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Origin and properties of microbial hotspots in top- and subsoil

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Abbreviations

^{13}C ..... *Stable Carbon Isotope*
^{14}C ..... *Radioactive Carbon Isotope*
ACL ..... *Average Chain Length*
AM ..... *Arbuscular Mycorrhiza*
C ..... *Carbon*
C_{mic} ..... *Microbial Biomass Carbon*
C_{org} ..... *Organic Carbon*
CPI ..... *Carbon Preference Index*
DOC ..... *Dissolved Organic Carbon*
E_H ..... *Redox Potential*
EOC ..... *Extractable Organic Carbon*
FA ..... *Fatty Acids*
O_2 ..... *Oxygen*
OM ..... *Organic Matter*
pO_2 ..... *Oxygen Partial Pressure*
SOC ..... *Soil Organic Carbon*
SP ..... *Subsoil Properties*
TLE ..... *Total Lipid Extract Contents*
TOC ..... *Total Organic Carbon*
TP ..... *Topsoil Properties*

Summary

Microbial hotspots, defined by increased abundance and activity of microorganisms make up only a small percentage of the total soil volume but most microbially-mediated biogeochemical processes that are relevant for element-cycling take place there. Microbial hotspots are characterized by much higher process rates like increased organic matter (OM) turnover and nutrient mobilization compared to bulk soil. The higher availability of easily decomposable substrate compared to bulk soil increases microbial abundance and activity. Processes that lead to increased substrate availability like rhizodeposition, root litter deposition, the input of nutrient rich material by soil fauna and the leaching of organics from topsoil OM create microbial hotspots like the rhizosphere or biopores in soil. Especially in nutrient-poor subsoil, microbial hotspots are important for improving nutrient availability to plants.

This thesis aims at 1) separating microbial hotspots based on molecular proxies; 2) assessing the ability of taprooted precrops that are known to deeply grow into soil in creating and maintaining microbial hotspots in subsoil; 3) determining the microbial utilization of root carbon (C) along a depth gradient down to 105 cm; and 4) determining the lateral extent of microbial hotspots in top- and subsoil based on the distribution and turnover of root-derived C and gradients of pH, oxygen (O_2) and redoxpotential from the root surface towards bulk soil.

In a field experiment alfalfa (*Medicago sativa* L.) was cultivated on a Haplic Luvisol for two years. Drilosphere, rhizosphere and bulk soil were sampled in 15 cm intervals down to 105 cm depth from three replicate alfalfa plots, to differentiate microbial hotspots in soil based on molecular proxies. Free extractable fatty acid contents of the samples were determined after purification of fatty acids from the total lipid extract by solid phase extraction. Separation between drilosphere, rhizosphere and bulk soil OM was performed by linear discriminant analysis. Additionally, three replicate plots of alfalfa and chicory (*Cichorium intybus* L.*)* were *in situ* pulse labeled with $^{13}CO_2$ after 110 days of growth, to determine C input along a depth gradient. Tracing of ^{13}C in plant and soil C pools enabled the determination of C input into soil and C uptake by microorganisms down to 105 cm depth. In an experiment under controlled conditions, alfalfa was grown in three-compartment pots on either top- or subsoil to determine the lateral extent of microbial hotspots. Nylon gauze avoided either roots or roots and arbuscular mycorrhizal hyphae to penetrate into the rhizosphere compartment. After $^{14}CO_2$ pulse labeling, the dynamic and distribution of root derived-C by diffusion alone or by

a combination of diffusion, root hair and hyphal transport was determined by quantifying ^{14}C incorporation in dissolved (DOC) and total organic carbon (TOC) in the rhizosphere. The activity of extracellular enzymes was determined in the rhizosphere to assess differences in microbial decompostition between top- and subsoil. By use of a microsensor and miniaturized platinum electrodes, O_2 and redoxpotential gradients within the top- and subsoil rhizosphere at differing matric potential ranges were quantified.

The relative contents of dicarboxylic fatty acids differed in reverse order between drilosphere, rhizosphere and bulk soil OM and these differences were not affected by soil depth. Depth independency and differences between drilosphere, rhizosphere and bulk soil OM indicated the suitability of the relative contents of unsaturated and dicarboxylic fatty acids for the separation of microbial hotspot OM. Linear discriminant analysis enabled the separation of drilosphere, rhizosphere and bulk soil OM based on a linear combination of the relative contents of unsaturated and dicarboxylic acids. The results of the classical molecular proxy analysis reflect the complexity of hotspot-forming processes leading to OM with various source materials transfomed intensively by the microbial community. To assess these processes of hotspot formation, the ability of taprooted precrops in creating and maintaining microbial hotspots in subsoil during their first vegetation period was investigated. C input by root biomass and rhizodeposition was quantified and the microbial response down to 105 cm soil depth was determined. The results showed that the ability of alfalfa to create and maintain microbial hotspots in subsoil is higher compared to chicory due to 1) higher overall growth rates and 2) higher investment of C into root growth and rhizodepostition in subsoil by alfalfa that exceeded that of chicory 8 times. The easily available C released by alfalfa roots enabled microbial growth and accelerated turnover of microbial biomass C, suggesting higher nutrient cycling rates and thus availability for plant uptake. Crops that reuse former root channels of alfalfa in subsoil can profit from improved nutrient supply. In contrast, the main part of chicory root biomass and rhizodeposition were found in topsoil suggesting that chicory is not recommendable as precrop to enhance nutrient supply from the subsoil, at least not in the first year after sowing. To assess the relevance of microbial hotspots, their spatial extend and the gradients with which the enhanced process rates decrease towards bulk soil have to be determined. The experiment under controlled conditions demonstrated that the exudation in topsoil was higher than in subsoil but the gradients of ^{14}C-labeled root exudates in DOC from the root surface towards bulk soil were steeper in topsoil. Additionally, higher enzyme activities in the topsoil rhizosphere indicated faster microbial decomposition of the root

exudates compared to the subsoil rhizosphere. Although it was expected that higher microbial decomposition would lower the exudate diffusion into topsoil compared to subsoil, the determined distances were equal. Therefore, higher microbial decomposition and higher root exudation into the topsoil rhizosphere were equaled by lower microbial decomposition and lower root exudation and therefore led to a similar rhizosphere extent in top- and subsoil. ^{14}C-labeled root exudates were found in 28 mm distance from the root surface in DOC and 20 mm distance from the root surface in TOC. The O_2 concentration decreased towards the root surface but was not affected by top- and subsoil properties but by the matric potential. A matric potential below -200 hPa enabled O_2 supply towards the root and facilitated aerobic respiration. A rhizosphere effect on the O_2 concentration was found up to 20 mm distance to the root surface. Changes in redoxpotential resulted of changing O_2 concentrations up to 2 mm distance from the root surface. The redoxpotential reached moderately reducing values in the rhizosphere only under prolonged water saturated conditions.

This dissertation demonstrated that the lateral extend of microbial hotspots like the rhizosphere exceeded estimations of previous studies. It could be shown that microbial hotspots play a crucial role for the enhancement of C and nutrient cycling in soils. This suggests that the cultivation of deep rooting precrops that allocate C into subsoil, as for example alfalfa, increases nutrient availability from subsoils in agroecosystems.

Zusammenfassung

Mikrobielle Hotspots zeichnen sich durch erhöhte mikrobielle Biomasse und Aktivität im Vergleich zum Gesamtboden aus. Sie umfassen nur einen sehr kleinen Teil des Bodenvolumens, indem jedoch die meisten für Stoffkreisläufe relevanten mikrobiellen biogeochemischen Prozesse ablaufen. In mikrobielle Hotspots sind Prozessraten erhöht, wie beispielsweise verkürzte Umsatzzeiten der organischen Substanz und eine erhöhte Nährstoffmobilisierung im Vergleich zum Gesamtboden. Eine verbesserte Verfügbarkeit von niedermolekularen organischen Substanzen im Vergleich zum Gesamtboden stimuliert das Wachstum und die Aktivität von Bodenmikroorganismen. Mikrobielle Hotspots entstehen in Bodenkompartimenten, die durch Rhizodeposition, den Eintrag von Wurzelstreu, den Eintrag von nährstoffreichem Material von der Bodenfauna und die Auswaschung von organischen Verbindungen aus dem Oberboden einen erhöhten Substrateintrag aufweisen. Im Boden stellen die Rhizosphäre sowie Bioporen wichtige mikrobielle Hotspots dar. Speziell im nährstoffarmen Unterboden sind mikrobielle Hotspots wichtig, da die Nährstoffe in den Hotspots im Vergleich zum Gesamtboden für Pflanzen besser verfügbar sind.

Im Rahmen dieser Dissertation wurden 1) mikrobielle Hotspots anhand molekularer Proxies unterschieden; und 2) das Potential von Vorfrüchten mit Pfahlwurzelsystem zur Ausbildung und Aufrechterhaltung von mikrobiellen Hotspots im Unterboden untersucht; darüber hinaus wurde 3) die mikrobielle Umsetzung des wurzelbürtigen Kohlenstoffs entlag eines Teifengradienten bis in 105 cm Tiefe bestimmt; und 4) die räumliche Ausdehnung der mikrobiellen Hotspots im Ober- und Unterboden anhand der Verteilung und anhand des Umsatzes von wurzelbürtigem Kohlenstoff, sowie anhand von sich ausbildenden pH, Sauerstoff- und Redoxpotentialgradienten von der Wurzeloberfläche in den Gesamtboden bestimmt.

Im Rahmen eines Feldexperimentes wurde Luzerne (*Medicago sativa L.*) zwei Jahre lang auf einem Haplic Luvisol angebaut. Drilsophäre, Rhizosphäre und der Gesamtboden wurden in 15 cm Intervallen bis in eine Tiefe von 105 cm beprobt, um mikrobielle Hotspots anhand von molekularen Proxies zur unterscheiden. Die Proben wurden auf ihre Gehalte an freien extrahierbaren Fettsäuren untersucht. Dafür wurden aus dem Gesamtlipidextrakt die Fettsäuren durch Festphasenextraktion abgetrennt. Die Differenzierung des organischen Materials aus der Drilosphäre, der Rhizosphäre und dem Gesamtboden wurde mittels einer linearen Diskriminanzanalyse durchgeführt. Desweiteren wurde auf der Versuchsfläche neben

Luzerne auch Wegwarte (*Cichorium intybus* L.) angebaut. Um den Kohlenstoffeintrag von Luzerne und Wegwarte in den Boden entlag eines Tiefengradientens zu vergleichen, wurden je drei Luzerne und drei Wegwarteparzellen nach 110 Tage Wachstum *in situ* mittels $^{13}CO_2$ pulsmarkiert. Die Verteilung des assimilierten ^{13}C in Spross, Wurzeln und Bodenkohlenstoffpools sowie die Bestimmungen der jeweiligen Kohlenstoffpoolgrößen ermöglichte es, den Kohlenstoffeintrag bis in eine Bodentiefe von 105 cm zu quantifizieren. In einer Laborstudie wurde die räumliche Ausdehnung mikrobieller Hotspots untersucht. Zu diesem Zweck wurde Luzerne in T-förmigen Gefäßen mit drei Kompartimenten zwei Monate lang kultiviert. Die Gefäße waren entweder mit Ober- oder Unterboden gefüllt. Die Wurzeln konnten nur im mittleren Teil der Gefäße wachsen, da eine Nylongaze sie daran hinderte in die seitlichen Rhizosphärenkompartimente vorzudringen. Unterschiedliche Maschenweiten der Gaze verhinderten entweder nur das Wurzelwachstum oder sowohl das Wurzelwachstum als auch das Eindringen der Hyphen von arbuskulären Mykorrhizapilzen in die Rhizosphärenkompartimente. Die Dynamik und Verteilung von wurzelbürtigem Kohlenstoff in der Rhizosphäre, wurde durch die Markierung der Luzerne mit $^{14}CO_2$ und anschließende Messung der ^{14}C-Aktivität im gelösten organischen Kohlenstoff und im gesamten organischen Kohlenstoff ermittelt. Um Unterschiede im mikrobiellen Abbau der abgegebenen Substanzen in der Ober- und Unterbodenrhizosphäre zu bestimmen, wurden die Aktivitäten extrazellulärer Enzyme gemessen. Zur Messung von Sauerstoff- und Redoxpotentialgradienten bei unterschiedlichem Matrixpotenzial in der Ober- und Unterbodenrhizosphäre wurden ein Sauerstoffmikrosensor und Platinelektroden verwendet.

Während die relativen Gehalte an ungesättigten Fettsäuren von Drilosphäre, über Rhizosphäre bis zum Gesamtboden abnahmen, verhielten sich die Dicarbonfettsäuren genau umgekehrt. Da diese Unterschiede unabhängig von der Bodentiefe waren, konnten diese Proxies zur Unterscheidung der Herkunft des organischen Materials verwendet werden. Mittels einer linearen Diskriminanzanalyse konnte so das organsiche Material mikrobieller Hotspots von dem des Gesamtbodens durch eine lineare Kombination der relativen Gehalte an ungesättigten Fettsäuren und Dicarbonsäuren unterschieden werden. Die unterschiedlichen Quellen des organischen Materials und dessen intensive mikrobielle Überformung veranschaulichen die Komplexität der Prozessse, die zur Entstehung von mikrobiellen Hotspots beitragen. Um diese Prozesse zu untersuchen, wurde das Potential von Vorfrüchten mit Pfahlwurzelsystem zur Ausbildung und Aufrechterhaltung mikrobieller Hotspots im Unterboden analysiert. Dafür wurde der Kohlenstoffeintrag über die Wurzelbiomasse und durch Rhizodeposition sowie die

mikrobielle Aufnahme bis in eine Tiefe von 105 cm quantifiziert. Die Ergebnisse zeigten, dass die Ausbildung mikrobieller Hotspots im Unterboden während der ersten Vegetationsperiode durch Luzerne stärker ist als durch Wegwarte. Die Gründe hierfür waren: 1) Ein höherer Biomassezuwachs von Luzerne und 2) eine 8 fach höherere Verlagerung des assimilierten Kohlenstoffs in das Wurzelwachstum sowie in Rhizodeposite im Unterboden durch Luzerne. Unter Luzerne wurde durch den erhöhten Eintrag von leichtverfügbarem Kohlenstoff das mikrobielle Wachstum und der Umsatz an mikrobiellem C im Unterboden erhöht. Dies weist auf höhere Nährstoffumsatzraten und damit auf deren höhere Pflanzenverfügbarkeit hin. Das könnte zu einer verbesserten Nährstoffversorgung der Hauptfrüchte beitragen, wenn deren Wurzeln durch die ehemaligen Luzernewurzelporen im Unterboden wachsen. Im Gegensatz zur Luzerne bildete die Wegwarte den größten Teil ihrer Wurzelbiomasse im Oberboden aus wohin sie auch den größten Teil ihrer Rhizodeposite exsudierte. Aus diesem Grund ist die Wegwarte zumindest in der ersten Vegetationsperiode nicht als Vorfrucht zu empfehlen, um die Nährstoffverfügbarkeit im Unterboden zu verbessern.

Um die Relevanz von mikrobiellen Hotspots für Nährstoffkreisläufe besser zu verstehen, ist es notwendig die Ausdehnung des Bodenvolumens mit erhöhten Prozessraten und die Gradienten mit denen diese Prozessraten zum Gesamtboden hin abnehmen zu untersuchen. Dies ermöglichte das oben beschriebene Experiment, bei dem Luzerne in den kompartimentierten Wachstumsgefäßen angezogen wurde. Hierbei zeigte sich, dass die Wurzelexsudation in die Oberbodenrhizosphäre verglichen mit der Exsudation in die Unterbodenrhizosphäre deutlich höher war. Allerdings waren die Gradienten der ^{14}C markierten Wurzelexsudate im gelösten organischen Kohlenstoff von der Wurzeloberfläche in Richtung Gesamtboden steiler als im Unterboden. Da zusätzlich zu dem erhöhten Eintrag und den steileren Gradienten auch die Enzymaktivitäten im Oberboden höher waren, kann von einem erhöhtem mikrobiellem Abbau der Wurzelexsudate im Vergleich zur Unterbodenrhizosphäre ausgegeangen werden. Obwohl erwartet wurde, dass erhöhter mikrobieller Abbau zu einer geringeren diffusiven Ausdehnung der Wurzelexsudate in der Oberbodenrhizosphäre führen würde, war dies nicht der Fall. Sowohl in der Oberboden- als auch in der Unterbodenrhizosphäre wurde ^{14}C aus Exsudaten bis in eine Entfernung von 28 mm im DOC und 20 mm im TOC zur Wurzeloberfläche nachgewiesen. Die Sauerstoffkonzentration nahm in Richtung zur Wurzeloberfläche ab, wobei der Gradient in Ober- und Unterbodenrhizosphäre identisch war. Ein Rhizosphäreneffekt auf die

Sauerstoffkonzentration konnte bis in 20 mm Entfernung zur Wurzeloberfläche gemessen werden. Das Matrixpotenzial war ausschlaggebend für die diffusive Nachlieferung von Sauerstoff, und damit für die Aufrechterhaltung der aeroben Respiration in der Rhizosphäre. Bei einem Matrixpotenzial von -200 hPa oder weniger fand keine Hemmung der Respirationsprozesse durch mangelnde O_2 Nachlieferung zur Wurzeloberfläche statt. Die auf der Sauerstoffkonzentration beruhenden Veränderungen des Redoxpotentials konnten bis in eine Entfernung von 2 mm zur Wurzeloberfläche erfasst werden. Nur unter ständiger Wassersättigung wurden in der Rhizosphäre schwach reduzierende Bedingungen erreicht.

