the opposed effect. Specific RPE increased between the tillering and head emergence stages to positive values, and even exceeds the RPE of the wild type. The inefficiency of the hairless mutant in nutrient uptake may have increased rhizodeposition due to a faster decay of roots, induced by insufficient supply of nutrients.

Root hairs may contribute 70−90% to total root surface area (Bucher, 2007) root hairs are crucial for nutrient uptake of the plant. Especially the uptake of phosphorus, which is highly immobile in soils, is promoted by root hairs. Phosphorus is quantitatively the second most limiting nutrient for plant growth after N (Lambers et al., 2006). Gahoonia and Nielsen (2003) showed a much stronger P-depletion zone around the root hairs of a barley wild type than for the root hairless mutant. Under P limitation, higher phosphatase activity in the soil was shown to increase the transformation of organic phosphates into available forms (Gahoonia et al., 2001; Paterson 2003; Olander and Vitousek, 2000). In our study, the activity of acid phosphatase at tillering is lower for the wild type but higher for the mutant without root hairs compared to the unplanted soil. This pattern diminished at the head emergence stage where phosphatase activity did not differ between unplanted soil and barley with and without root hairs. These contradictory results are likely explained by the high P availability of our soil.

The activity of the C-cycling associated enzymes β-glucosidase and β-cellobiohydrolase, responsible for the decomposition of relatively labile C molecules (simple sugars, starch, cellulose) was lower for the wild type compared to the unplanted soil and the root hairless mutant at T1. Moreover, β-glucosidase decreased at the head emergence stage for both

barley types indicating lower microbial investments in C-cycling enzymes (Allison et al., 2011).

Planting increased the activity of ß-xylosidase and chitinase at both growth stages. The chitinase (N-acetyl-glucosaminidase) degrades chitin (unbranched polymer of N-acetyl-D-glucosamine), which is found in bacterial and fungal cells (Beier and Bertilsson, 2013). Chitin is an important source of organic N in soil, as it is one of the most abundant polymers on earth and contains about 6% N in relatively recalcitrant form (Ekenler and Tabatabai, 2002; Duo-Chuan, 2006; Kelly et al., 2011). In N-poor microsites, i.e. at low concentrations of mineral N, the production of N-acquiring exoenzymes such as amino-peptidases (e.g. LAP) and chitinases (e.g. N-acetyl-glucosaminidase) are stimulated to obtain more N from organic forms (Olander and Vitousek, 2000; Weintraub and Schimel, 2005; Kelly et al., 2011). When mineral N is becoming scarce, microbes decompose more labile forms of N-containing organic matter first because less energy is required (Kelly et al., 2011). Thereafter, according to microbial life strategy, several microbial guilds may shift their enzyme production (Schimel and Schaeffer, 2012) from enzymes responsible for degradation of relatively labile substrate (e.g. LAP, cleaving of peptide bonds in proteins) to enzymes decomposing relatively recalcitrant substrates (e.g. chitinase, hydrolysis of chitooligosaccharides into N-acetylglucosamine) to meet the metabolic N demand (Kelly et al., 2011). In this experiment, the LAP activity was reduced for both barley types between T1 and T2 (statistically significant only for WT). In contrast, chitin is produced by microorganisms and chitinase activity was shown to be enhanced by the presence of the rhizosphere (Geisseler et al., 2010). Microbial biomass was higher at T2 than at T1 for the planted soils. Thus, a higher microbial turnover led to the release of chitin into the soil, which induced the high chitinase activities of both barley types at T2. The shift from enzymes degrading labile substrates to enzymes that decompose more recalcitrant forms of N is a strong indication for priming effects on SOM decomposition.

6.5 Conclusions

In conclusion, rhizosphere priming effects are intimately linked to root morphology, e.g. root hairs. While the barley wild type with root hairs induced positive priming during tillering (69% above unplanted soil) the mutant without root hairs suppressed SOM decomposition by 28%. At head emergence, microbial biomass increased for both planted soils compared to the unplanted soil and barley types with and without root hairs accelerated SOM decomposition through priming. The SOM decomposition rate under the hairless mutant barley even exceeds that of the wild type despite lower plant biomass (72% priming for the wild type, 209% priming for the mutant). In case of positive priming, the chitinase and ß-xylosidase activities increased indicating decomposition of stable SOM. Future research on the effects of root morphology on rhizosphere priming effects are needed, especially on the effect of root branching, the number of tips and the diameter (which are expected to largely impact rhizodeposition). Special emphasis should be placed on potential mechanisms linking root morphology and microbial activity with rhizosphere priming effects.

Acknowledgements

This study was supported by grants from the German Research Foundation (PA 2377 1/1; FOR 918) and German Academic Exchange Service (DAAD) within a PPP program (57051794), and a grant from the US National Science Foundation (Grant No. 1354659). The seeds for the experiment were kindly supplied by Dr. Timothy George (Ecological Sciences, The James Hutton Institute, Dundee, Scotland UK). We would like to thank Jenna Merrilees, Susann Enzmann, and Karin Schmidt for their help during the setup and analyses of the experiment.

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7. Additional research

7.1 Food choice and chemical sensing experiment

Protists regulate bacterial abundances in soils, whereas fungi are mainly consumed by arthropods and mycophagous nematodes. Nevertheless, some protist taxa have been found to consume fungi and despise bacteria. Since these protists are difficult to culture little is known about their (a) ecological impact, e.g. grazing selectivity, growth rates and preferred prey and (b) adaptation to fungal food sources, e.g. enzyme production and chemical sensing.