Im Rahmen dieses Dissertation konnte gezeigt werden, dass mikrobielle Hotspots im Boden eine größere laterale Ausdehnung erreichen als bislang angenommen. Darüber hinaus konnte gezeigt werden, dass diese Hotspots eine Schlüsselfunktion bei der Erhöhung von Kohlenstoff- und Nährstoffumsätzen besitzen. Daher empfiehlt sich der Anbau von tiefwurzelnden Vorfrüchten mit ausgeprägter C-Verlagerung in den Unterboden, wie beispielsweise Luzerne, um die Nährstoffverfügbarkeit aus dem Unterboden in Agrarökosystemen zu verbessern.

Chapter 1

1 Extended Summary

1.1 Introduction

Microbial hotspots in soil are defined as microsites with increased process rates compared to bulk soil as for example increased organic matter (OM) turnover and nutrient mobilization (Cheng 2009; Kuzyakov 2010; Kuzyakov and Blagodatskaya 2015). These hotspots make up only a small percentage of the total soil volume, but represent the place where most microbial-mediated biogeochemical processes relevant for element-cycling take place (Kuzyakov and Blagodatskaya 2015). Higher microbial abundance and activity in microbial hotspots are caused by increased availability of easily decomposable organic carbon (C) used as substrate (De Nobili et al. 2001; Kuzyakov 2002). The C and energy sources for microbial growth are derived from shoot and root detritus, soil fauna and microbial necromass, rhizodeoposits and C allocated to preferential flow pathways by leaching. As these sources are distributed inhomogenously throughout the pedon, the resulting hotspots also show a heterogenous distribution (Beare et al. 1995; Kuzyakov and Blagodatskaya 2015). Besides the detritussphere and aggregate surfaces, the rhizosphere and biopores are the most important microbial hotspots in soil (Kuzyakov and Blagodatskaya 2015).

The rhizosphere is defined as the soil volume affected by root activity (Darrah 1993; Hinsinger et al. 2005; Gregory 2006). Plant roots growing through soil affect soil properties in their direct vicinity. Water and nutrient uptake, root respiration and rhizodeposition modify microbial abundance and actitiy, physical, chemical and biochemical conditions and processes in the soil surrounding the root compared to bulk soil (Hinsinger et al. 2005; Gregory 2006). Due to root growth and differing potential of root zones for water and nutrient uptake or for rhizodeposition (Luster et al. 2009), the rhizosphere and its properties are temporarily and spatially dynamic (Watt et al. 2006).

In plant nutrition, the rhizosphere plays a crucial role, as it displays the area in soil where plants acquire nutrients (Darrah 1993). The availability of these nutrients is affected by rhizodeposition (Dakora and Phillips 2002; Dilkes et al. 2004), which is defined as the release of volatile, soluble and particulate substances from the root into the soil (Uren 2007; Wichern et al. 2008). The amount of released rhizodeposits, which comprise a wide range of organic compounds, depends on plant species, plant developmental stage and environmental conditions (Rovira 1956; Pinton et al. 2007). Disregarding the process of exudation, released organic compounds can be divided into high molecular weight organic substances comprising mucilage, lysates, exoenzymes and low molecular weight organic substances comprising

sugars, amino acids, organic acids lipids and phenols (Kraffczyk et al. 1984; Marschner 1995; Wichern et al. 2008; Fischer et al. 2010). Low molecular weight concentrations in the rhizosphere exceed the concentrations in bulk soil by one order of magnitude (Strobel 2001; van Hees et al. 2002; Fischer et al. 2007; Fischer et al. 2010).

The released compounds have different functions in affecting plant nutrient acquisition (Dakora and Phillips 2002). Rhizodeposits directly affect nutrient availability (Paterson 2003) through root induced pH changes (Marschner et al. 1986; Dakora and Phillips 2002; Jones 1998; Gahoonia and Nielsen 1991; Marschner and Römheld 1983; Kirk 1999), the exudation of phytosiderophores improving Fe, Zn, Cu and Mn availability (Treeby et al. 1989; Marschner et al. 1986; Cakmak et al. 1998), the exudation of phenolics improving the solubility of Fe and P (Dakora and Phillips 2002) or the exudation of extracellular enzymes hydrolizing organic N and P (Tarafdar and Jungk 1987). Indirect effects of rhizodeposits on nutrient mobilization are related to the attraction of microorganisms by chemoattractants such as flavonoids, aromatic acids, amino acids and dicarboxylic acids (Dakora and Phillips 2002). The increased availability of substrate stimulates microbial growth and activity, causing the accumulation of microorganisms in the rhizosphere (Lynch and Whipps 1990; Jones et al. 2009). The abundance of microorganisms in the rhizosphere is between twice up to more than 1000 times as high compared to bulk soil (Rouatt 1959; Westover et al. 1997).

Microbial nutrient mobilization by decomposition of soil organic matter (SOM) and mobilization of nutrients from clay minerals and sesquioxides increases nutrient availability for plants, due to favourable living conditions for microorganisms in the rhizosphere (Kuzyakov 2002; Paterson 2003; Blagodatskaya et al. 2007).

Plants invest a high amount of photosynthetically fixed C into rhizodeposition, indicating the importance of the interactions with microbes including nutrient mobilization for their nutrition. Up to 50% of photosynthetically C fixed by grasses or cereals is allocated belowground, whereof approximately 50% is invested into root growth and 30% is rhizodeposited (Kuzyakov and Domanski 2000; Kuzyakov 2002; Johnson et al. 2006).

Further microbial hotspots in soil are biopores. Biopores develop when roots are being decomposed leaving a pore with increased OM content compared to bulk soil. Besides ancient root channels, the burrowing activity of the soil fauna, especially the activity of earthworms create stable biopores (Tiunov and Scheu 1999; Kautz et al. 2013a). The effect of earthworm activity on soil properties depends on earthworm species and ecological categories (Brown et

al. 2000). Anecic earthworms (e.g. *Lumbricus terrestris)* produce burrows that extent from the soil surface deep into the soil. They feed on particulate OM mixed with soil particles and transport surface litter into deeper soil (Brown et al. 2000). Nutrient-rich material is found in the burrow walls due to coatings of mucus and egested nutrient-rich material. The material of the burrow wall is enriched in soil flora and fauna compared to bulk soil (Brown et al. 2000). Therefore, earthworms increase the OM content in the environment through their activity. The soil that is affected by earthworms is called drilosphere that can be defined as the 2 mm thick soil layer surrounding the earthworm burrow (Bouché 1975)(Figure 1.1-1d). Due to the enrichment in substrate and the subsequentially increased microbial activity and turnover of SOM compared to bulk soil, the drilosphere soil is an important microbial hotspot in soil.

The conditions in root- and earthworm derived biopores are of special interest in nutrient poor subsoil horizons. With increasing soil depth, SOM content, nutrient availability, rooting density, microbial biomass and mycorrhizal infection decrease (Jobbagy and Jackson 2001; Fierer et al. 2003; Salomé et al. 2010). SOM in subsoil originates from bioturbation, root litter, rhizodeposition and leaching of organics from topsoil OM (Rumpel and Kögel-Knabner 2011; Kaiser and Kalbitz 2012). Its distribution is more heterogeneous compared to topsoil due to a lower amount of roots, which grow more isolated from each other, lower bioturbation and preferential flow pathways (Rumpel and Kögel-Knabner 2011). Thus, in subsoil, biopores represent hotspots in subsoil with increased SOM content and microbial activity (Tiunov and Scheu 1999; Bundt et al. 2001). Compared to the very low OM contents in subsoil, microbial hotspots represent locally restricted microenvironments with extremely high C availability. Mineralisation of SOM by microorganisms can release nutrients into the soil that then become available for plants. Higher substrate availability in subsoil biopores enables increased OM turnover and microbial nutrient mobilization in biopores compared to bulk soil (Cheng 2009; Kuzyakov 2010). Thus, biopores in subsoil can provide increased nutrient availability for

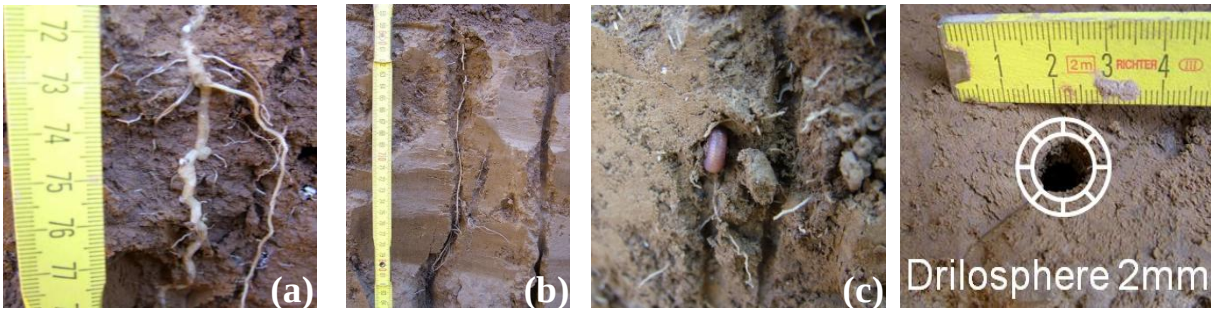

Figure 1.1-1: Taproot of chicory growing (a) through bulk soil in 75 cm depth creating a pore when beeing decomposed and; (b) in a preexisting biopore in 60-70 cm depth. Earthworm creating burrows (c). The 2 mm thick layer that surrounds and earthworm burrow is defined here as drilosphere soil (Bouché 1975)(d).

plants.

Besides of the availability, the accessibility of subsoil resources is improved by biopores (Kautz et al. 2013a). Once a biopore is present, it can be reused by plant roots to easily grow into subsoil. Roots growing in biopores benefit from lower mechanical impedance, higher oxygen (O_2) availability and increased nutrient content in pore walls compared to bulk soil (Böhm and Köppke 1977; Stewart et al. 1999; Watt et al. 2006). Another benefical effect results of the root's contact to other living or dead roots inside a pore and the already existing microbial community (Watt et al. 2006). Next to being re-used by roots growing in the subsoil without physical impedance, biopores might become colonized by soil fauna. Therefore, the OM in biopores can originate from different sources. In addition to root- and soil fauna-derived OM, the enhanced microbial activity leads to a high contribution of microbial bio- and necromass to the OM in biopores.

In arable subsoil the development of biopore systems is influenced by soil properties, the root system of a crop and the associated activity of the soil fauna (Kautz et al. 2013a). Crops that build a taproot (allorhizous root system), in contrast to crops that have a fibrous root system (homorhizous root system), are known to increase biopore abundance in subsoil (McCallum et al. 2004). As biopores can improve nutrient availability for plants, the use of taprooted precrops in a cropping sequence positively affects the growth of the subsequent crops. Nutrient uptake from arable subsoil can especially be relevant for plant nutrition, under nutrient-poor topsoils and during drought periods (Kautz et al. 2013a; Marschner 1995).

1.2 Objectives

Microbial hotspots drive nutrient cycling in soil and therefore affect nutrient availability for crops. Especially in subsoil, the rhizosphere and biopores are an important microhabitate for microorganisms, as their conditions highly contrast bulk soil. Taprooted precrops in contrast to crops with fibrous root system are known to form roots that deeply penetrate into the soil and therefore increase biopore abundance in subsoil.

This thesis targets to assess the ability of two taprooted precrops alfalfa (*Medicago sativa* L.) and chicory (*Cichorium intybus* L.) to create microbial hotspots in top- and subsoil.

Therefore, the following objectives were aimed at:

(1) Quantification of C input by root biomass and rhizodeposition into top- and subsoil within the first growing season of these precrops and

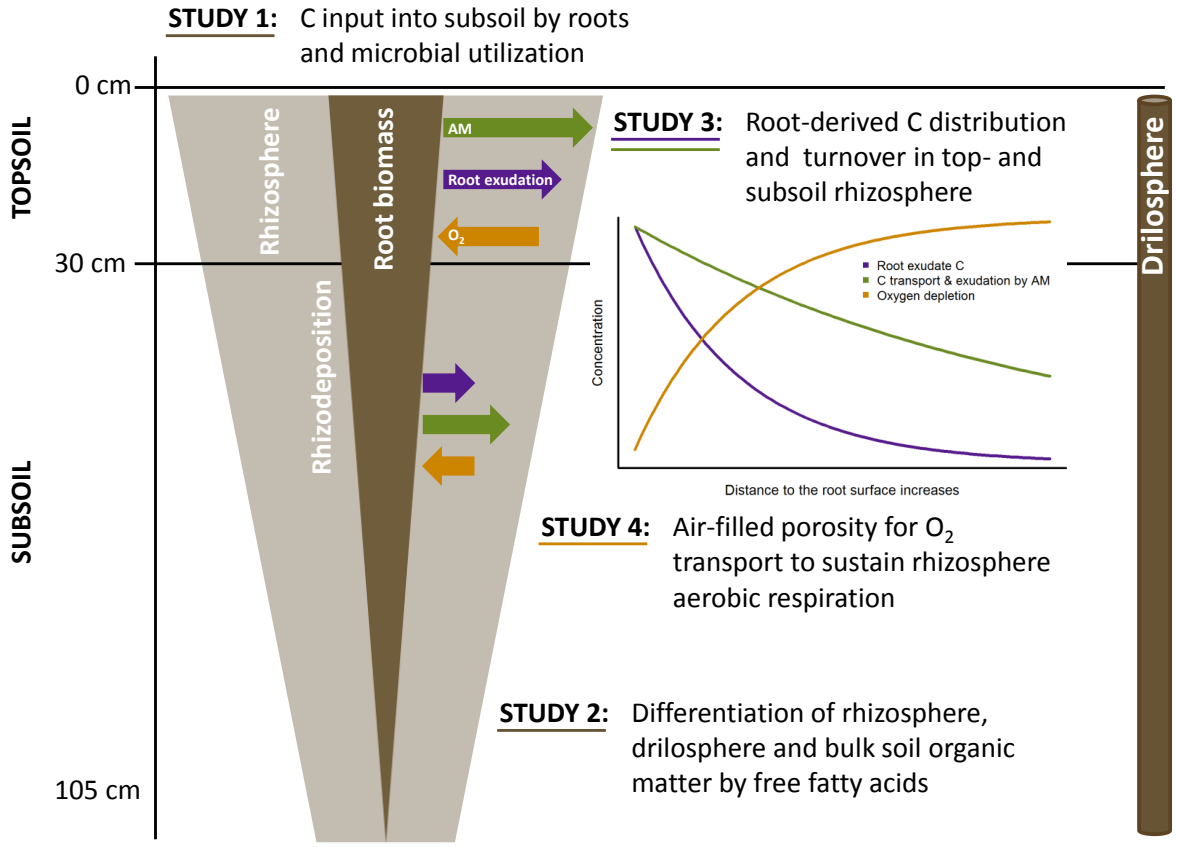

Figure 1.2-1: Schematic overview of the individual aims of the studies conducted within this thesis

(2) Determination of microbial biomass and microbial utilization of root-derived C along the depth gradient down to 105 cm depth (Figure 1.2-1, Study 1).

(3) Characterization of the molecular pattern of free fatty acids to identify molecular proxies for differentiation of root- versus earthworm-derived biopores along the depth gradient down to 105 cm (Figure 1.2-1, Study 2).

In addition to biopore characterization along depth gradients, the lateral extension of biopores should be investigated exemplarily at a rhizosphere in this dissertation. Therefore, the processes occuring during the formation of the root-derived biopore along a lateral gradient of increasing distance to the root were investigated with the following aims:

(4) Quantification of C input, microbial utilization and turnover with increasing distance to the root (Figure 1.2-1, Study 3).

(5) Determination of the effect of arbuscular mycorrhiza on the extension of the rhizosphere (Figure 1.2-1, Study 3).

(6) Assessment of oxygen- and redox-gradients along the lateral gradient starting at the root surface (Figure 1.2-1, Study 4).

1.3 Material and Methods

1.3.1 Field site description

To gain more insight into subsoil processes in arable soils the DFG-research group 1320 "*Crop Sequences and the Nutrient Acquisition from the Subsoil*" was founded. The objective was to determine the importance of biological macropores for nutrient accession and acquisition from the subsoil by different crops. Subsoil is defined here as the rootable part (unconsolidated mineral soil) of the soil beneath the plowed or formerly plowed topsoil horizon, where soil structure persists due to the absence of plowing (Kautz et al. 2013a). To clarify this questions a field trial was established in Klein-Altendorf near the city of Bonn (Germany; 50°37'21'' N, 6°59'29'' E). Precrops with differing root systems (taproot vs. fibrous root system) and cultivation time (1, 2 and 3 years) were cultivated. The climate can be described as maritime temperate (Cfb Köppen climate classification) with a mean annual precipitation of 625 mm and a mean annual temperature of 9.6 °C (Gaiser et al. 2012).