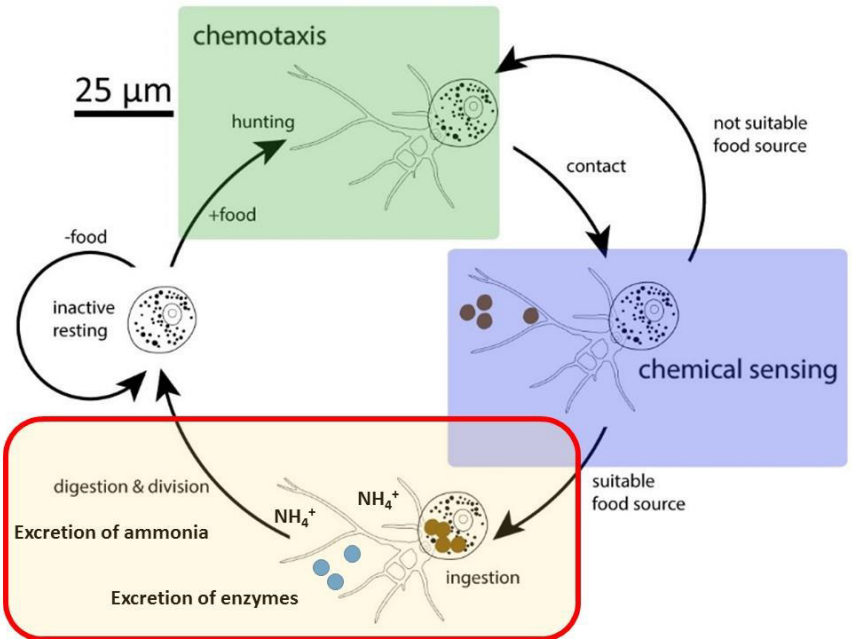

Figure II.7/34 Illustration of a simplified hypothesised hunting cycle of *L. terrestris*. Addition of suitable food induces change from inactive resting stages to grazing. This recognition of prey is supposed to be the initial process of the cycle.The prey might be found due to chemotaxis. In contact with the prey a second process or prey recognition takes place while sensing. If the food source is recognized as suitable ingestion takes place. Excretion of enzyme and ammonia takes place (modified after Dumack *et al.*, 2016).

A food choice experiment, including the amoeba *Lecythium terrestris* and three offered eukaryotes as potential prey: 1) yeast species (*Saccharomyces cerevisiae*), 2) green alga (*Chlorella vulgaris*) and 3) diatom (*Navicula sp.*) was conducted.

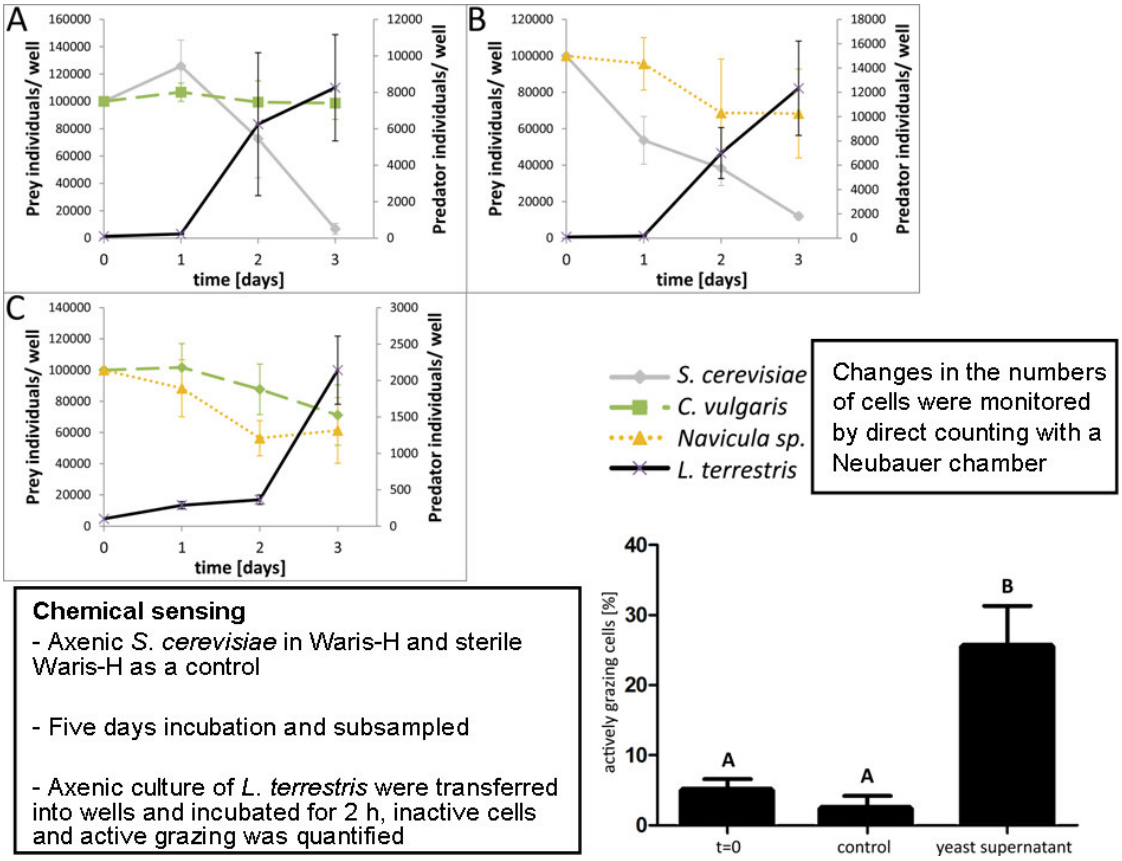

Figure II.7/35 a), b), c) Predator and prey individuals during 3 days and the quantification of actively grazing cells

Despite the consumption of all offered food sources, the predator exhibited the highest growth rate when taking up the fungal food source (yeast) (Figure II.7/35). We demonstated that *L. terrestris* senses chemically fungal abundances and excreted enzymes that are able to degrade C and N sources (Figure II.7/36).

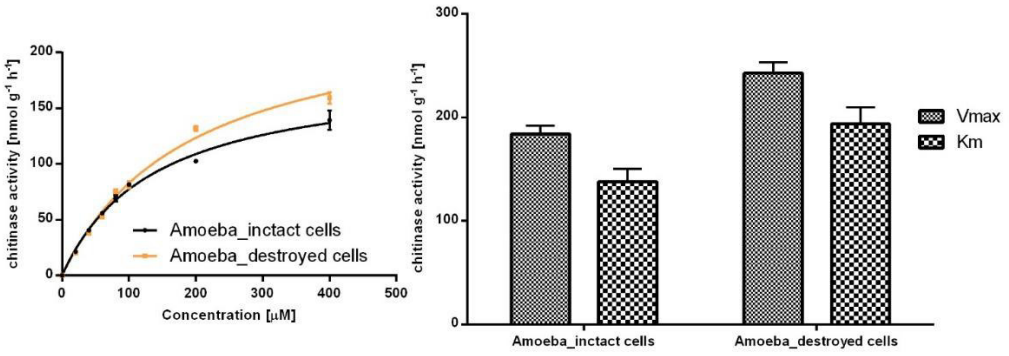

Figure II.2/36 Chitinase activity (left) of intact and destroyed amoeba cells; Catalytic properties of chitinase (right).

7.2 Binary links

There is lack of knowledge on C transfer between microbial and faunal food web, especially on the identification of binary links for bacterial and fungal feeders. We focused on investigation of grazing of the micro- and mesofauna (shown for colembola) on the C transfer of their fungal prey in model systems.

The objective of the study was the assessment of fungal activity in model systems in order to calculate the C budget of microorganisms added as prey to the soil based on $\delta^{13}C$ of microbial biomass, DOC, SOM and CO_2. Moreover, the evaluation of respiratory activities of prey depending on predators based on total and $\delta^{13}C$ of CO_2.

First results showed fungi with highest MBC (Figure II.7/38) and lowest CO_2 production (Figure II.7/37) among all treatments, indicating a highly efficient C use. However, when colembola and fungi were present in soil, the CO_2, EOC, and MBC were highest (Figure II.7/37).

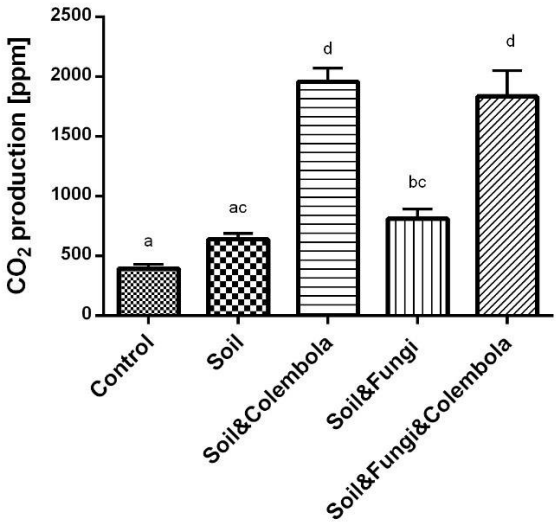

Figure II.7/37 CO_2 production during predator vs. prey interaction including controls.

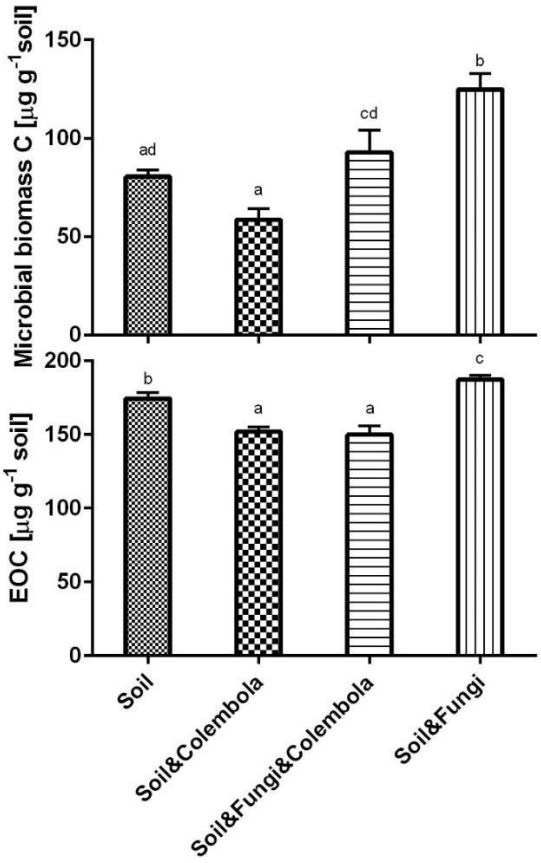

Figure II.7/38 Microbial biomass and extractable organic C

7.3 Microbial loop

The soil microbial food webs are affected by the complexity and accessibility of C in two major decomposition pathways: 1) rhizodeposits and 2) root litter. Thus, the amount and quality of substrates entering the soil mainly control microbial processes in the rhizosphere and detritusphere. The predation of soil fauna on microorganisms is another factor controlling microbial decomposition of organics, since protozoan grazing is suggested to affect microbial activity.

To investigate the contribution of protists to the priming effect we labelled root exudates (^{14}C) in corn -planted (*Zea mays*) microcosms and duplicated all treatments and introduced a model protist (*Acanthamoebae castellani*) to the resprective replicates. For further identification of C resources fueling microbial-protozoan interactions, $^{13}C/^{15}N$ labelled *Lolium perenne* root litter was added to the system.

Identical plant biomass patterns were exhibited in soil with combined substrate input (rhizodeposits and detritus) in presence and absence of protists. However, the ^{15}N (detritus-N) root to shoot ratio was significantly higher (by 24%, $P < 0.05$) for the combined substrate input in absence of protozoan grazing compared to grazed soil, indicating lower detritus-N translocation into shoots. All together, this clearly suggested an increased N uptake into the shoots in relation to the roots of corn during protozoan predation. The N uptake by plants was strongly connected to increased root exudation, which led to higher incorporation of maize-derived C into microbial biomass during amoebaean grazing. In conclusion, microfaunal grazing induced specific process chains between benefiting plants and microorganisms, caused by proliferation of N. Thus, protozoan grazing drives rhizophere processes and increases microbial activity.

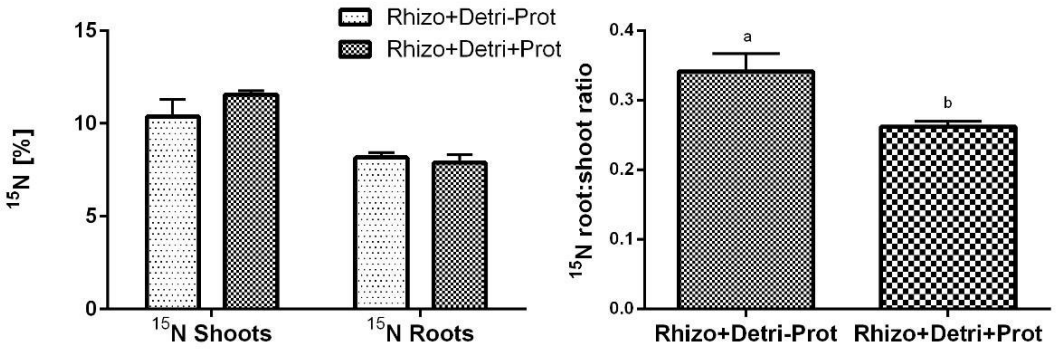

Figure II.7/39 Root litter-derived ^{15}N (left) in the shoots and the roots of corn. ^{15}N root to shoot ratio in the presence of Acanthamoeba in rhizosphere (Rhizo) and detritusphere (Detri). Significant ($P<0.05$) differences between with and without protozoan predation were given by lower-case letters.