The soil at the experimental site developed from loess (silt loam) and was classified as Haplic Luvisol (Hypereutric, Siltic) (WRB, IUSS-ISRIC-FAO 2006; Gaiser et al. 2012; Kautz et al. 2013b). The soil is characterized by a silt loam to silty clay loam texture, pH values ≥ 6.5 ($CaCl_2$) and a high base saturation throughout the whole soil profile, a maximum bulk density of 1.52 g cm^{-3} in the Bt2 and Bt3 horizon and a calcium carbonate rich C horizon > 127 cm (Table 1.3-1). The plowing layer (A_p horizon) with a thickness of 30 cm was followed by an eluvial E/B horizon down to 45 cm. The E/B horizon was underlain by the diagnostic illuvial argic subsurface horizons that had a texture of 1) silty clay loam with 27% clay in the fine

Table 1.3-1: Soil properties of the reference soil profile at the field trial of the DFG research group FOR 1320 in Klein Altendorf (Athmann et al. 2013; Kautz et al. 2014).

Depth	**Horizon**	**Texture**				**Bulk density**	**pH**		**$CaCO_3$**	**SOC**	**Ntot**	**CEC**
(cm)	(WRB)	S (%)	U (%)	T (%)	WRB	g cm^{-3}	$CaCl_2$	H_2O	g kg^{-1}	g kg^{-1}	g kg^{-1}	$cmol_c$ kg^{-1}
0-27	Ap	8	77	15	SiL	1.29	6.5	7.9	< 1	10.0	1.02	12.01
27-41	E/B	5	74	20	SiL	1.32	6.9	7.8	< 1	4.6	0.55	11.91
41-75	Bt1	4	69	27	SiCL	1.42	6.9	8.1	< 1	4.5	0.51	15.68
75-87	Bt2	4	65	30	SiCL	1.52	6.9	7.7	< 1	3.9	0.5	18.48
87-115	Bt3	5	70	25	SiL	1.52	7.1	7.2	< 1	2.5	0.34	15.49
115-127	Bw	5	72	23	SiL	1.46	7.3	8.2	< 1	2.6	0.34	14.35
127-140+	C	8	75	13	SiL	1.47	7.4	8.3	127	n.d.	> 0	

Soil organic carbon (SOC), Total Nitrogen (Ntot), Cation exchange capacity (CEC)

earth fraction (Bt1); 2) silty clay loam with 30% clay in the fine earth fraction (Bt2) and; 3) silt loam with 25% clay in the fine earth fraction (Bt3). The ratio of clay in the argic to the overlying coarser E/B horizon was ≥ 1.2 indicating the illuvial clay accumuation.

At the field site 60 m^2 plots were planted with the forage precrops alfalfa (*Medicago sativa* L.) and chicory (*Cichorium intybus* L.) with allorhizous root system and tall fescue (*Festuca arundinacea* Schreb) with homorhizous root system (Gaiser et al. 2012; Kautz et al. 2013b). The cultivation time of the precrops was one, two or three years followed by the cultivation of the main crops. Precrops were cut and chopped with a mulcher (3 - 4 times per year) (Gaiser et al. 2012). All experiments presented in this thesis were conducted before the cultivation of the main crops. C fluxes between plant and soil C pools and the comparison of C input into subsoil between alfalfa and chicory were determined within the first year of cultivation without cutting and chopping within the 150 days of growth (see Study 1). To differentiate the OM between rhizosphere, drilosphere and bulk soil based on free extractable fatty acids three replicate plots that were cultivated for two years with alfalfa were sampled (see Study 2).The homogenized topsoil (sampled from 0-30 cm, A_p horizon) or subsoil (45-75 cm, B_t horizon) used in the laboratory experiment to determine 1) the spatial distribution and turnover of root-derived C in alfalfa rhizosphere depending on top- and subsoil properties and mycorrhization (see Study 3) and; 2) the oxygen and redox potential gradients in the rhizosphere of alfalfa (see Study 4) was taken from three replicate plots of alfalfa cultivated in the second year.

1.3.2 Experimental setup and realization of study 1

C input into top- and subsoil by root biomass and rhizodeposition and its uptake by microorganisms was studied by *in situ* $^{13}CO_2$ pulse labeling and C pool measurements. Alfalfa and chicory were labeled with ^{13}C after 110 days of growth. ^{13}C was traced in above- and belowground biomass, CO_2 efflux (only 1 day after labeling), rhizosphere and bulk soil and in microbial biomass 1 and 40 days after labeling. C pool sizes and $\delta^{13}C$ signature were determined in 15 cm intervals from the soil surface down to 105 cm depth (Figure 1.3-2). A summary of the material and methods used to realize the experiment is given in Table 1.3-2.

Table 1.3-2: Summary of the material and methods used in study 1

Aims	Methods and analyzes
Partitioning of photosynthetically fixed C between plant, soil, and microbial biomass C pools and soil respiration (Figure 1.3-1).	*In situ* $^{13}CO_2$ pulse labeling of alfalfa and chicory (Riederer et al. 2015; Hafner et al. 2012).
$\delta^{13}C$ signature of plant biomass, rhizosphere and bulk soil, microbial biomass 1 and 40 days after labeling.	Samples were measured by the isotope ratio mass spectrometer (Thermo Fischer, Bremen, Germany) coupled with and elemental analyzer (Eurovector, Milan, Italy) via a ConFlo III interface (Thermo-Fischer, Bremen, Germany) at the Centre for Stable Isotope Research and Analysis, University of Göttingen.
Soil respiration and the amount of recent assimilates invested into root respiration and used for rhizomicrobial respiration ($\delta^{13}C$ signature of $SrCO_3$) 1 day after the labeling.	Static alkali absorption method (Lundegardh 1921; Kirita 1971; Singh and Gupta 1977) was used to determine soil respiration. The amount of recent assimilates recovered in soil respiration was determined by adding $SrCl_2$ to NaOH to precipitate $SrCO_3$. The extracts were freeze dried and $\delta^{13}C$ signature was determined in $SrCO_3$.
Determination of microbial biomass carbon	Chloroform-fumigation-extraction method modified after Brookes et al. (1985) and Vance et al. (1987). Extractable organic carbon in the fumigated and non-fumigated samples was measured by catalytic oxidation (Multi N/C 2100 S, Analytik Jena, Germany).
$\delta^{13}C$ of microbial biomass C	K_2SO_4 extracts were freeze dried. Dried extracts were weighed into tin capsules (> 15 µg C per capsule) for $\delta^{13}C$ analysis.

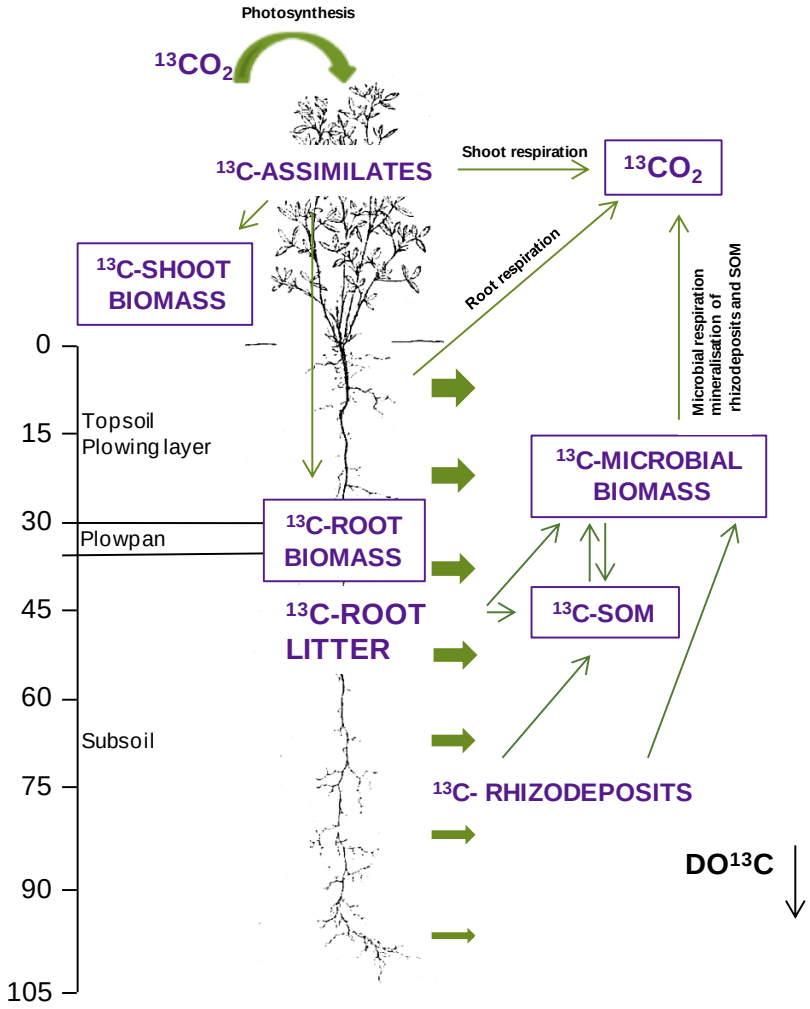

Figure 1.3-1: Partitioning of photosynthetically fixed C (^{13}C) between plant and soil C pools and respired as CO_2. The partitioning of ^{13}C was determined between shoot-, root-,and microbial biomass, rhizosphere and bulk soil and CO_2 efflux (lila bordered boxes) 1 and 40 days after labeling to determine the incorporation of assimilated C at the time of the labeling into the mentioned C pools.

Figure 1.3-2: Chambers that were used for simultaneous *in situ* $^{13}CO_2$ pulse labeling of three replicate 1 m^2 plots of chicory (a). Alfalfa plot after the pulse labeling with two closed chambers for determining total soil respiration and the contribution of recently assimlated C (b). 15 cm soil cores taken subsequently from the soil surface down to 105 cm depth (c).

1.3.3 Experimental setup and realization of study 2

Drilosphere, rhizosphere and bulk soil was sampled from three replicate plots that were cultivated for two years with alfalfa at the filed site in Klein Altendorf. After the preparation of a profile wall biopores and roots were search within four depth intervals down to 105 cm depth using a spatula. The 2 mm soil layer surrounding an earthworm burrow was sampled as drilosphere soil using micro spoons (Figure 1.3-3 a). Rhizosphere soil was sampled by extracting roots growing directly attached to the soil not in a preexisting pore and then carefully remove rhizosphere soil by brushes (Figure 1.3-3 b). Bulk soil was sampled away from visible pores or roots.To differentiate the OM between rhizosphere, drilosphere and bulk soil the free extractable fatty acid content was determined. Separation was conducted by linear discriminant analysis. A summary of the material and methods used to realize the experiment is given in Table 1.3-3.

Table 1.3-3: Summary of the material and methods used in study 2

Aims	Methods and analyzes
Sampling drilosphere, rhizosphere and bulk soil in: 0 - 30 cm (Ap) 30 - 45 cm (E/B) 45 - 75 cm (Bt1) 75 - 105 cm (Bt2 and Bt3) depth	Samples were taken from profile walls at three replicate alfalfa plots in four depth intervals *Bulk soil:* was sampled away from visible biopores *Drilosphere soil:* 2 mm thick soil layer surrounding earthworm burrows was sampled with a micro spoon. Mainly vertical oriented pores were sampled as drilosphere, if coatings and earthworm feaces were present but no roots. *Rhizosphere soil:* Soil remaining at the root after shaking (max. 2 mm) was removed from the roots with fine brushes. For sampling rhizosphere soil only roots growing through soil not in preexisting pores were used. *Roots:* after removing rhizosphere soil root samples remained. During sampling, samples were cooled and frozen thereafter.

Determining free extractable fatty acids in soil and root samples	Samples were freeze dried, crushed or ball milled (root samples) (Retsch MM 200). Lipid extracts were obtained by soxhlet extraction, (DCM/MeOH) 93:7 (v:v) followed by solid phase extraction on KOH coated SiO_2 columns to separate fatty acids (Wiesenberg et al. 2010) after methylation using BF_3/MeOH
GC-FID measurement of free extractable fatty acids for quantification	Agilent 7890 GC with flame ionization detector (30 m DB5 columns (0.32 mm inner diameter and 0.25 µm film thickness))
GC-MS measurment of free extractable fatty acids for compound identification	Hewlett Packard 5890 GC Series II (30 m DB5 columns (0.32 mm inner diameter and 0.25 µm film thickness)) coupled to Hewlett Packard 5871 mass spectrometer
Determination of organic carbon content	Analytik Jena N/C analyzer equipped with an oven for solid samples
Separation between drilosphere, rhizosphere and bulk soil organic matter	Linear discriminant analysis was conducted using unsaturated and dicarboxylic acid contents as predicting variables and the soil compartment was used as categorical variable.

Figure 1.3-3: Illustration of the biopores sampled as drilsophere soil (a) and the roots not growing in a preexisting pore that were used to sample rhizosphere soil (b). Biopores where both roots and earthworms contributed to the OM were not sampled (c).

1.3.4 Experimental setup and realization of study 3

Alfalfa plants were grown for two months in three-compartment pots filled with either homogenized topsoil (sampled from 0-30 cm, A_p horizon) or subsoil (45-75 cm, B_t horizon) of a Haplic Luvisol (WRB IUSS-ISRIC-FAO 2006) (Figure 1.3-4 a and Figure 2.3-1). Alfalfa roots grew in the root compartment but were hindered to grow into the rhizosphere compartment by nylon gauze (Figure 1.3-4 c and Figure 1.3-4 d). Two months after sowing, alfalfa plants were pulse labeled with $^{14}CO_2$. The distribution and dynamic of root-derived C in dissolved and total organic carbon in the rhizosphere of alfalfa was determined by tracing ^{14}C in the soil solution and soil slices after cutting the rhizosphere compartments (Figure 1.3-4 b). A summary of the material and methods used to realize the experiment is given in Table 1.3-4.

Figure 1.3-4: Alfalfa plants growing in the three-compartment pots used as experimental design in study 2 (a). After the $^{14}CO_2$ pulse labeling of alfalfa soil solution was sampled using micro suction cups (PI Ceramic, Lederhose, Germany) (Göttlein et al. 1996) (b). Alfalfa roots that covered the nylon gauze (c) and the surface of the associated rhizosphere compartment (d).

Table 1.3-4: Summary of the material and methods used in study 3

Aims	Methods and analyzes
Determine the distribution and dynamic of root-derived C in alfalfa rhizosphere by (1) diffusion or (2) diffusion, root hair and hyphal transport	$^{14}CO_2$ pulse labeling of alfalfa grown in three compartment pots (Cheng et al. 1993; Gocke et al. 2011). Nylon gauze with a mesh size of (1) 1 µm or (2) 30 µm was used to separate the root compartment from the rhizosphere compartment
Determine the distribution and dynamic of root exudates and C released by arbuscular mycorhhizal fungi in dissolved organic C.	Soil solution sampling in 3, 6, 9, 13, 19 and 28 mm distance from the root surface by micro suction cups (PI Ceramic, Lederhose, Germany) (Göttlein et al. 1996). Measuring ^{14}C activity in the soil solution by Liquid Scintillation Counting (LS 6500 Multi-154 Purpose Scintillation Counter, 217 Beckman, USA).
Determine the distribution and dynamic of root exudates and C released by arbuscular mycorhhizal fungi in total organic C.	Rhizosphere compartments were cut into slices at distances of 2, 4, 6, 8, 10, 12, 14, 16, and 20 mm Soil was homogenized and combusted. Evolving CO_2 was trapped in NaOH. ^{14}C activity was measured in NaOH by Liquid Scintillation Counting (LS 6500 Multi-154 Purpose Scintillation Counter, 217 Beckman, USA)
Determine the colonization of alfalfa roots by arbuscular mycorrhizal fungi	Staining of arbuscular mycorrhizal fungi structures in root tissue by the ink and vinegar method (Vierheilig et al. 1998). Determine the proportion of alfalfa root length colonized (McGonigle et al. 1990)
Determine the activity of leucin-amino-peptidase (EC 3.4.11.1), ß-glucosidase (EC 3.2.1.21) and ß-N-acetylglucosaminidase (EC 3.2.1.52)	A microplate fluorimetric enzyme assay based on methylumbelliferone substrates was used (Marx et al. 2001; German et al. 2011; Merz 2011).

1.3.5 Experimental setup and realization of study 4

Alfalfa was grown in three-compartment pots for two month (Figure 1.3-5). The experimental setup was comparable to study 3. A summary of the material and methods used to realize the experiment is given in Table 1.3-5.

Figure 1.3-5: Realization of the oxygen and redoxpotential measurement in the rhizosphere of alfalfa.