7.4 Microbial activity and rhizosphere priming in top- and subsoil

Rhizodeposits increase microbial activity, biomass and growth rates compared to a bare fallow soil. We hyphothesied that RPEs strongly depend on the quantity of the primer. Furthermore, the increasing amounts of root exudates with higher root biomass will increase the intensity of the RPE and microbial activity. Negative priming effects are more often pronounced in subsoil horizons compared to the topsoil, since less decomposable C is available therefore different enzyme systems are produced. To prove this assumption, treatments with topsoil and subsoil were established. Moreover, the influence of the quantity of root exudates as primer was assessed by increasing the plant density in top- and subsoil treatments. The following planting densities of *Trititcum spp*. were established: unplanted soil (0 plants pot^{-1}), super low plant density (2 plants pot^{-1}), low plant density (5 plants pot^{-1}), plant density common for agricultural systems (10 plants pot-1), and high plant density (20 plants pot^{-1}) (Figure II.7/39). Besides, further pots with mineral N fertilizer addition were prepared for the common plant density since RPE may be affected by the concentration of mineral N. Soil of two different horizons was used in this experiment: 1) topsoil (0−20 cm, Ap horizon), 2) subsoil (70−90 cm, B horizon). The soil was taken from a farm on the campus reserves of the University of California, Santa Cruz. The soil texture was classified as a sandy loam (Mollisol). To minimize the influence of a high

CO_2 efflux due to soil disturbance by sieving on our priming results, the soil was pre-incubated in the greenhouse for 2 weeks (Figure II.7/39).

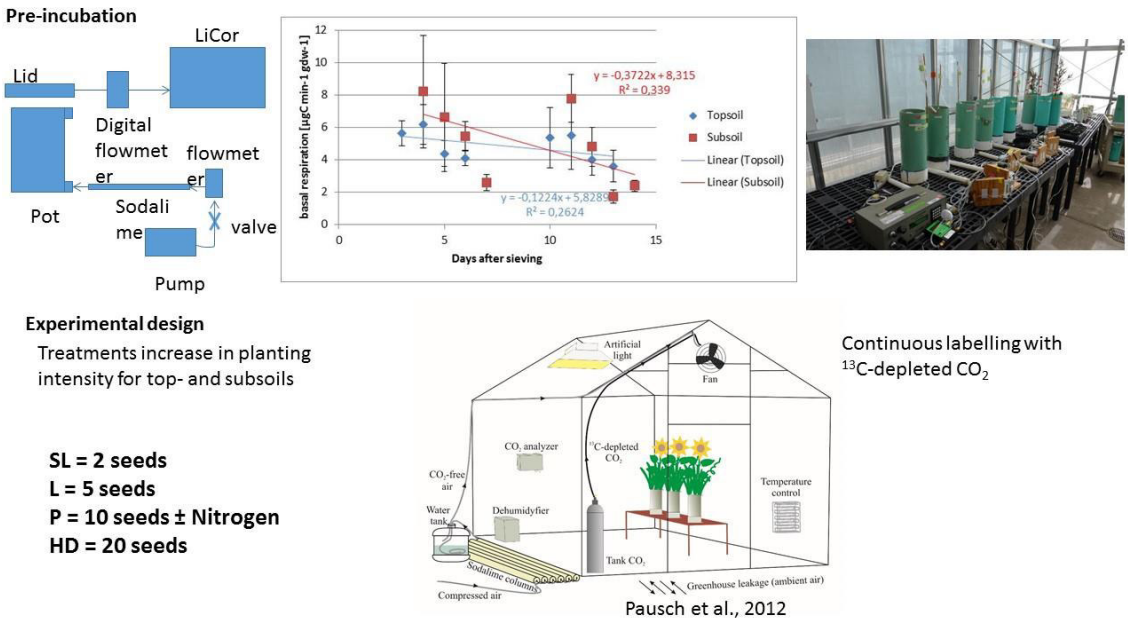

Figure II.7/40 Soil pre-incubation and experimental design (planting intensities, greenhouse)

Shoot and root biomass reflected the plant density pattern for top- and subsoil treatments (II.7/41). The root-derived CO_2 in relation to root biomass increased stronger in the rhizosphere of topsoils than in the rhizophere of subsoils (II.7/42). Furthermore, enzyme activities where higher in the rhizosphere of topsoils than those of subsoils (II.7/42). First results showed strong effects of planted top- and subsoils together with clear differences on plant densities.

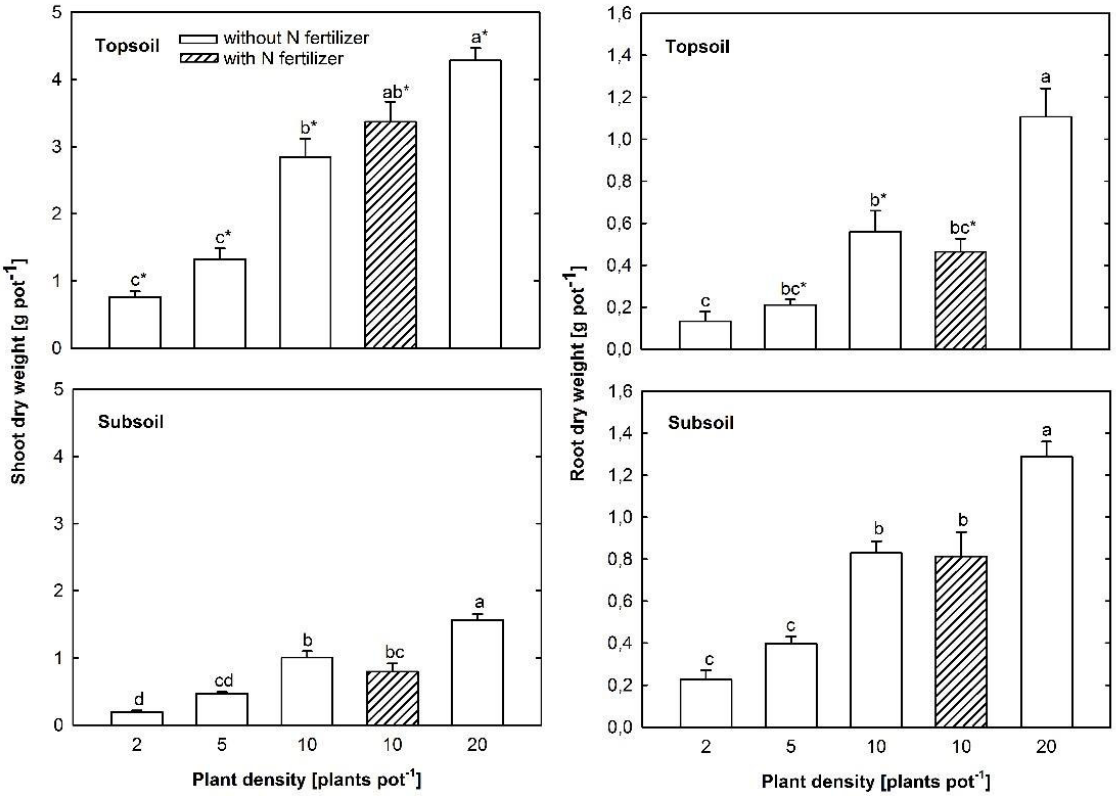

Figure II.7/41 Shoot and root biomass with increasing plant density in top- and subsoil. Significant differences are given in lower-case letters (P<0.05).

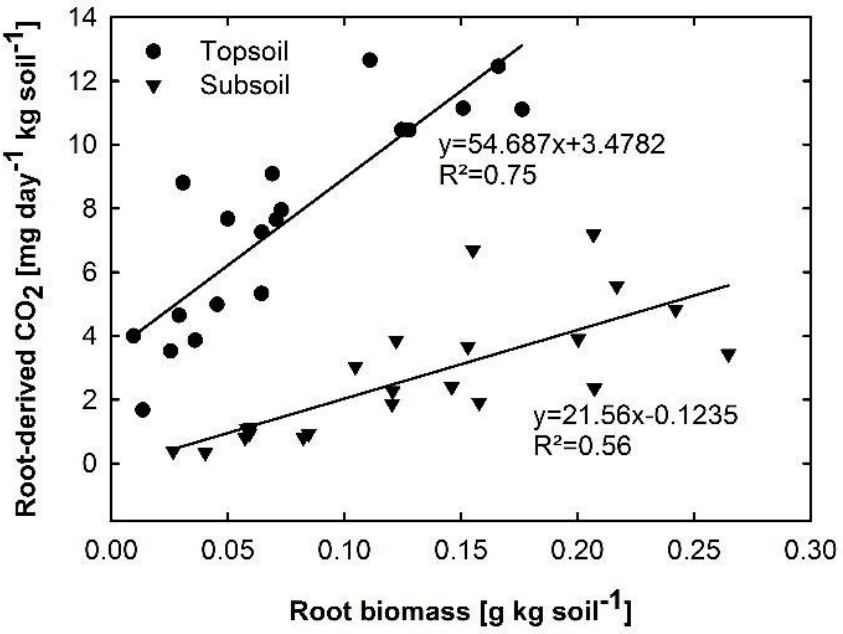

Figure II.7/42 Root-derived CO_2 in relation to root biomass for top- and subsoil treatments.

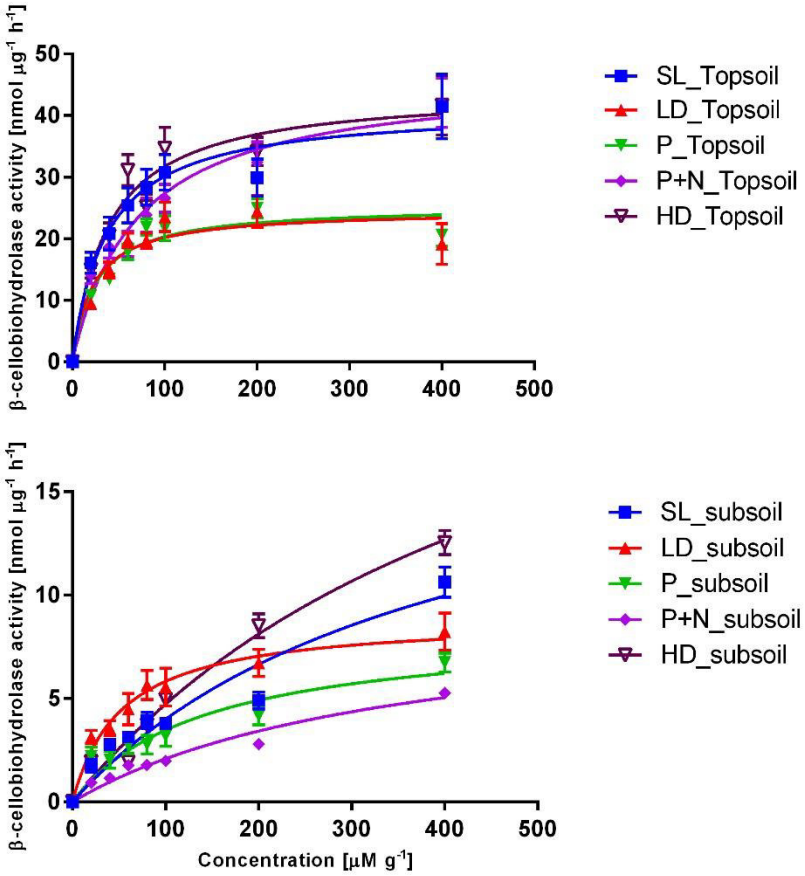

Figure II.7/43 Beta-cellobiodydrolase activity in top- (upper) and subsoil with increasing plant densities and ± nitrogen.

7.5 Soil zymography: Microbial activity in situ

Despite hot spots occupy only a very small volume of soil, most element-cylcling processes prevailed in these microsites (Spohn and Kuzyakov, 2014). The microbial and enzyme activities are suggested to increase in these habitats, such as rhizosphere and detritusphere (Nannipieri *et al.*, 2012; Blagodatskaya and Kuzyakov, 2013). However, the spatial organization of microbial activity in the rhizosphere is poorly understood. Soil zymography enables the visualization of the distribution of enzyme activities, thereby reflecting hot spots of microbial activity in situ (Spohn and Kuzyakov, 2013).

We hypothesized that the protease activity increases when additional easily available substrate is present in the rhizosphere, because microorganisms will start to mine for N to promote their growth. A rhizobox experiment with maize was conducted to study the leucine-aminopeptidase activity in the rhizosphere. For this purpose, we measured the leucine-aminopeptidase activity before and after the addition of glucose. This is the first study visualizing the relative changes of enzyme activity in time and space in the complex rhizosphere of maize.