Table 1.3-5: Summary of the material and methods used in study 4

Aims	Methods and analyzes
Determine the redoxpotential (E_H) and the spatial distribution of oxygen (O_2) from bulk soil towards the root surface of alfalfa at four different matric potential ranges.	The O_2 concentration was measured by an O_2 Clark-type microsensor (100 µm diameter tip, Unisense A/S, Aarhus, Denmark) The E_H was measured by miniaturized platinum electrodes (100 µm diameter tip, Unisense A/S, Aarhus, Denmark). The microsensors were inserted into the rhizosphere compartment and pushed gently from bulk soil towards the root surface (0.5 mm steps, compartment length 4 cm) (Figure 1.3-5 and Figure 2.4-1).
Determine the dynamic of the O_2 concentration and the E_H at the root surface	The microsensors were inserted up to the root surface where O_2 and E_H were measured for 20 hours under different conditions.
Monitoring of the matric potential was monitored by a ceramic minitensiometer installed in the lateral pot at 10 mm distance from the root surface.	Ceramic mini tensiometers that were installed in the rhizosphere compartment at a distance of 10 mm from the root surface.

pH gradients	The rhizosphere compartment was sliced parallel to the root surface in 2, 4, 7, 10, 15, 20, 25, 30, 40 mm pH was measured for every soil slice in 0.01 M $CaCl_2$-solution
Texture of top- and subsoil	Wet sieving and precipitation (USDA 2011)
Estimation of the air-filled porosity	Sand, silt and clay contents were used to estimate the van Genuchten water retention parameters by pedotransferfunctions (Schaap et al. 2001; van Genuchten 1980). These were used to estimate the volumetric water content at each matric potential. Air-filled porosity: difference between total porosity and the corresponding water contents
Determine the O_2 consumption in top- and subsoil rhizosphere compartments	Calculating: the O_2 relative diffusion coefficient (Moldrup et al. 1997). the O_2 concentration (Gliński and Stępniewski 1985) the O_2 consumption rate per unit soil ($g\ m^{-3}\ s^{-1}$) (Gliński and Stępniewski 1985)

1.4 Results

Objectives and main results of the 4 studies are summarized in Table 1.4-1.

Table 1.4-1: Overview of the objectives and main results of the studies conducted

Objectives	Main results
Study 1: **Carbon input and partitioning in subsoil by chicory and alfalfa**	
– Estimation and comparison of C input into top- and subsoil by root biomass and by rhizodeposition between two common taprooted precrops alfalfa (*Medicago sativa* L.) and chicory (*Cichorium intybus* L.) during the first vegetation period – Determination of the partitioning of photosynthetically fixed C between plant and soil C pools and the uptake by microorganisms down to a depth of 105 cm.	– Alfalfa showed a higher growth during the experiment than chicory – Alfalfa root biomass was more homogeneously distributed throughout the soil profile, whereas the main part of chicory root biomass was found in topsoil – C input by alfalfa into topsoil, containing root and rhizodeposited C, was 1.6 times (3940 kg C ha^{-1}) and into subsoil 8 times higher (3820 kg C ha^{-1}) compared to chicory → Especially C input into subsoil was higher under alfalfa – the recovery of assimilated C in microbial biomass differed slightly between top- and subsoil → subsoil microorganisms incorporated a higher portion of released C due to substrate scarcity → topsoil microorganisms incorporated less as higher substrate availability caused higher mineralization → Higher C input and microbial growth in subsoil can improve physico-chemical and biological properties under alfalfa cultivation and therefore enhance root growth and consequently the water and nutrient uptake from subsoil compared to chicory

Study 2

Molecular differentiation between root- and earthworm-derived biopores in soil based on free extractable fatty acids

– Differentiation of rhizosphere, drilosphere and bulk soil OM based on free fatty acids – Identification of the source of OM in biopores based on molecular proxies	– The relative amounts of unsaturated FA and dicarboxylic acids differed in reverse order between bulk soil, rhizosphere and drilosphere OM but did not change with depth – Therefore, a linear combination of the relative content of unsaturated fatty acids and dicarboxylic acids separated OM between the rhizosphere, drilosphere and bulk soil (Linear discriminant analysis). – A universal application of the linear combination of relative contents of unsaturated FA and dicarboxylic acids to categorize OM of unknown origin into bulk soil, rhizosphere and drilosphere OM requests the evaluation of the discriminant model based on samples from other sites and ecosystems as well as a validation using biopore OM produced under controlled conditions – The source identification of OM in biopores failed using classical molecular proxies – Organic carbon content was highest in drilosphere, intermediate in rhizosphere and lowest in bulk soil in every soil depth

Study 3

Spatial distribution and turnover of root-derived carbon in alfalfa rhizosphere depending on top- and subsoil properties and mycorrhization

– Effect of top- and subsoil properties on the extent to which root exudates diffuse from the root surface of alfalfa towards the soil – Effect of arbuscular mycorrhizal fungal hyphae on root-derived C distribution in alfalfa rhizosphere	– Extent of exudate diffusion in the rhizosphere with topsoil properties was equal to the rhizosphere with subsoil properties – Exudates extended up to 28 mm in DOC and 20 mm in TOC from the root surface into soil – Despite the generally low concentrations of root exudates in the soil, the high tracer amount (^{14}C) used in this study enabled the detection of root exudates at larger distances than previously reported

	– Higher root exudation into the rhizosphere but steeper gradients of ^{14}C in DOC and higher activity of extracellular enzymes indicated increased microbial decomposition of root exudates under topsoil compared to subsoil properties → Higher root exudation into the rhizosphere with topsoil properties but higher microbial decomposition resulted in equal exudate extent compared to lower exudation into the rhizosphere with subsoil properties and lower microbial decomposition – No effect of arbuscular mycorrhizal fungal hyphae on root-derived C distribution in the rhizosphere was found → colonization of roots by arbuscular mycorrhizal fungi was low and thus a low arbuscular mycorrhizal fungi hyphae biomass can be assumed to account for the lack of the effect of mycorrhiza on rhizosphere extension

Study 4

Oxygen and redox potential gradients in the rhizosphere of alfalfa grown on a loamy soil

– Determination of spatial O_2 distribution in the rhizosphere as a function of root and rhizomicrobial respiration and matric potential – Estimation of the required air-filled porosity to sustain aerobic conditions in the rhizosphere – Physicochemical extension of the rhizosphere should be determined based on its E_h and pH gradients	– O_2 concentration decreased towards the root surface. Rhizosphere effect was determined up to 20 mm for O_2 and up to 2 mm for E_h – O_2 concentration decreased from bulk soil to the root surface due to root and rhizomicrobial respiration but O_2 gradients were equal in the rhizosphere with topsoil compared to subsoil properies → matric potential was the main parameter affecting O_2 supply to sustain aerobic respiration → O_2 supply from bulk soil up to the root surface was not limited below -200 hPa – 9 - 12 % air-filled porosity was sufficient to transport O_2 for rhizosphere aerobic respiration at more saturated conditions O_2 consumption rates decreased

1.5 Conclusions

Microbial hotspots represent the soil volume where process rates are increased compared to bulk soil. Therefore, microbial hotspots drive nutrient cycling in soil and affect nutrient availability for crops.

Due to their high relevance a direct identification and characterization of microbial hotspots would be desirable, but often they cannot be well identified morphologically. Therefore, molecular apporaches, which enable not only the quantification of microbial hotspots versus bulk soil volume but also the differentiation of single hotspots would be favorable. In this dissertation a classical biomarker class, the fatty acids, were investigated in microbial hotspots. It could be demonstrated that classical molecular proxies like the average chain length or the carbon preference index did not reveal a clear differentiation of microbial hotspots, because complex processes during the formation of microbial hotspots lead to an overprinting of the fatty acid fingerpring of the original C source by various transformations that finally form the hotspot OM. However, using multivariate statistical approaches, a linear discriminant model, enabled a siginificant differentiation of distinct microbial hotspot OM with unsaturated fatty acids and dicarboxylic acids being most important. However, to finally categorize OM of unknown origin into drilosphere, rhizosphere and bulk soil OM requires the evaluation of the linear discriminant model based on various soils from other sites and ecosystems. To understand the molecular pattern underlying the separation power of linear discrimant functions request a validation using biopore OM produced under controlled conditions.

This complexity in biopore OM sources and transformation demonstrates the complex interactions of processes occurring in microbial hotspots such as biopores. This process complexity can even be observed if only a single type of a microbial hotspot, e.g. the rhizosphere, is investigated along depth gradients or between different plant species. The investigation of rhizosphere properties along a depth gradient demonstrated that the ability of individual plant species to maintain microbial hotspots is strongly deviating. The comparison between taprooted plants, alfalfa and chicory, clearly suggests that the ability to form microbial hotspots is related to growth and especially to belowground C allocation. The investment of recent C into belowground biomass growth and rhizodepostition by alfalfa exceeded that of chicory 8 times. The continuous C investment into subsoil not just stimulates microbial growth and accelerated turnover of microbial biomass C but also maintained

microbial decomposition functions in subsoil due to the input of more substantial C sources. This was not found in chicory subsoil as the main part of root biomass and the investment of recent C into root growth and rhizodeposition were focused in topsoil.

Besides of net growth also the shape of the root system strongly affects the hotspot distribution in soil. Deeper rooting of plants and the subsequent increase in subsoil plant biomass strongly reduced the generally decreasing gradient in microbial biomass with depth. To evaluate whether this increase in hotspot abundance and biopore formation in deep subsoil by precrops will lead to an increased nutrition from subsoil-mobilized nutrients requires further experiments with controlled crop sequencing and multiple-isotope labeling approaches for tracing input and re-mobilization of subsoil OM.

To understand the relevance and role of microbial hotspots for nutrient cycling it is crucial to understand the extend of the soil volume with increased process rates and to know the gradients with which the process rates decrease towards bulk soil. Therefore, this dissertation aimed not only at describing depth-related changes in hotspot properties but also their lateral extend in top- and subsoil. The diffusion distance of root exudates was equal in top- and subsoil rhizosphere although it was expected that root exudates will be decomposed faster under topsoil properties due to higher microbial activity resulting in lower exudate extent. Concluding, the spatial distribution and therefore the soil volume affected by root exudates is equal in top- and subsoil as higher root exudation into topsoil rhizosphere is compensated by higher microbial decomposition. Root exudates were found at a distance of 28 mm ($DO^{14}C$) and 20 mm ($TO^{14}C$) from the root surface and therefore exceeded previously reported distances. Higher ^{14}C activity used for labeling compared with previous studies enabled the detection of low exudate concentrations at longer distances from the root surface.

The maintenance of microbial aerobic respiration and the related process rates depend on O_2 supply towards the root surface. Within this thesis it was shown that the rhizosphere effect on O_2 gradients was independent from microbial activity. Soil water content clearly governed O_2 supply for sustaining aerobic respiration. No limitation in O_2 supply towards the root surface was found below a matric potential of -200 hPa.

Although it is known that arbuscular mycorrhizal symbioses extends the soil volume affected by root activity the experimental setup failed in determining this effect, presumably due to a lack in mycorrhization. To determine the effect of the extent of the rhizosphere into the mycorrhizosphere, experiments based on inoculation of the plants with arbuscular

mycorrhizal fungi and the measurement of root-derived C in external hyphae extracted from soil are required. Generally, the parameters of the lateral gradients determined by T-pot model systems would be desirable to measure in different soil depth directly at distinct functional root parts at roots growing through soil to finally assess hotspot extend and dynamic under field conditions.

Finally, this dissertation demonstrated the complexity in hotspot processes and dynamics: An intensification of studies is needed to assess C and nutrient inputs, its microbially-mediated transformation dynamics and the resulting nutrient mobilization. This is especially important as these processes strongly depend on biotic factors like plant root properties and abiotic factors like water potential. To finally assess and predict the role of such hotspots for soil process modelling, more detailed investigations on their spatial distribution, their extend, their lifespan and their C and nutrient dynamic are required.

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Chapter 2

2 Publications and Manuscripts

Study 1

2.1 Carbon input and partitioning in subsoil by chicory and alfalfa

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Abstract

Background and Aims: Input of organic matter into soil creates microbial hotspots. Due to the low organic matter content in subsoil, microbial hotspots can improve nutrient availability to plants. Therefore, carbon (C) input of root biomass and rhizodeposition and the microbial utilization of root C by alfalfa and chicory, both deep-rooting taprooted preceding crops, was determined.

Methods: Three replicate plots of alfalfa and chicory grown on a Haplic Luvisol were $^{13}CO_2$ pulse labeled after 110 days of growth. ^{13}C was traced in plant biomass, rhizosphere, bulk soil and in microbial biomass after 1 and 40 days. C stocks and $\delta^{13}C$ signature were quantified in 15 cm intervals down to 105 cm depth.

Results: Alfalfa plant biomass was higher and root biomass was more homogeneously distributed between top- (0 - 30 cm) and subsoil (30 - 105 cm) compared to chicory. C input into subsoil by alfalfa, including roots and rhizodeposited C, was 8 times higher (3820 kg C ha^{-1}) into subsoil compared to chicory after 150 days of growth. Microbial biomass in subsoil increased with alfalfa but decreased with chicory.

Conclusions: Despite their general ability to build biopores, taprooted preceding crops differ in creating microbial hotspots in subsoil. Higher C input and microbial growth in subsoil under alfalfa cultivation can improve physico-chemical and biological properties, and so enhance root growth and consequently the water and nutrient uptake from subsoil compared to chicory.

Keywords: Microbial hotspots, Plant-soil-microorganism interactions, Rhizosphere, Subsoil, C input, $^{13}CO_2$ pulse labeling

2.1.1 Introduction

Crops with a taproot system form vertical stable macropores extending from topsoil into subsoil (Mitchell et al. 1995; McCallum et al. 2004). These biopores can be used by subsequent crops to easily grow into the subsoil, due to lower mechanical impedance, higher oxygen and water availability, and higher soil organic matter (SOM) content compared to bulk soil (Böhm and Köpke 1977; Stewart et al. 1999; Rasse and Smucker 1998). The increased SOM in biopores compared to bulk soil mainly results from rhizodeposition, root litter and leaching of organics from topsoil SOM (Kaiser and Kalbitz 2012; Kautz et al. 2013). Higher resource availability enables increased organic matter turnover and microbial nutrient mobilization in biopores compared to bulk soil due to higher microbial activity and abundance (Cheng 2009; Kuzyakov 2010). Decreasing SOM content and nutrient availability with increasing soil depth make biopore conditions especially relevant for nutrient acquisition from subsoil. Nutrient uptake from arable subsoil, i.e. the soil below the plough layer, can be relevant for plant nutrition (Marschner 1995). It is especially important under dry or nutrient-poor topsoil conditions and during drought periods (Fleige et al. 1983; Kuhlmann and Baumgärtel 1991). Therefore, crop sequences using taproot preceding crops can enhance the exploration of subsoil resources for the subsequent crops. In turn, better knowledge of biopore characteristics and input of SOC into arable subsoil is needed.

Rhizodeposits translocated by plant roots into the soil are of ecological importance as they affect nutrient availability for plant growth (Dakora and Phillips 2002; Dilkes et al. 2004). Rhizodeposits are one of the preferred substrates for microorganisms (Blagodatskaya et al. 2009), which are responsible for most biochemical reactions that mobilize nutrients from SOM. More heterogeneous distribution (Rumpel and Kögel-Knabner 2011) and lower content of SOM in subsoil (Salomé et al. 2010) strengthen the contrast between the rhizosphere and bulk soil. Therefore, the importance of rhizodeposits for microbial nutrient mobilization is assumed to be higher in subsoil compared to topsoil (Kautz et al. 2013). Knowledge of the amounts of organic substances added by plant roots into the soil and especially into subsoil is crucial for evaluating mobilization of nutrients. Nearly all previous studies estimated carbon (C) input within the top 30 cm of the soil (Kuzyakov and Domanski 2000; Amos and Walters, D. T. 2006). Despite various studies on root depth distribution (Böhm 1979; Jackson et al. 1996), C input by rhizodeposition into deeper soil horizons remains largely unconsidered.

To determine the input of photosynthetically fixed C into soil by roots, both root biomass and rhizodeposition need to be considered (Johnson et al. 2006; Pausch et al. 2013). Up to 50% of photosynthetically fixed C by grasses including cereals is allocated belowground, whereof approximately 50% is invested into root growth and 30% is rhizodeposited (Kuzyakov and Domanski 2000; Kuzyakov 2002; Johnson et al. 2006). However, there are few studies that include rhizodeposition of agricultural crops to assess C input into soil, especially into subsoil.

The aims of our study were (1) to estimate the amount of photosynthetically fixed C invested in building up root biomass and released into soil by rhizodeposition down to 105 cm depth and; (2) to compare C input into top- and subsoil between two common taprooted preceding crops chicory (*Cichorium intybus* L.) and alfalfa (*Medicago sativa* L.). Root biomass of alfalfa and chicory plants was determined down to 105 cm depth, 110 and 150 days after sowing. The partitioning of recently assimilated C between plant and soil C pools was determined by *in situ* $^{13}CO_2$ pulse labeling of alfalfa and chicory plants. Pulse labeling enabled the amount of photosynthetically fixed C invested into root biomass and rhizodeposition to be compared between alfalfa and chicory.

2.1.2 Material and Methods

2.1.2.1 Site description

The agricultural field site is located at the Klein Altendorf experimental station of the University of Bonn (50°37‘21‘‘N, 06°59‘29‘‘E). The climate is maritime temperate (Cfb Köppen climate classification) with a mean annual precipitation of 625 mm and a mean annual temperature of 9.6 °C (Gaiser et al. 2012).