The experimental set-up for soil zymography is explained elsewhere (Spohn and Kuzyakov, 2014). After the incubation of the membrane (1 h) images where taken under UV-light. The camera setup, distance and angle of the camera were kept constant, while taking images of calibration and after incubation of the membrane. The images of enzyme activities before and after glucose addition as well as the RGB image were referenced by image to image registration (Figure II.7/44). Sole the blue channel of the original RGB-image was analyzed and further converted into its grey- scale values. We calculated the difference image of the zymography images before and after glucose amendment to show the relative changes of enzyme activity due to the glucose addition (Figure II.7/47). Moreover, the root architecture was identified by segmenting the roots according to thresholding analysis (Figure II.7/46).

First results demonstrated higher leucine-aminopeptidase activity in the rhizosphere after glucose addition (Figure II.7/45). This suggests higher production of leucine-aminopep-

tidases in the rhizosphere when increasing amount of easily available substrates is present. In particular, main roots areas showed increased activities relative to control, indicating strong spatial variation of proteases activities in the complex rhizosphere of corn in presence of additional substrate.

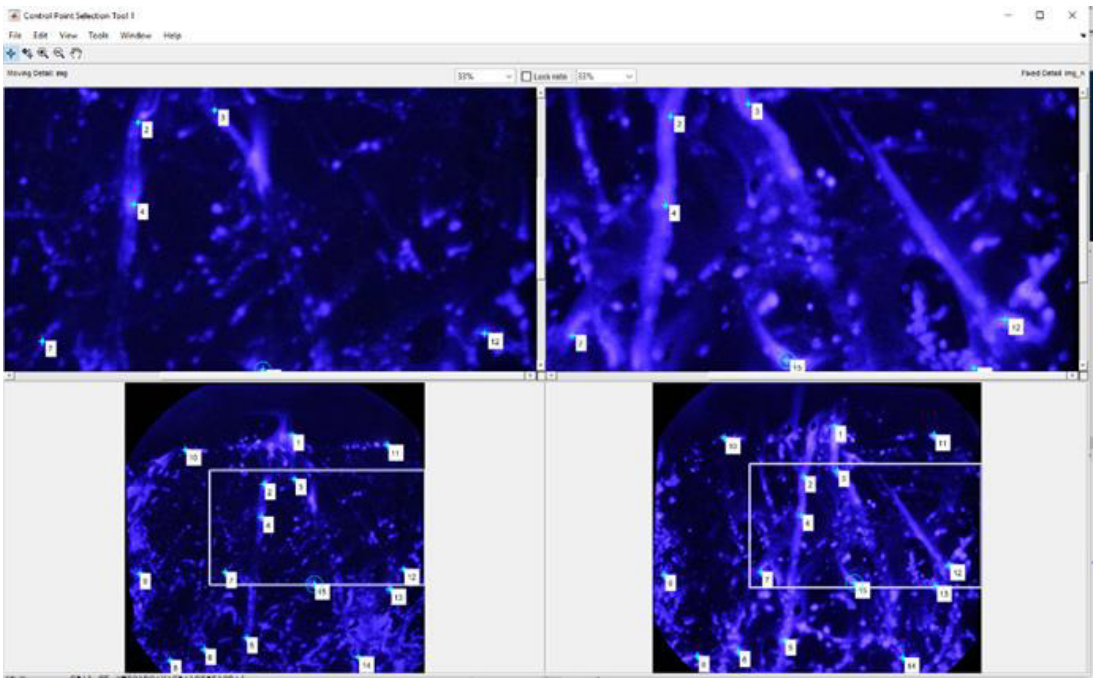

Figure II.7/44 Zymography images before (left) and after (right) the addition of glucose. The upper images represent zoomed views of the control points; Images below show the whole rhizosphere. Image referencing was perfomed with MatLabs (The MathWorks, Germany).

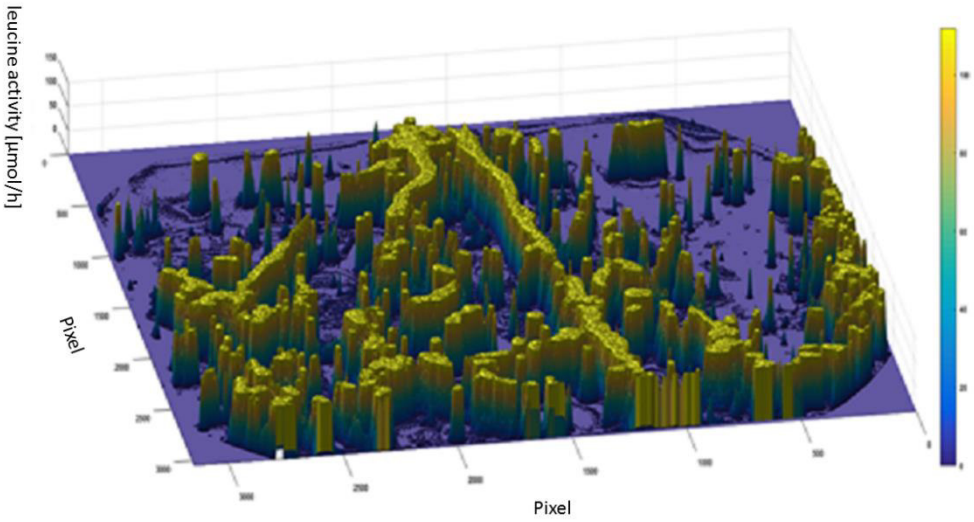

Figure II.7/45 Leucine-aminopeptidase activity of the zymography image after glucoase addition.

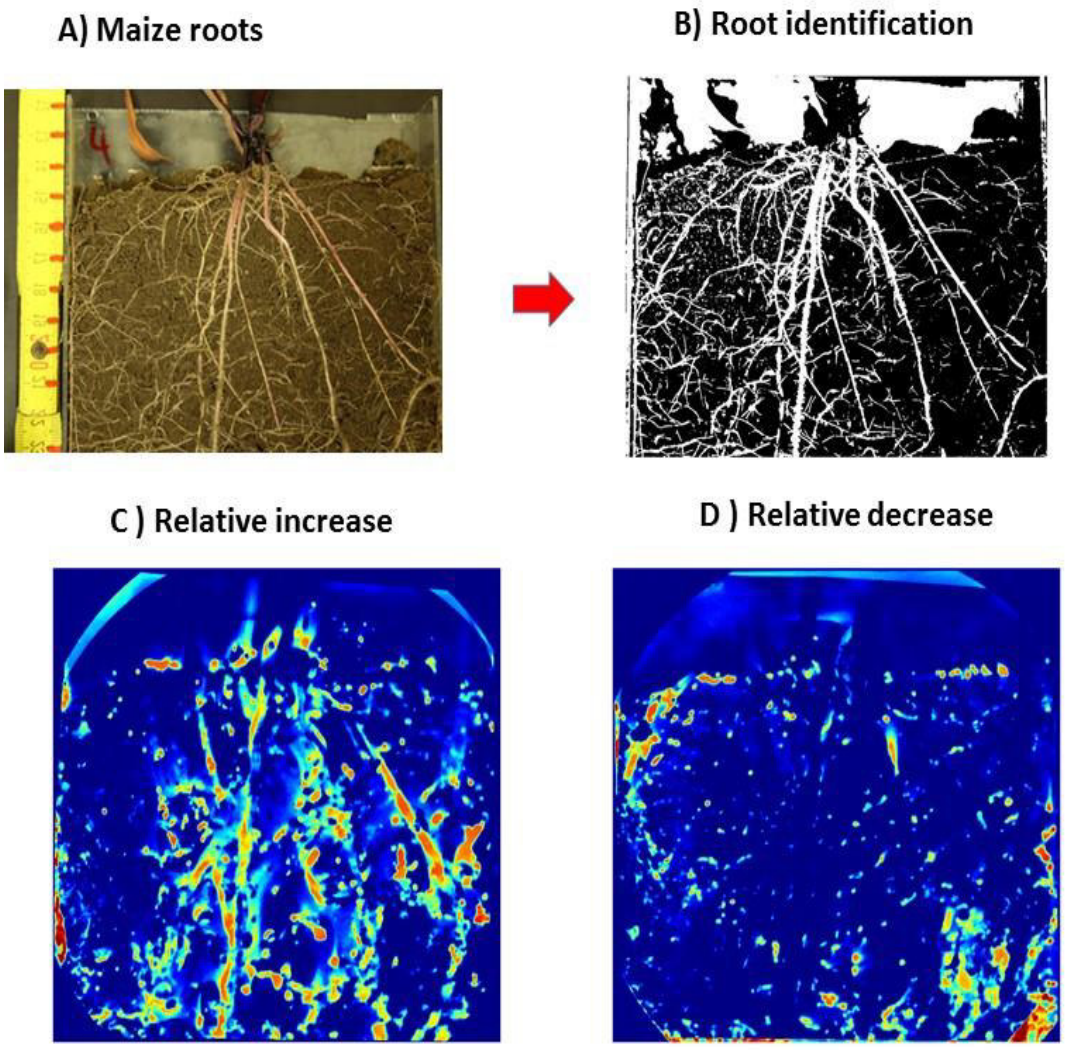

Figure II.7/46 A) RGB image ot maiz roots; B) Root identification; C) Relative increase of leucine-aminopeptidase in the rhizosphere of maize during 24 h after the addition of glucose; red highest D) Relative decrease of leucine-aminopeptidase in the rhizosphere of maize during 24 h after the addition of glucose.

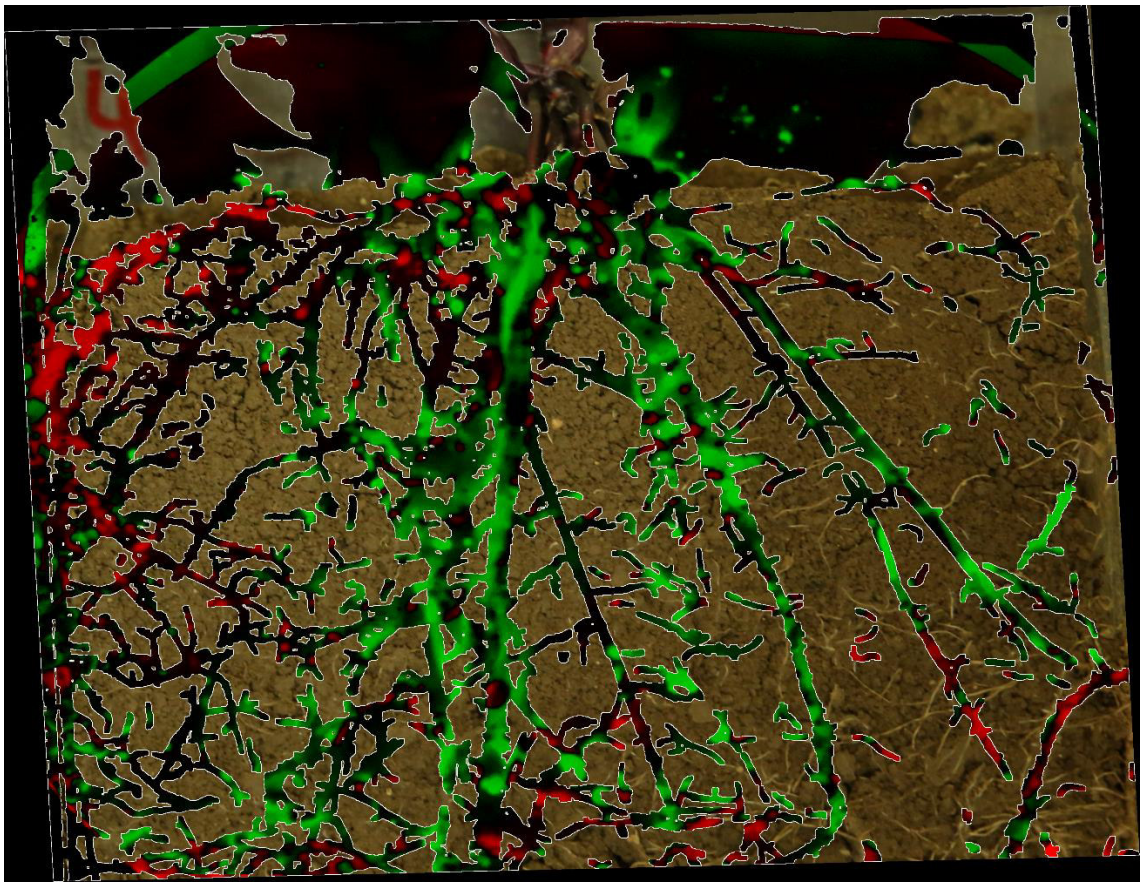

Figure II.7/47 Relative change of leucine-aminopeptidase activity after the addition of glucose. Green areas show increases, whereas red areas depict decreases of enzyme activity and black areas indicate no changes.