The soil at the experimental site developed from loess and is classified as loamy Haplic Luvisol WRB (IUSS-ISRIC-FAO 2006) having an A_p horizon of 30 cm, followed by an E/B horizon down to 45 cm. Accumulation of clay was found from 45 cm down to 95 cm (Gaiser et al. 2012).

Alfalfa (*Medicago sativa* L.) and chicory (*Cichorium intybus* L.) were sown on the 15th of April 2011 with a seeding density of 25 kg ha^{-1} (alfalfa) and 5 kg ha^{-1} (cichory) (Gaiser et al. 2012). The plots for alfalfa and chicory were 60 m^2 each. Neither the alfalfa nor the chicory plots were fertilized before or during the experiment.

2.1.2.2 $^{13}CO_2$ pulse labeling

The $^{13}CO_2$ pulse labeling of chicory and alfalfa was performed after 110 days of growth, on the 1st of August 2011 (alfalfa) and on the 2nd of August 2011 (chicory) (Riederer et al. 2015; Hafner et al. 2012). Three replicate plots (1 m^2 each) of chicory and alfalfa were pulse labeled. The $^{13}CO_2$ pulses for each crop replicate were applied simultaneously. The chambers were 1 m long, 1 m wide and 0.5 m high. 100 ml of the labeling solution containing 15 g sodium carbonate ($Na_2{}^{13}CO_2$) enriched with ^{13}C to 99 atom% was placed inside the chamber. After closing the chamber, 80 ml of 5 M sulphuric acid (H_2SO_4) was injected into the labeling solution from the outside, using a syringe. A 12-volt fan ensured a uniform distribution of $^{13}CO_2$ inside the chamber. The temperature inside the chamber was measured during labeling. The CO_2 concentration inside the chamber was monitored by a CO_2 sensor (GM 70, Vaisala, Helsinki, Finland). Plants assimilated the label for 5 h before the chamber was removed.

2.1.2.3 Sampling and sample preparation

Samples were taken 1 and 40 days after labeling, which corresponded to 110 and 150 days of plant growth, respectively. The partitioning of assimilated C was determined as ^{13}C in shoots, roots, rhizosphere, bulk soil and microbial biomass. Alfalfa and chicory shoots were sampled by cutting 2 plants directly at the soil surface at each of the three replicate plots. The shoot samples of each plot were combined thereafter. To sample soil and roots a root auger with a diameter of 84 mm was used. Soil cores with a length of 15 cm were taken successively from the soil surface down to 105 cm depth. At each replicate plot, soil cores were sampled: 1) exactly at the place where the shoot was cut (including the main root biomass of the taproot crops) after 1 and 40 days and; 2) between rows (after 40 days). Roots were manually removed from the soil cores and carefully shaken to separate bulk soil from rhizosphere soil. Roots and the attached rhizosphere soil were put into a beaker containing deionized water. To improve separation, the beaker was put into an ultrasonic bath for five minutes (35 kHz, 320W, 3L). After removing the roots and rhizosphere soil, the bulk soil was sieved to 2 mm. Shoots, roots, rhizosphere and bulk soil were freeze dried, weighed and ball milled (ball mill, Retsch MM2). Before the bulk soil was freeze dried, gravimetric water content was determined for each soil depth in three replicates and soil for the determination of microbial biomass (see below) was removed. Soil respiration and the amount of recent assimilates recovered in soil respiration 1 day after labeling was determined by the static alkali absorption method (Lundegardh 1921; Kirita 1971; Singh and Gupta 1977). $SrCl_2$ was added to the NaOH to precipitate $SrCO_3$. The extracts were freeze dried and $\delta^{13}C$ signature was determined in $SrCO_3$.

2.1.2.4 Reference samples

To determine ^{13}C assimilation during the $^{13}CO_2$ pulse labeling period two replicate samples of shoot, root, rhizosphere and bulk soil samples down to 50 cm depth were taken directly after removing the labeling chamber from all three replicate plots of alfalfa and chicory, respectively. Sampling and sample preparation was done according to the procedure described above. The sum of the ^{13}C recovered directly after removing the chamber was used as a reference for the samplings after 1 and 40 days.

2.1.2.5 Microbial biomass carbon

Microbial biomass C (C_{mic}) was determined by the chloroform fumigation-extraction method modified after Brookes et al. (1985) and Vance et al. (1987), in each case using 10 g of fresh bulk soil (sieved to < 2 mm) from every depth interval. Samples were fumigated in a chloroform atmosphere for one week. For the extraction of the fumigated and non-fumigated samples, 30 ml of 0.05 M K_2SO_4 was used. Extractable organic carbon (EOC) was measured by catalytic oxidation (Multi N/C 2100 S, Analytik Jena, Germany). The difference in EOC between fumigated and non-fumigated samples was divided by the k_{EC} (0.45) value, defining the extractable part of microbial biomass C, after Jörgensen (1996), to estimate total C_{mic}.

To measure the $\delta^{13}C$ signature of C_{mic}, the K_2SO_4 extracts of both fumigated and non-fumigated samples were freeze dried. Dried extracts were weighed into tin capsules (> 15 µg C per capsule) for $\delta^{13}C$ analysis.

2.1.2.6 Natural abundance samples

To determine the natural abundance of ^{13}C in shoots, roots, rhizosphere, bulk soil and microbial biomass down to 105 cm depth, these C pools were sampled once before the $^{13}CO_2$ pulse labeling. For the natural abundance samples the same sampling and sample preparation was performed as for the enriched samples described before.

2.1.2.7 C stock calculation

To compare the above- and belowground C stocks in top- and subsoil between alfalfa and chicory, C stocks (kg C ha^{-1}) of shoots, roots, rhizosphere, bulk soil and microbial biomass were calculated. Shoot C stocks were calculated by the following equation:

$$ShootC = P \cdot \frac{S}{2} \cdot C \cdot 10 \quad (1)$$

where P is the number of plants on a plot (1 m^2), S (g) is the dry weight that was divided by 2 because the dry weight was measured on two plants and C (%) is the C content of the shoots.

C stocks of roots, rhizosphere, bulk soil and microbial biomass were calculated for each soil layer using the following equations:

$$RootC = \frac{R}{V} \cdot z \cdot C \cdot 1000 \quad (2)$$

$$RhizosphereC = \frac{RS}{V} \cdot z \cdot C \cdot 1000 \quad (3)$$

$$BulksoilC = z \cdot \rho \cdot C \cdot 1000 \quad (4)$$

$$MBC = C_{mic} \cdot z \cdot \rho \cdot 100 \quad (5)$$

where R is the dry weight of root biomass (g), V (cm^3) is the volume of the root auger, z (cm) is the length of the soil core, C (%) is the C content, ρ (g cm^{-3}) is the bulk density and C_{mic} (mg g^{-1}) is the microbial biomass C content.

The planting of alfalfa and chicory in rows results in differing C stocks between the rows and the interrows. The ratio of plant-covered to interrrow C stocks in every soil depth determined after 150 days was used to calculate interrow C stocks after 110 days of growth. To calculate total C stocks, the plot area was divided into (1) the area covered with plants and (2) the interrow area. The area covered with plants was calculated by multiplying the diameter of the root auger by the number of plants per plot, giving 52%. Total C stocks were calculated as area-weighted averages of plant-covered and interrow C stocks.

2.1.2.8 $\delta^{13}C$ analysis and stable isotope calculations

The $\delta^{13}C$ signature and C content of shoots, roots, rhizosphere soil and bulk soil and the $\delta^{13}C$ signature of EOC of the fumigated and non-fumigated samples ($\delta^{13}C$ signature of C_{mic}) and of natural abundance control samples were determined with an isotope ratio mass spectrometer (Thermo Fischer, Bremen, Germany) coupled with an elemental analyzer (Eurovector, Milan, Italy) via a ConFlo III interface (Thermo-Fischer, Bremen, Germany) at the Centre for Stable Isotope Research and Analysis, University of Göttingen.

The ^{13}C excess in a C pool (% of total C atoms) caused by the $^{13}CO_2$ pulse labeling was determined as ^{13}C excess compared to the natural abundance samples

$$^{13}C_{atom\,\%\,excess} = {}^{13}C_{atom\,\%\,sample} - {}^{13}C_{atom\,\%\,NA} \quad (6)$$

The ^{13}C excess in a C pool was used to estimate the amount of ^{13}C (g ^{13}C m^{-2}) that was incorporated into that pool (g C m^{-2}).

$$^{13}C_{amount} = \frac{^{13}C_{atom\,\%\,excess}}{100} \cdot C_{pool} \quad (7)$$

The sum of the ^{13}C recovered in shoots, roots, rhizosphere and bulk soil of the reference samples ($^{13}C_{ref}$) was used as 100% of ^{13}C assimilated by plants. To calculate the percentage of ^{13}C recovery in a C pool ($^{13}C_{rec}$) at time t (1 and 40 days) after labeling, the ^{13}C amount was related to the reference ^{13}C amount ($^{13}C_{ref}$).

$$^{13}C_{rec\,t} = \frac{^{13}C_{amount\,t}}{^{13}C_{ref}} \cdot 100 \quad (8)$$

According to C stock calculations, total ^{13}C recoveries in C pools were calculated as area-weighted averages of plant-covered and interrow ^{13}C recoveries.

2.1.2.9 Estimation of net rhizodeposition

To estimate net rhizodeposition $netC_E$ (kg C ha^{-1}) into top- and subsoil, the ratio between C released into soil and C retained in root biomass was calculated. The sum of ^{13}C recovered in rhizosphere soil $^{13}C_{RS}$ and in bulk soil $^{13}C_{BU}$ was divided by the ^{13}C recovered in roots. This ratio was calculated for topsoil (0 - 30 cm) and for subsoil (30 - 105 cm) 1 day after labeling. The topsoil ratio was multiplied by the measured root C stocks C_{root} (kg C ha^{-1}) 110 or 150 days after sowing in 0 - 15 cm and 15 - 30 cm depth. The subsoil ratio was multiplied by the measured root C stocks C_{root} (kg C ha^{-1}) in every sampling interval from 30 - 105 cm depth.

$$netC_E = \frac{(^{13}C_{RS} + {}^{13}C_{BU})}{^{13}C_{root}} \cdot C_{root} \quad (9)$$

2.1.2.10 Statistics

All results are presented as means of 3 field replicates ± standard error of the mean (SEM). Only the significant differences between crops or between depths are described in the text.

We tested if root C stocks, rhizosphere C stocks, microbial biomass C, or the distribution of ^{13}C between roots, rhizosphere and microbial biomass differed between the soil depths or between the preceding crops and if there were interactions between these effects. The test was a 2x7 factorial analysis of variance (ANOVA) (2 cultivars x 7 soil depths) at a significance

level of $p<0.05$, using R version 3.0.2 (R Core Team 2013). Normal distribution of the residuals was tested using the Shapiro-Wilk normality test. Levene's test was conducted to test for homogeneity of variances using the R package car (Fox and Weisberg 2011). The 2x7 ANOVA was calculated using log-transformed data. The residuals of the ANOVA model for all variables were then normally distributed and homoscedasticity was improved.

Kruskal-Wallis ANOVA was conducted to test for significant differences in shoot C stock, top- and subsoil root, rhizosphere and microbial biomass C stock between alfalfa and chicory ($p<0.05$) and between the sampling times ($p<0.05$). Kruskal-Wallis ANOVA was also applied to test for significant differences in ^{13}C recovery in shoots, top- and subsoil roots, rhizosphere soil and microbial biomass between alfalfa and chicory ($p<0.05$) and between the sampling times ($p<0.05$).

2.1.3 Results

2.1.3.1 Above- and belowground carbon stocks

Alfalfa and chicory shoot C stocks were equal 110 days after sowing (Table 2.1-1). The increase in aboveground biomass of alfalfa during the following 40 days was higher than of chicory, resulting in the alfalfa shoot C stock being more than twice that of chicory after 150 days of growth.

The average alfalfa root C stock from 0 to 105 cm depth was lower than the chicory root C

Table 2.1-1: Above- and belowground C stocks (kg C ha^{-1}) 110 and 150 days after sowing of alfalfa and chicory. Belowground C stocks are presented for topsoil (0 - 30 cm) and subsoil (30 - 105 cm).

C pool	C stock (kg C ha^{-1}) [e]																			
	Alfalfa 110 d					Chicory 110 d					Alfalfa 150 d					Chicory 150 d				
Shoot	528.4	±	32.9	a [f]	* [g]	468.1	±	4.3	a	*	1961.3	±	194.9	a	**	817.8	±	126.0	b	**
Soil respiration	204.8	±	22.6	a		93.3	±	8.3	b											
(kg C $ha^{-1}d^{-1}$) [e]																				
0 - 30 cm																				
Root	662.6	±	66.3	a	*	1902.9	±	350.6	b		2640.9	±	814.9	a	**	2238.3	±	436.7	a	
Rhizosphere	64.8	±	17.9	a		301.1	±	221.2	a		81.9	±	19.6	a		116.2	±	36.3	a	
Microbial biomass	92.5	±	15.3	a		101.6	±	13.2	a		76.0	±	6.4	a		80.5	±	5.4	a	
Bulk soil	36,975.6	±	2005.8			47,275.9	±	2873.8												
30 - 105 cm																				
Root	109.1	±	44.1	a	*	67.3	±	7.4	a	*	662.9	±	40.2	a	**	211.1	±	48.9	b	**
Rhizosphere	27.3	±	3.8	a		56.5	±	35.6	a		36.8	±	1.7	a		29.6	±	5.4	a	
Microbial biomass	81.6	±	9.6	a		128.7	±	12.5	a	*	114.5	±	16.5	a		76.0	±	2.9	b	**
Bulk soil	56,718.0	±	337.0			57,016.7	±	1397.6												

[e] Values are given as means and standard errors of the mean.

[f] Different letters indicate significant differences between alfalfa and chicory 1 day after labeling or 40 days after labeling (Kruskal-Wallis test; $p < 0.05$).

[g] Asterisks indicate significant differences between 110 and 150 days after sowing for alfalfa or chicory (Kruskal-Wallis test; $p < 0.05$).

stock at the beginning of the observation period, but higher at the end. (Figure 2.1-1 a, Table 2.1-1). The increase in alfalfa root biomass resulted in equal C amounts being stored in alfalfa topsoil roots and three times more C being stored in alfalfa subsoil roots compared to chicory after 150 days (Table 2.1-1). Root C stock was highest in the upper 15 cm and decreased with soil depth at both observation dates (Figure 2.1-1 a, Table 2.1-1).

Microbial biomass decreased throughout the entire profile with depth and was higher under chicory than under alfalfa after 110 days (Figure 2.1-1 c). In contrast, the microbial biomass C stock was higher under alfalfa compared to that under chicory after 150 days (Table 2.1-1). In topsoil, microbial biomass was equal between the chicory and alfalfa cultivation and between the beginning and end of the observation period (Table 2.1-1). In subsoil, however, microbial

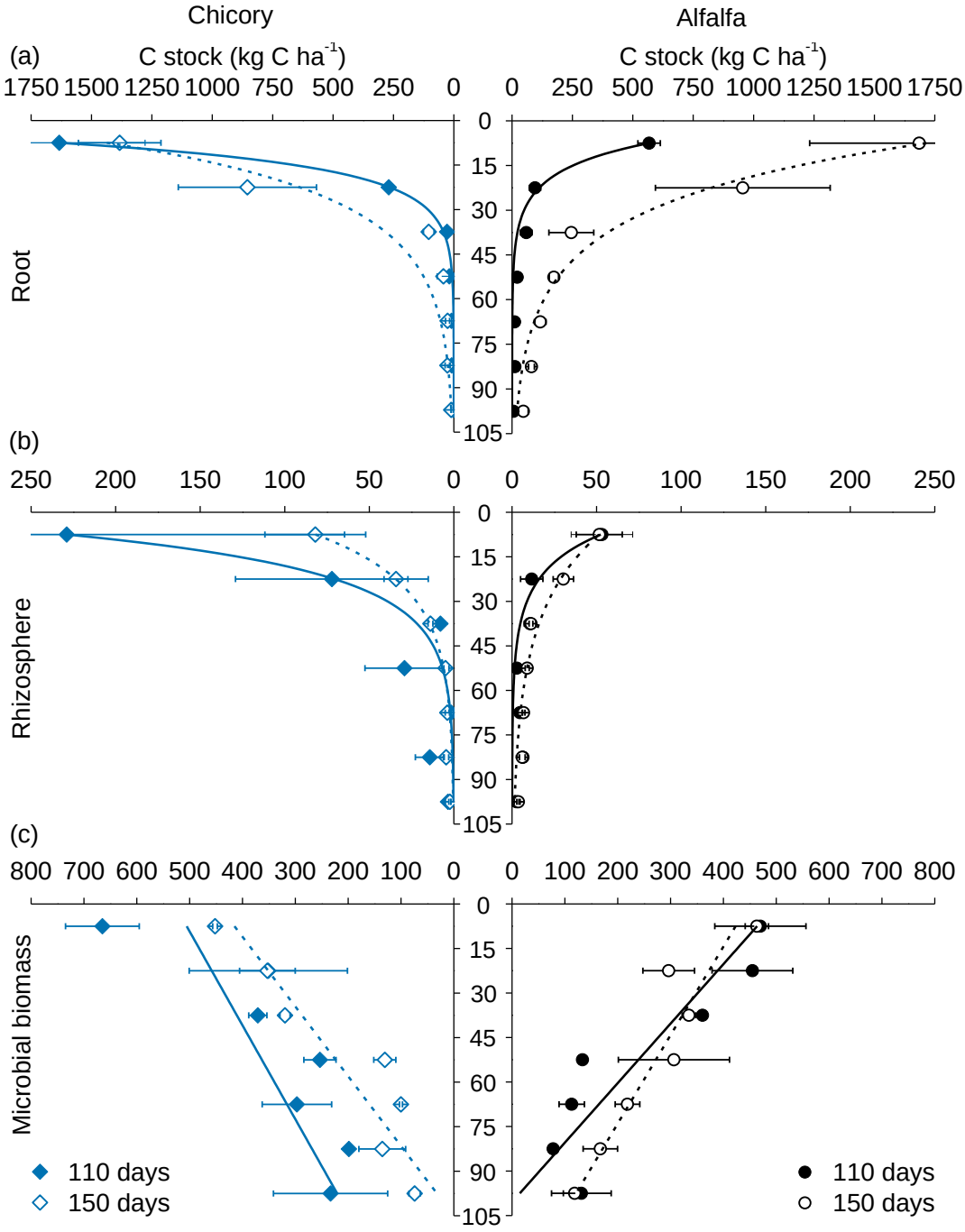

Figure 2.1-1: Root, rhizosphere and microbial biomass C stocks under alfalfa and chicory, measured in 15 cm intervals down to 105 cm soil depth 110 and 150 days after sowing. Error bars represent standard errors of the mean (n=3).

biomass decreased under chicory from day 110 to day 150, resulting in lower microbial biomass under chicory than under alfalfa.

In summary, the increase in alfalfa above- and belowground plant biomass over 40 days resulted in higher plant C stocks than for chicory. The main differences were found in subsoil root C stocks and microbial biomass.

2.1.3.2 Isotopic signature after $^{13}CO_2$ labeling and of natural abundance samples

The isotopic signature of roots, rhizosphere soil and microbial biomass indicated strong ^{13}C enrichment after the $^{13}CO_2$ pulse labeling of alfalfa and chicory plants (Figure 2.1-2 a-c). The ^{13}C enrichment was found for roots, rhizosphere soil and microbial biomass in every depth down to 105 cm, 1 day and 40 days after labeling. This ^{13}C enrichment allowed the recently assimilated C to be partitioned between shoots, roots, rhizosphere soil and microbial biomass. Bulk soil was excluded from the calculations, due to the low ^{13}C enrichment relative to the natural abundance reference samples (Figure 2.1-2 d).

The $\delta^{13}C$ values of roots, rhizosphere soil and microbial biomass under chicory tended to decrease with depth 1 and 40 days after labeling (Figure 2.1-2 a-c). In contrast, $\delta^{13}C$ values of roots, rhizosphere soil and microbial biomass under alfalfa increased with depth. Increasing ^{13}C enrichment with soil depth indicated that the percentage of recently assimilated C in total C present was higher under alfalfa.

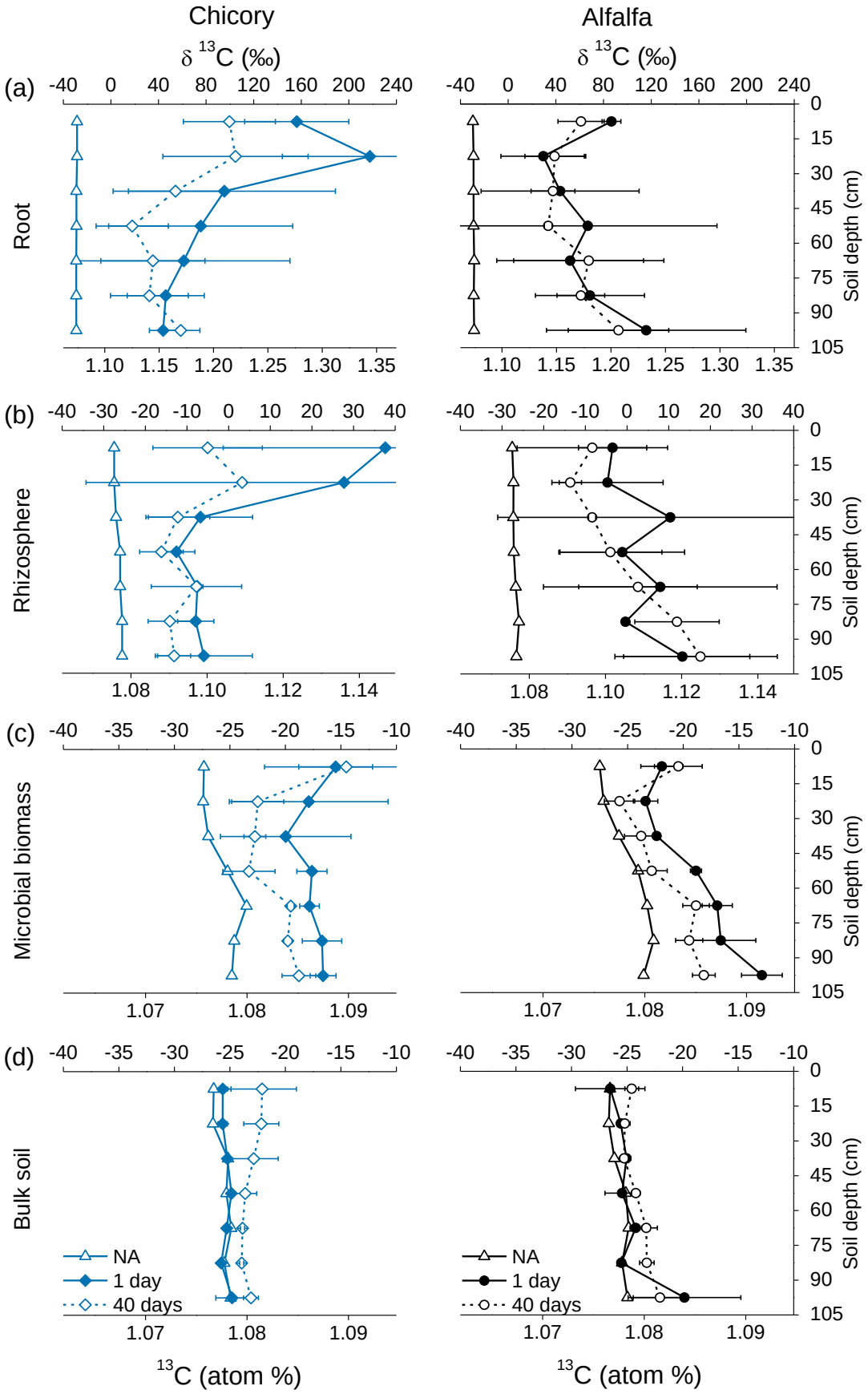

Figure 2.1-2: ^{13}C enrichment (atom%^{13}C) and the corresponding isotopic signature $\delta^{13}C$ (‰) of roots, rhizosphere, bulk soil and microbial biomass down to 105 cm depth. Values are given for the natural abundance control samples (white triangles) and for the samples taken at the 1st and 40th day after the *in situ* $^{13}CO_2$ pulse labeling of chicory and alfalfa. Error bars represent standard errors of the mean (n=3).

2.1.3.3 Budget of assimilated ^{13}C

The recovery of ^{13}C in the reference samples amounted to 69 ± 5 % and 76 ± 13 % of the applied ^{13}C in the alfalfa and chicory plots, respectively.

^{13}C recovery in shoots of alfalfa was higher compared to chicory one day after labeling (Table 2.1-2). However, chicory allocated half of the assimilated C belowground, compared to only one third allocated by alfalfa. At the end of the 40-day chase period, 29% and 22% of assimilated ^{13}C was incorporated into shoots of alfalfa and chicory, respectively. Equal ^{13}C amounts incorporated into shoots, but lower ^{13}C incorporation into alfalfa belowground C pools indicated that ^{13}C losses by shoot and soil respiration within the chase period were higher under alfalfa (Table 2.1-2).

Table 2.1-2: Partitioning of assimilated ^{13}C between C pools, 1 and 40 days after the labeling.

C pool	^{13}C recovery (% of assimilated ^{13}C) [e]																	
	Alfalfa 1 d				Chicory 1 d				Alfalfa 40 d				Chicory 40 d					
Shoot	66.9	±	2.3	a [f] * [g]	38.1	±	5.5	b *	29.4	±	3.9	a **	21.5	±	2.6	a **		
Soil respiration	11.6	±	1.1	a	5.9	±	1.6	b										
0 - 30 cm																		
Root	6.0	±	0.6	a *	28.2	±	4.0	b	18.0	±	5.8	a **	28.0	±	12.7	a		
Rhizosphere	0.15	±	0.05	a	0.7	±	0.2	b	0.1	±	0.01	a	0.2	±	0.1	a		
Microbial biomass	0.45	±	0.08	a	1.25	±	0.3	b	0.49	±	0.05	a	0.89	±	0.25	a		
30 - 105 cm																		
Root	0.9	±	0.6	a *	0.5	±	0.2	a *	4.1	±	0.6	a **	1.2	±	0.1	b **		
Rhizosphere	0.07	±	0.03	a	0.05	±	0.02	a	0.07	±	0.003	a	0.03	±	0.004	b		
Microbial biomass	0.26				0.69	±	0.11		0.52	±	0.15	a	0.59	±	0.15	a		

[e] Values are given as means and standard errors of the mean.

[f] Different letters indicate significant differences between alfalfa and chicory 1 day after labeling or 40 days after labeling (Kruskal-Wallis test; $p < 0.05$).

[g] Asterisks indicate significant differences between 1 and 40 days after labeling for alfalfa or chicory (Kruskal-Wallis test; $p < 0.05$).

In topsoil, five times less ^{13}C was recovered in alfalfa roots compared to chicory after one day (Table 2.1-2, Table 2.1-5 supplementary material). Despite an increase of ^{13}C in alfalfa topsoil roots during the chase period, total ^{13}C incorporation remained lower after 40 days. Similar to roots, the ^{13}C recoveries in topsoil rhizosphere soil and microbial biomass were lower under alfalfa than those of chicory.

At the end of the chase period, 4.1% of assimilated ^{13}C was incorporated into alfalfa subsoil roots. In contrast, only 1.2% was incorporated into chicory subsoil roots (Table 2.1-2, Table 2.1-6 supplementary material). A higher incorporation of assimilated ^{13}C into alfalfa subsoil roots was found at every soil depth after 40 days (Figure 2.1-3, Table 2.1-6 supplementary material). Despite the higher recovery in alfalfa subsoil roots, the incorporation of ^{13}C into the microbial biomass remained lower.

The ^{13}C budget indicated that the allocation of assimilated C to belowground C pools was faster in chicory than in alfalfa. Despite the higher ^{13}C incorporation into belowground C pools under chicory, more than twice as much ^{13}C was incorporated into subsoil C pools under alfalfa.

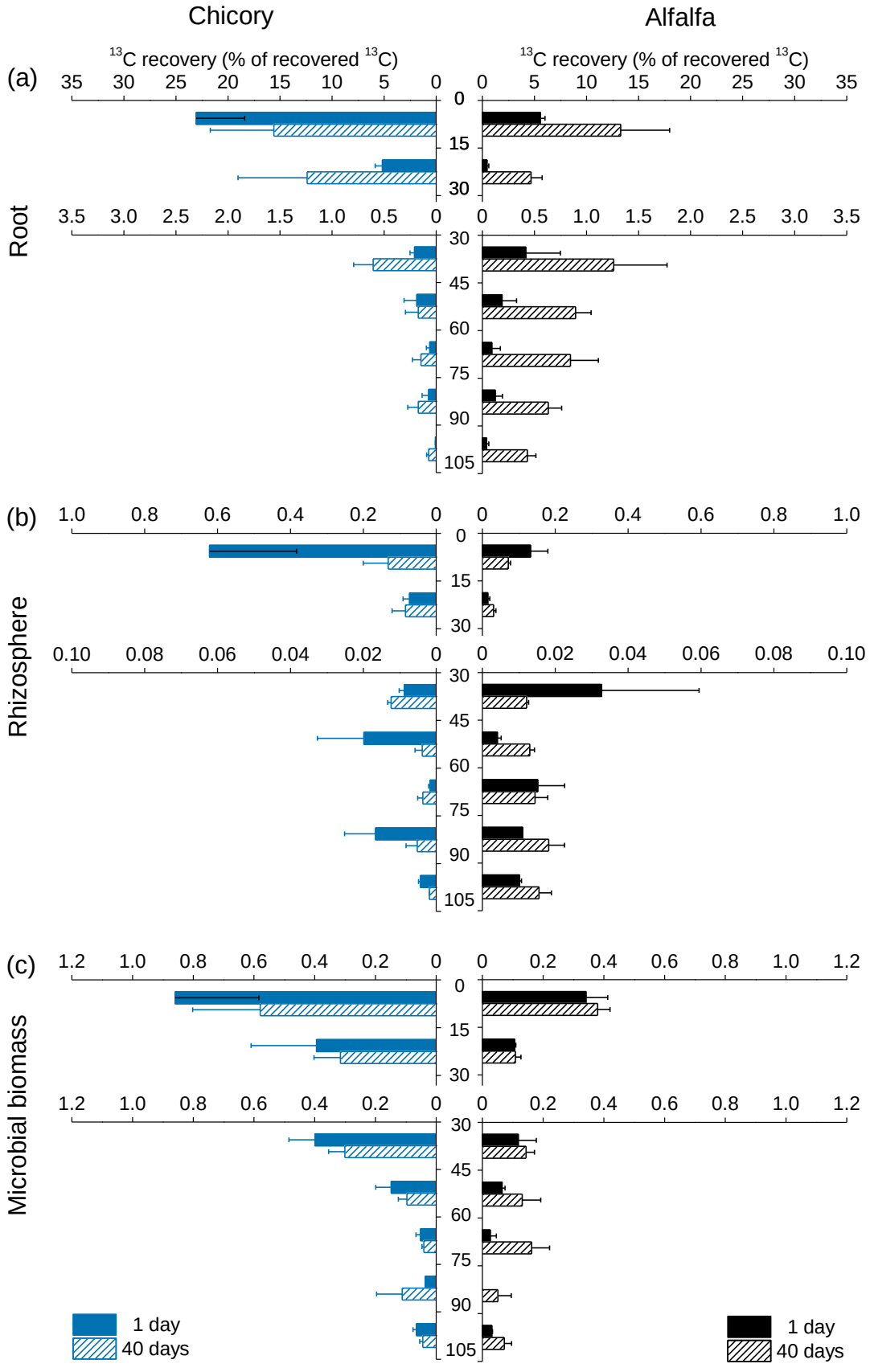

Figure 2.1-3: ^{13}C recovery in root biomass, rhizosphere soil and microbial biomass in top- and subsoil of the alfalfa and chicory plots, 1 and 40 days after the $^{13}CO_2$ pulse labeling. Topsoil and subsoil are separated by horizontal lines. Note much higher resolution of the x-axis for the subsoil (a, b). Error bars represent standard errors of the mean (n=3).

2.1.3.4 Differences in C stocks and assimilate partitioning between top- and subsoil

Root C stocks in topsoil were 8 times higher for alfalfa and 28 times higher for chicory than in subsoil 110 days after sowing (Table 2.1-3). Over the 40-day observation period, the ratio of root C stock in topsoil to root C stock in subsoil decreased. At the end of the observation period, the alfalfa root C stock in topsoil was only 4 times higher than in subsoil, whereas for chicory plants it was still 12.5 times higher than in subsoil. The ^{13}C recovery in topsoil roots of chicory was 67 times higher than in subsoil roots, indicating that chicory plants allocated and incorporated more assimilated C into topsoil roots compared to alfalfa plants (Table 2.1-3). Smallest differences between top- and subsoil were found for microbial biomass C stocks and microbial biomass ^{13}C recoveries under both plants. Microbial biomass C stocks under alfalfa were even higher in subsoil compared to topsoil 150 days after sowing.

2.1.3.5 Estimation of rhizodeposition

To estimate net rhizodeposition down to 105 cm depth, we assumed that ^{13}C recovered in rhizosphere and bulk soil reflects assimilated C released into soil. Our estimation therefore excludes the amount of assimilated C that was respired by roots or microorganisms during the first day. The ratio of ^{13}C released into soil to ^{13}C recovered in roots was smaller in topsoil (0.5 and 0.1 for alfalfa and chicory, respectively) than in subsoil (4.8 and 1.2 for alfalfa and

Table 2.1-3: Topsoil (0 - 30 cm) to subsoil (30 - 105 cm) root, rhizosphere and microbial biomass C stock ratio and ^{13}C recovery ratio for alfalfa and chicory plots.