Curriculum vitae

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Studies

1/2013 − 5/2016	Title of PhD thesis: "*Microbial activity: Indicators & Drivers*" at the Department of Soil Science of Temperate Ecosystems, Georg-August-University, Göttingen, Germany
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2/1998 – 5/2002 Apprenticeship as a laboratory assistant at InfraServ Company, Burgkirchen, Germany

Workshops and courses

International Field Course and Soil Judging Contest, Gödöllö, Hungary (Food and Agriculture Organization of the United Nation, FAO, 2015).

SOMFrac: Evaluation of soil organic matter fractions methods – towards standardization and model compatibility, Göttingen, Germany (2015).

Application of Isotopes in Soil Science Göttingen, Germany (2013).

Publications and presentations

Publications:

Loeppmann, S., Blagodatskaya, E., Pausch, J., Kuzyakov, Y. (2016). Enzyme properties down the soil depth – A matter of substrate quality in rhizosphere and detritusphere. *Soil Biology and Biochemistry* 103, 274−283.

Loeppmann, S., Blagodatskaya, E., Pausch, J., Kuzyakov, Y. (2016). Substrate quality affects kinetics and catalytic efficiency of exo-enzymes in rhizosphere and detritusphere. *Soil Biology and Biochemistry* 92, 111−118.

Loeppmann, S., Semenov, M., Blagodatskaya, E., Kuzyakov, Y. (2015). Substrate quality affects microbial-and enzyme activities in rooted soil. *Journal of Plant Nutrition and Soil Science* 179, 39−47.

Mueller, C., Rethemeyer, J., Kao-Kniffin, J., Löppmann, S., Hinkel, K., Bockheim, J. (2015). Large amount of labile organic carbon in permafrost soils of northern Alaska. *Global Change Biology* 21, 2804−2817.

Pausch, J., Loeppmann, S., Kühnel, A., Forbush, K., Kuzyakov, Y., Cheng, W. (2016). Rhizosphere priming of barley with and without root hairs. Accepted *Soil Biology and Biochemistry,* 10.1016/j.soilbio.2016.05.009.

Oral presentations:

Loeppmann, S., Blagodatskaya, E., Kuzyakov, Y. Microbial respiration and kinetics of extracellular enzymes activities through rhizosphere and detritusphere at agricultural field. EGU, Vienna, Austria. (30.04.2014).

Loeppmann, S., Clissmann, F., Gunina, A., Pausch, J., Koller, R., Bonkowski, M., Kuzyakov, Y. Effects of protozoan grazing on carbon flow and enzyme activities in rhizosphere and detritusphere. SOM, Göttingen, Germany (22.09.15).

Invited talks:

Loeppmann, S. Blagodatskaya, E., Pausch, J., Kuzyakov, Y. Microbial respiration and enzyme kinetics in two main hot spots: Rhizosphere and detritusphere. Thünen-Institute Braunschweig, Germany (24.06.13).

Loeppmann, S., Mueller, C., Rethemeyer, J., Kao-Kniffin, J., Hinkel, K., Bockheim, J. Large amount of labile organic carbon in permafrost soils of northern Alaska. ETH Zürich, Switzerland (12.11.2012).

Poster pitches (short talks):

Loeppmann, S., Blagodatskaya, E., Kuzyakov, Y. Microbial respiration and enzyme kinetics in two main hot spots: Rhizosphere and Detritusphere. DBG Workshop (Kommission II, III, VII), Weihenstephan, Freising, Germany (04.05. −06.05.2014).

Loeppmann, S., Clissmann, F., Gunina, A., Pausch, J., Koller, R., Bonkowski, M., Kuzyakov, Y. Effects of protozoan grazing on carbon flow and enzyme activities in rhizosphere and detritusphere. Rhizosphere 4, Maastricht, Netherlands (21.06. −25.06.2015).

Loeppmann, S., Müller, C.W., Kao-Kniffin, J., Bockheim, J., Rögner, K., Kögel-Knabner, I. Soil organic carbon distribution in Cryosol-cores of drained thaw lake basins in Barrow, Northern Alaska. 4. Meeting of AK Permafrost, Bonn/Rolandseck, Gemany (31.10. −2.11.2011).

Poster presentations:

Loeppmann, S., Blagodatskaya, E., Kuzyakov, Y. Microbial respiration and enzyme kinetics in two main hot spots: Rhizosphere and Detritusphere. Biogeomon 2014, 8th International Symposium on Ecosystem Behavior, Bayreuth, Germany (13.07. −17.07.2014).

Loeppmann, S., Blagodatskaya, E., Pausch, J., Kuzyakov, Y. Linking microbial activity parameters with the turnover of microorganisms and of soil organic matter. DBG, Rostock (08.09. −12.9.13).

Loeppmann, S., Clissmann, F., Gunina, A., Pausch, J., Koller, R., Bonkowski, M., Kuzyakov, Y. Effects of protozoan grazing on carbon flow and enzyme activities in rhizosphere and detritusphere. DBG, Munich, Germany (05.09. −10.09.2015).

Loeppmann, S., Müller, C.W., Kao-Kniffin, J., Bockheim, J., Rögner, K., Kögel-Knabner, I. Soil organic carbon distribution in Cryosol-cores of drained thaw lake basins in Barrow, Northern Alaska. International Polar Year (IPY) – From knowledge to action, Montréal, Canada (22.04. −27.04.2012).

Grants and Fellowships

Congress Travel Grant Eurosoil, Istanbul, Turkey donated by Georg-August University, Göttingen (17.07. −24.07.2016).

Research at University of Santa Cruz, California, USA (01.01. −15.03.2015) provided by the German Academic Exchange Service (DAAD) within a ppp program (57051794).

Young Career Scientist Grant IPY Conference Montréal, Canada, (22.04.−27.04.2012) donated by the Association of Polar Early Career Scientists (APECS).

Research Grant field trip, Norway, (11.08.−27.08.2008) donated by Deutscher Akademischer Austauschdienst (DAAD).

Declaration

I hereby declare, to the best of my knowledge and belief, that this thesis contains no material previously published or written by another person, except where due reference has been made in the text of the thesis. This thesis contains no material which has been accepted or definitely rejected for theaward of any other doctoral degree at any university.

Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbst verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie alle wörtlich und sinngemäß übernommenen Stellen in der Arbeit gekennzeichnet zu haben. Ferne erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.