	C pool	Ratio Topsoil/Subsoil [e]			
		Alfalfa 1d	Chicory 1d	Alfalfa 40d	Chicory 40d
^{13}C recovery	Root	17.6 ± 7.2	67.4 ± 25.1	4.2 ± 0.7	25.6 ± 11.8
	Rhizosphere	3.3 ± 1.4	15.7 ± 5.4	1.4 ± 0.1	7.9 ± 3.0
	Microbial biomass	1.7 ±	1.9 ± 0.5	1.1 ± 0.3	1.5 ± 0.3
C stock	Root	7.8 ± 2.5	28.2 ± 4.0	3.9 ± 1.1	12.5 ± 4.1
	Rhizosphere	2.6 ± 1.0	4.7 ± 0.7	2.3 ± 0.6	4.2 ± 1.4
	Microbial biomass	1.4 ±	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.1

[e] Values are given as means and standard errors of the mean.

chicory, respectively). To estimate the amount of assimilated C released into soil, we assumed that the ratio of ^{13}C released into soil and ^{13}C recovered in roots is constant over time. This ratio, determined 110 days after sowing, was multiplied with the root C stock at 110 and 150 days. We estimated that alfalfa released 325 kg C ha^{-1} into topsoil and 521 kg C ha^{-1} into subsoil during 110 days of growth (Figure 2.1-4 b). The higher alfalfa root biomass 150 days after sowing resulted in 1294 kg C ha^{-1} released into topsoil and 3166 kg C ha^{-1} into subsoil (Figure 2.1-4 ab). Chicory rhizodeposition was lower. We estimated 203 kg C ha^{-1} and 82 kg C ha^{-1} into top- and subsoil, respectively, during 110 days of growth, and 239 kg C ha^{-1} and 256 kg C ha^{-1} into top- and subsoil, respectively, during 150 days of growth.

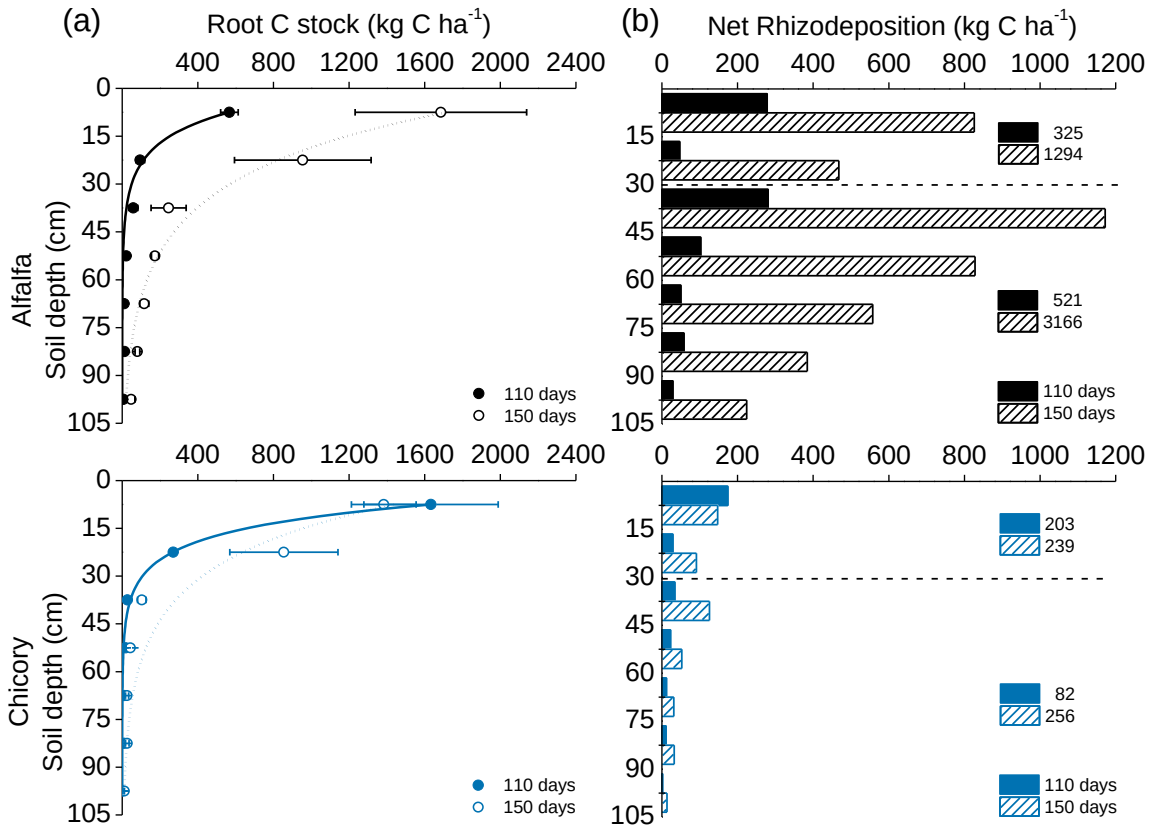

Figure 2.1-4: Estimation of alfalfa and chicory net rhizodeposition from 0 - 105 cm depth in 15 cm intervals, 110 and 150 days after sowing (b). The numbers of the filled (110 days) and dashed (150 days) boxes represent the sum of rhizodeposited-C (kg C ha^{-1}) into top- or subsoil. Root C stock 110 and 150 days after sowing (a) was multiplied by the ratio of recent C (^{13}C) recovered in soil to recent C recovered in roots, calculated for top- and subsoil separately.

2.1.4 Discussion

2.1.4.1 C input into top- and subsoil by alfalfa and chicory

The comparison of C input into top- and subsoil between alfalfa and chicory revealed higher C input, including roots and net rhizodeposition, under alfalfa during 150 days of growth. The partitioning of photosynthetically fixed C revealed that alfalfa invested more into building up subsoil roots compared to chicory (Table 2.1-2). As a consequence, alfalfa root biomass was more evenly distributed between top- and subsoil compared to chicory (Table 2.1-3). Root distribution is affected by plant species, period of growth and environmental factors (Lamba et al. 1949), causing varying distribution of root biomass throughout the soil profile. Previous studies of alfalfa root distribution reported a fast development of deeply penetrating taproots, which agrees with the increase in alfalfa subsoil root C stock in the current study (Upchurch and Lovvorn 1951; Bell 2005). Chicory, however, incorporated 26 times more assimilated C into topsoil roots than subsoil roots (Table 2.1-3). The higher investment into topsoil root biomass found in the current study is related to the developmental stage, as chicory was reported to develop a deep root system after 2 years of growth (Perkons et al. 2014).

In addition to root biomass, C released into soil needs to be determined in order to estimate total C input into soil (Johnson et al. 2006; Pausch et al. 2013). However, the quantification of rhizodeposition is difficult, as rhizodeposits are easy to decompose (Johnson et al. 2006; Pausch et al. 2013). To estimate rhizodeposition at the field scale, Pausch et al. (2013) determined the ratio of rhizodeposited C to root C in a lab study, which was then applied to root C determined in the field. To estimate net rhizodeposition in the current study, we determined the rhizodeposited-C-to-root-C ratio in top- and subsoil. C lost by soil respiration was excluded from our calculation due to experimental difficulties in determining *in situ* respiration down to 105 cm depth. Therefore, the actual ratio of rhizodeposited C to root C would be higher. Net rhizodeposition of alfalfa was estimated to be 1290 kg C ha^{-1} and 3160 kg C ha^{-1} into top- and subsoil, respectively, during 150 days of growth, and therefore much higher than of chicory. Rhizodeposition increases the activity of microorganisms in the rhizosphere (De Nobili et al. 2001). This, in turn, increases organic matter turnover and nutrient mineralization (Cheng 2009). The requirement of nutrients, i.e. P and micronutrients, for N_2 fixation of alfalfa (O'Hara 2001) and the higher increase in above- and belowground plant biomass of alfalfa than chicory resulted in higher investment of recent C into soil (Table 2.1-1, Table 2.1-2 and Figure 2.1-4). Moreover, N_2 fixation from the atmosphere leads to a

higher C demand by rhizosphere microorganisms (Vance and Heichel 1991; Herridge et al. 2008) under legumes compared to non-leguminous herbs. This is another reason why alfalfa allocated more C into the soil than chicory. In conclusion, total C input including root biomass and net rhizodeposition was estimated to be 3940 kg C ha^{-1} into topsoil and 3830 kg C ha^{-1} into subsoil by alfalfa and only 2480 kg C ha^{-1} into topsoil and 470 kg C ha^{-1} into subsoil by chicory within 150 days of growth.

The estimation of net rhizodeposition into subsoil could be improved by a series of $^{13}CO_2$ pulse labelings accompanied by root biomass determination throughout the vegetation period. It has been shown that C input into soil as root biomass and exudation depends on the developmental stage of the crop (Swinnen et al. 1994; Kuzyakov et al. 1999; Kuzyakov et al. 2001). A series of $^{13}CO_2$ pulse labelings throughout the vegetation period of alfalfa and chicory would account for changes in the partitioning of recently assimilated C between root biomass and soil depending on depth and developmental stage of the plants. Furthermore, the time necessary for plant roots to explore deeper soil would be considered.

2.1.4.2 The response of microorganisms to C input into subsoil

Microbial biomass in subsoil is limited in energy due to a lower supply of fresh C (Fontaine et al. 2007) than in topsoil. Easily available C that is released into soil via rhizodeposition stimulates microbial activity (De Nobili et al. 2001). Especially in subsoil, rhizodeposits are important for microorganisms due to the usually scarce substrate supply. The amount of released C taken up by microorganisms in subsoil was similar to that in topsoil under alfalfa and chicory. Although C input into roots and C released into soil strongly decreased with depth, the uptake of C by microorganisms was only slightly affected (Figure 2.1-3). This suggests that in subsoil, microorganisms used a higher proportion of the substrate supplied by rhizodeposition, as a result of C limitation. In contrast, continuous input of plant litter ensures substrate availability for microorganisms in topsoil. Sufficient substrate availability caused higher mineralization of rhizodeposits, resulting in similar uptake of released C into topsoil compared to subsoil microbial biomass.

During the observation period, the microbial biomass in subsoil increased under alfalfa but decreased under chicory (Table 2.1-1, Figure 2.1-1 c). However, the absolute incorporation of released ^{13}C into microbial biomass under chicory was higher after 40 days (Figure 2.1-3 c). The increase in alfalfa root biomass and associated rhizodeposition indicates a continuous

supply of substrate for microorganisms, enabling their growth (De Nobili et al. 2001). This suggest that the higher and sustained availability of easily available C under alfalfa caused an accelerated turnover of microbial biomass C (Dorodnikov et al. 2009; Blagodatskaya et al. 2011). In contrast, an insufficient substrate supply under chicory could not even maintain microbial biomass. In conclusion, accelerated turnover of microbial C resulted in lower total ^{13}C incorporation into the microbial biomass in subsoil under alfalfa compared to chicory.

Furthermore, lower ^{13}C incorporation into the subsoil microbial biomass under alfalfa plants could have been affected by root distribution. Alfalfa taproots can branch up to the fifth order, whereas chicory taproots can branch up to the fourth order (Kutschera et al. 2009). The increase in alfalfa root biomass and lateral root development could have caused a more dispersed root distribution, leading to rhizodeposition of ^{13}C throughout a larger volume of subsoil. Due to the low levels of microbial biomass in the subsoil, a greater proportion of the rhizodeposited C of the alfalfa roots did not come into contact with the microbial biomass and was therefore not incorporated.

2.1.4.3 Relevance of carbon input into subsoil

Generally, SOM content and microbial biomass decrease exponentially with soil depth (Fierer et al. 2003; Castellazzi et al. 2004). The SOC stock of the chicory and alfalfa plots decreased exponentially down the entire soil profile (data not shown) but we found a slower decrease of microbial biomass that followed a linear rather than exponential decline (Figure 2.1-1). We think that the distribution of the microbial biomass was a function of the root distribution over the soil profile. In particular, the exploration of the subsoil by alfalfa taproots and the release of easily available C enabled microbial growth and the development of microbial hotspots (Kuzyakov and Blagodatskaya 2015; Spohn and Kuzyakov 2014). The input of a diversity of organic compounds, including both low and high molecular weight organic substances maintains a broad capability in microbial decomposition functions (De Nobili et al. 2001). This biochemical ability to decompose various substrates also enables decomposition of various SOM compounds and thus the opportunity to access immobilized nutrients.

2.1.5 Conclusions

C input into soil, including root biomass and net rhizodeposition, by two taprooted preceding crops, alfalfa and chicory, was determined over 150 days of growth down to 105 cm depth. C input into the topsoil (0 - 30 cm) by alfalfa was 1.6 times higher (3940 kg C ha^{-1}) than by chicory (2480 kg C ha^{-1}) and C input into subsoil (30 - 105 cm depth) by alfalfa was 8.2 times higher (3830 kg C ha^{-1}) than by chicory (470 kg C ha^{-1}). The higher C input into soil resulted from a larger increase in alfalfa above- and belowground biomass during the vegetation period. Especially the C input into subsoil was higher under alfalfa. The root system of alfalfa was more equally distributed between top- and subsoil, whereas the main part of chicory root biomass was found in the topsoil. The *in situ* $^{13}CO_2$ pulse labeling of alfalfa and chicory plants enabled the determination of assimilated C partitioning between above- and belowground C pools down to 105 cm depth. In accordance with the increase in subsoil root biomass of alfalfa, the labeling revealed higher incorporation of assimilated C into subsoil roots. Tracing of ^{13}C in roots and soil enabled the estimation of net rhizodeposition into top and subsoil down to 105 cm. Net rhizodeposition of alfalfa was 5 times higher into topsoil and 12 times higher into subsoil compared to chicory. To account for the variability of root biomass and rhizodeposition during the vegetation period and at different soil depths, several pulse labelings would enable a more precise estimation of C input.

Although C allocation to roots and rhizodeposition decreased strongly from top- to subsoil, the uptake by microorganisms was similar in top- and subsoil. Our results suggest that subsoil microorganisms incorporated a higher proportion of released C due to scarce substrate supply, whereas in the topsoil, sufficient substrate availability caused higher mineralization of released C to CO_2. Because alfalfa invested more C into building up subsoil root biomass and into rhizodeposition during the observation period, microbial turnover was accelerated. Therefore, total ^{13}C incorporation by microorganisms was lower in subsoil under alfalfa than under chicory.

2.1.6 Acknowledgements

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2.1.7 Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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2.1.9 Supplementary material

Supplementary material for

Carbon input and partitioning in suboil by chicory and alfalfa

Plant and Soil

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Table 2.1-4: Results of the 2x7 analysis of variance showing the main and interaction effects of the factors crop and soil depth on the dependent variables root C stock and microbial biomass C stock 110 and 150 days after sowing. Degrees of freedom (df), F-values (F) and the significance level (p) are shown for crop, soil depth (depth) and the interaction between the factor levels of crop and soil depth (crop:depth)

factor	log(root C stock) 110 d			log(root C stock) 150 d			log(microbial C stock) 110 d			log(microbial C stock) 150 d		
	df	F	p	df	F	p	df	F	p	df	F	p
crop	1	0.74	n.s.	1	26.17	***	1	6.08	*	1	9.56	**
depth	6	21.21	***	6	39.12	***	6	4.22	**	6	19.52	***
crop:depth	6	0.41	n.s.	6	1.72	n.s.	6	1.79	n.s.	6	1.97	n.s.
Residuals	25			28			24			28		

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

Table 2.1-5: Results of the 2x2 analysis of variance showing the main and interaction effects of the factors crop and soil depth on the dependent variables ^{13}C recovery in roots, rhizosphere and microbial biomass 1 and 40 days after $^{13}CO_2$ pulse labeling in topsoil. Degrees of freedom (df), F-values (F) and the significance level (p) are shown for crop, soil depth (depth) and the interaction between the factor levels of crop and soil depth (crop:depth).

factor	log(root ^{13}C) 1 d			log(root ^{13}C) 40 d			log(rhizosphere ^{13}C) 1 d			log(rhizosphere ^{13}C) 40 d		
	df	F	p	df	F	p	df	F	p	df	F	p
crop	1	66.04	***	1	0.26	n.s.	1	12.40	**	1	2.61	n.s.
depth	1	71.83	***	1	2.29	n.s.	1	20.70	**	1	2.70	n.s.
crop:depth	1	6.14	*	1	0.09	n.s.	1	0.07	n.s.	1	0.53	n.s.
Residuals	8			8			8			8		
	log(microbial ^{13}C) 1 d			log(microbial ^{13}C) 40 d								
	df	F	p	df	F	p						
crop	1	0.01	n.s.	1	7.38	*						
depth	1	2.72	n.s.	1	16.22	**						
crop:depth	1	0.21	n.s.	1	6.44	*						
Residuals	8			8								

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

Table 2.1-6: Results of the 2x5 analysis of variance showing the main and interaction effects of the factors crop and soil depth on the dependent variables ^{13}C recovery in roots, rhizosphere and microbial biomass 1 and 40 days after $^{13}CO_2$ pulse labeling in subsoil. Degrees of freedom (df), F-values (F) and the significance level (p) are shown for crop, soil depth (depth) and the interaction between the factor levels of crop and soil depth (crop:depth).

factor	log(root ^{13}C) 1 d			log(root ^{13}C) 40 d			log(rhizosphere ^{13}C) 1 d			log(rhizosphere ^{13}C) 40 d		
	df	F	p	df	F	p	df	F	p	df	F	p
crop	1	0.04	n.s.	1	24.26	***	1	0.66	n.s.	1	33.07	***
depth	4	2.70	n.s.	4	1.78	n.s.	4	1.59	n.s.	4	1.53	n.s.
crop:depth	4	0.06	n.s.	4	0.80	n.s.	4	1.75	n.s.	4	2.28	n.s.
Residuals	17			20			16			20		
	log(microbial ^{13}C) 1 d			log(microbial ^{13}C) 40 d								
	df	F	p	df	F	p						
crop	1	19.66	***	1	0.24	n.s.						
depth	4	3.70	*	4	2.65	n.s.						
crop:depth	3	0.76	n.s.	4	1.22	n.s.						
Residuals	14			19								

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

Study 2

2.2 Molecular differentiation between root- and earthworm-derived biopores in soil based on free extractable fatty acids

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Abstract

Soil organic matter stabilization has been of rising interest with special emphasis to the identification of relevant processes and their locations within soil. Recently, biopores including root- and earthworm-derived ones were identified as channels by which organic matter can enter subsoil horizons and might be subsequentially stabilized. It is currently under debate, to which extent biopores of various origins contribute to carbon sequestration. However, to assess the origin and stability of organic matter in biopores, the identification of their sources is mandatory, which is difficult if the source biomass is not determined in the respective biopores.

The aims of this study were to test the suitability of free extractable fatty acids 1) to differentiate between rhizosphere, drilosphere and bulk soil organic matter, and 2) to identify the source of organic matter in biopores. For this purpose, top- and subsoil horizons of a temperate agricultural soil were sampled for bulk soil, free of visible root remains, rhizosphere and drilosphere samples and analysed for molecular marker composition in the fraction of free extractable fatty acids. Rhizosphere and drilosphere samples were enriched in organic C and lipids compared to bulk soil. Frequently used molecular proxies like the average chain length (ACL) and the carbon preference index of free extractable fatty acids were weak criteria to identify sources of biopore organic matter. ACL values suggested vertical translocation of organic matter into subsoil horizons especially in the mainly vertically oriented earthworm-derived biopores. The relative contribution of dicarboxylic acids and unsaturated fatty acids to total fatty acids allowed differing between rhizosphere, drilosphere and bulk soil organic matter. 10-20% dicarboxylic acids and > 10% unsaturated fatty acids were diagnostic for rhizosphere organic matter and < 10% dicarboxylic acids and > 10% unsaturated fatty acids indicated drilosphere organic matter, compared to bulk soils with > 15% dicarboxylic acids and < 10% unsaturated fatty acids. Discriminant analysis confirmed that the organic matter in the rhizosphere, drilosphere and bulk soil can be differentiated by a linear combination of relative contents of unsaturated fatty acids and dicarboxylic acids. This differentiation was depth independent as the relative differences in unsaturated fatty acid and dicarboxylic acid contents between bulk soil, rhizosphere and drilosphere OM did not change down to 105 cm soil depth. Concluding, this study highlights the potential to use free extractable fatty acids to differentiate between bulk soil, rhizosphere and drilosphere OM.

Keywords: Roots; earthworms; biomarkers; rhizosphere, drilosphere, organic matter

2.2.1 Introduction

Carbon (C) sequestration and preservation in soil became of rising interest during the past decades (Marschner et al., 2008; Schmidt et al., 2011) to cope with increasing atmospheric CO_2 concentration (Solomon et al., 2007). One of the strategies highlighted was to incorporate more C into the subsoil via deep rooting plants (Kell, 2012; Smith et al., 2007). This was supported by the findings that in general, radiocarbon ages of soil organic matter (OM) increase with depth (Bol et al., 1996) and root C is characterized by a slower turnover than aboveground litter C (Mendez-Millan et al., 2010b). This is in agreement with findings that roots contribute larger amounts to soil C than aboveground litter (Rasse et al., 2005). Furthermore, the significance of C stored in subsoils was frequently underestimated in former studies. Meanwhile, The proportion of C stored in subsoils (below 40 cm depth) was determined to account for at least 40% of the total C in the whole soil column (Rumpel and Kögel-Knabner, 2010). However, the incorporation and stabilisation mechanisms of freshly incorporated root C into the subsoil still remain largely unknown (Rumpel and Kögel-Knabner, 2010). Roots enter the subsoil by forming new pores or using already existing pore networks (Kautz et al., 2013). This mechanism strongly depends on the texture and density of the subsoil. Apart from physical swelling and shrinking processes, macropores are produces by burrowing soil animals and plant roots (Kautz et al., 2013). Such biopores are used by plants for rooting to access water and nutrients in subsoils. Biopores can be preserved for thousands of years in deep subsoils of several m depth below the present soil surface and thus provide a long-term opportunity for plant roots to access deeper soil horizons (Gocke et al., 2014a). However, little is known about the formation of biopores and their persistence in soils and subsoil horizons. In addition to roots, also earthworms and other burrowing soil animals produce biopores and thus contribute to the pore network in soils. Currently, the sources of OM in biopores can be accessed and differentiated only 1) morphologically in unaltered biopores, 2) if living roots or burrowing soil animals are present or 3) if remains like e.g. earthworm faeces or root remains are found if the source organism itself could not be determined. This becomes more difficult, if biopores are of higher age and the connection to their specific biogenic source cannot be determined or if biopores are of mixed origin, i.e. produced by burrowing soil animals or tap roots and reused by other roots afterwards. To understand the C dynamics in biopores, the source determination of organic matter therein is mandatory.

In addition to root- and soil organism-derived organic matter, microorganisms are a source of OM and contribute to OM transformation in biopores and bulk soil. The contribution to soil OM (SOM) in biopores differs spatially between the mentioned sources. The contribution of burrowing animals to SOM is restricted to the pore wall, only (Brown et al., 2000; Tiunov and Scheu, 1999). In opposite, rhizodeposition and mycorrhiza also contribute to SOM distant to the pore wall in bulk soil (Jones et al., 2009; Sauer et al., 2006). Therefore, the spatial extension of microbial activity in drilosphere and rhizosphere differs (Lambers et al., 2009).

Molecular proxies deriving from compound classes like lipids have been frequently used for source apportionment of organic matter in soils and sediments (Harwood and Russell, 1984; Hedges and Oades, 1997). Free extractable fatty acids (FA) and bound FA like phospholipid fatty acids (PLFA) have been used to differentiate sources of organic matter in soil, because the composition of these lipid fractions differs between plants, soil animals and microorganisms (Hansen and Czochanska, 1975; Harwood and Russell, 1984). While PLFA are sensitive to degradation and are characterized by fast turnover in soil, FA are characterized by a slower turnover (Marschner et al., 2008) and thus a larger potential to unravel different sources of organic matter in subsoils after millennia (Gocke et al., 2014b). The differing FA composition of various biological tissues is related to specific metabolic processes that cause the respective composition of saturated, unsaturated, straight chain, cyclic, branched, mono- and dicarboxylic acids. While the assessment of various groups of microorganisms can be accessed by the composition of saturated, unsaturated and branched short-chain FA (typically consisting of < 19 C atoms), plant-derived organic matter consists of saturated and unsaturated C_{16} and C_{18} FA as well as long-chain FA (> 19 C atoms) (Harwood and Russell, 1984). Furthermore, suberin as main component of root tissues (Kolattukudy, 1981) contributes large amounts of dicarboxylic acids (Mendez-Millan et al., 2010a). Due to their ubiquitous presence in living organisms, potential diagnostic character to differ between source organisms and potential of being preserved after the lifetime of the respective source organism, FA were assumed to allow a differentiation of biopores in soil produced by roots and earthworms. To evaluate the potential use of FA for the molecular differentiation of OM in bulk soil, rhizosphere and drilosphere, we tested biopores of known origin from an agricultural site for their FA composition using molecular proxies like average chain length (ACL), carbon preference index (CPI), the relative contribution of dicarboxylic and unsaturated FA, followed by statistical evaluation of the significance of the results.

2.2.2 Material and Methods

2.2.2.1 Sampling

Three plots that were cultivated for two years with alfalfa (*Medicago sativa* L.) were selected as field replicates from the field trial in Klein-Altendorf near the city of Bonn (Germany; 50°37’21’’ N, 6°59’29’’ E). Alfalfa has a taproot system and is well known for deep rooting up to 9 m depth (Carlson, 1925) and formation of biopores. The soil was described as Haplic Luvisol (Gaiser et al., 2012; WRB, 2007). A profile wall was prepared and sampled down to 105 cm depth at each of the three replicate alfalfa plots. Soil horizons were sampled according to depth intervals identified in a standard profile on one edge of the field trial with properties previously described in detail (Kautz et al., 2014). The sampling depths were 0-30 cm (Ap), 30-45 cm (E/B), 45-75 cm (Bt1) and 75-105 cm (Bt2 and Bt3). Thus, only the top layer is representing the topsoil, whereas all other depth intervals are corresponding to subsoil horizons. In the figures, the values are shown in the middle of the corresponding depth increment. Bulk soil was sampled from each depth interval distant to visible biopores at the profile wall. To assess biopores, soil was scratched off the profile wall by using a spatula until a biopore could be identified. A pore was determined as drilosphere, if wall coatings or earthworm faeces were found but no roots. Soil was scratched off at a 2 mm thick soil layer surrounding the earthworm burrow by help of a micro spoon, but faeces were not collected as they are not part of the biopore wall. The orientation of the earthworm burrows that were sampled was mainly vertical as horizontal burrows were not excavated in large quantities (Figure 2.2-1). Rhizosphere was defined as the soil remaining attached to the root until a distance of approximately 2 mm after root removal from the profile wall and shaking at the air. Only roots that were directly attached to the soil and therefore not visibly growing in a preexisting pore were used to sample rhizosphere soil (Figure 2.2-1). Rhizosphere soil was carefully removed from the roots using fine brushes. From each depth interval root samples were also obtained. Despite biopores are highly abundant in soils with numbers of several hundred m^{-2}, their abundance typically decreases with soil depth (Gocke et al., 2014a). Therefore, not all types of biopores could be detected in all soil profiles and depth intervals.

Bulk soil, rhizosphere and drilosphere soil and roots were cooled during sampling and frozen directly after sampling. Before further analyses, all samples were freeze-dried and crushed with pestle and mortar afterwards. Plant roots were milled in a ball mill (Retsch MM 200).

2.2.2.2 Organic carbon and free extractable fatty acid analysis

All soil samples were decarbonated using 3 M HCl and washed until neutral pH before measurement of C_{org} with an Analytik Jena N/C analyzer equipped with an oven (Feststoffmodul) for solid samples.

Depending on sample availability 0.8-10 g root or soil material was available for lipid analyses. Lipid extraction was performed via Soxhlet extractors at room temperature using a solvent mixture of DCM/MeOH (93:7; v:v) (Wiesenberg et al., 2010). After extraction, the lipid extract yield was obtained gravimetrically after solvent evaporation until dryness under atmospheric conditions in pre-weighed sample vials. Separation of FA was performed on KOH-coated SiO_2 columns (Wiesenberg et al., 2010). In brief, samples were re-dissolved in DCM and transferred to the columns. Neutral lipids were eluted with DCM and not further analyzed as it was assumed that alkanes as largely abundant compounds in aboveground plant tissues but commonly not in roots did not show significant changes in biopore walls compared to bulk soil. Afterwards, FA were flushed from the columns using DCM/formic acid (99:1; v:v). FA fractions were dried and weighed. If available, 1-2 mg FA were re-dissolved in DCM and 50 µL deuteriated (D_{39}) eicosanoic acid was added as internal standard. Quantification of

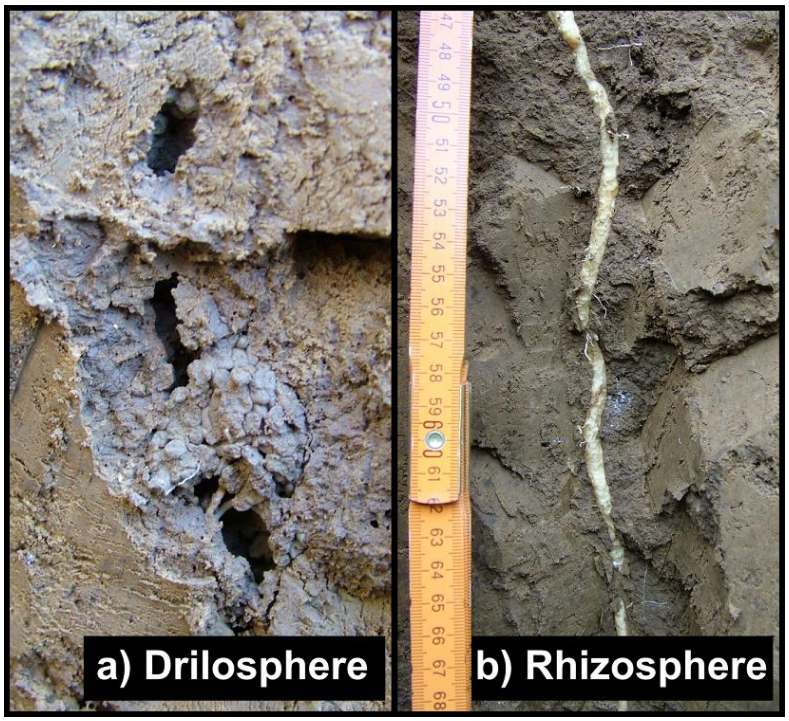

Figure 2.2-1: Illustration of the drilosphere (a) and rhizosphere soil (b) that was sampled for the fatty acid analyses. A 2 mm thick soil layer surrounding the earthworm burrow was defined as drilosphere, when coatings or faeces were found at the pore wall but no roots. Rhizosphere was defined as the soil remaining at the root after shaking up to a distance of 2 mm. Only soil attached to a root that was not growing in a preexisting pore was defined as rhizosphere soil.

FA was done on an Agilent 7890 GC equipped with flame ionization detector after methylation using BF_3/MeOH. Compound identification was performed on a Hewlett Packard 5890 GC Series II coupled to Hewlett Packard 5871 mass spectrometer. Both GC were equipped with 30 m DB5 columns (0.32 mm inner diameter and 0.25 µm film thickness; J&W) by comparison to spectral libraries (W8/N08) and comparison to standards. Splitless injection was performed at 40°C and isothermal conditions were kept for 2 min. Temperature was ramped at 5°C min^{-1} until 140°C, followed by 2°C min^{-1} until 210°C and 3°C min^{-1} until 300°C. The final temperature was kept constant for 20 min.

2.2.2.3 Molecular proxies

The average chain length (ACL) of FA serves as a measure of the source and/or degradation of FA (Harwood and Russell, 1984) in soils and was calculated as follows (Wiesenberg et al., 2012):

$$ACL = \sum (a \cdot A) \cdot n^{-1} \quad (1)$$

with *a* as the relative amount of individual FA with *A* carbon atoms and *n* as the sum of all FA. Low ACL values (< 18) typically indicate the microbial source of FA as microorganisms are dominated by FA with 16 and 18 carbon atoms in the alkyl chain and contain almost no FA with more than 19 carbon atoms (Harwood and Russell, 1984). Values > 18 are related to exclusive plant origin due to the large abundance of FA with > 18 carbon atoms, which is especially relevant for aboveground plant biomass. However, as in roots FA with 20 or more carbon atoms could be almost absent, the plant-derived ACL value could also range between 17-18. Degradation of plant biomass leads to an increase of the ACL value as preferentially short-chain FA are degraded.

In addition to the ACL, the carbon preference index (CPI) has been frequently used as measure for source apportionment and degradation in soils and sediments (Naraoka and Ishiwatari, 2000; Wiesenberg et al., 2012; Xie et al., 2003), which was calculated as follows:

$$\text{CPI} = \frac{\left(\frac{(C_{20}+C_{22}+C_{24}+C_{26}+C_{28}+C_{30})}{(C_{19}+C_{21}+C_{23}+C_{25}+C_{27}+C_{29})} + \frac{(C_{20}+C_{22}+C_{24}+C_{26}+C_{28}+C_{30})}{(C_{21}+C_{23}+C_{25}+C_{27}+C_{29}+C_{31})}\right)}{2} \quad (2)$$

with C_x as the relative amounts of *n*-FA with x carbon atoms. Plant wax-derived FA are characterized by long-chain (> 18) FA with a predominance of even over odd carbon numbered homologues (Harwood and Russell, 1984). In contrast, microbial biomass is characterized by almost equal values of odd and even numbered homologues, if the latter are present. Consequently, incorporation of microorganism-derived FA and degradation leads to a decrease in CPI values in soils and sediments. Therefore, especially the CPI of long-chain FA (*n*-$C_{20\text{-}30}$) has been proven to be of diagnostic character of C sources (Naraoka and Ishiwatari, 2000; Wiesenberg et al., 2012).

The relative contribution of unsaturated FA to total FA can provide insight into the contribution of plant- and microorganism-derived FA, which contain unsaturated compounds

(Harwood and Russell, 1984), whereas degradation of OM leads to a decrease in these compounds and a selective enrichment of saturated FA.

Dicarboxylic acids are regarded as molecular markers of root-derived FA in soils as they are part of the suberin macromolecule (Kolattukudy, 1981). However, due to their presence in macromolecules, cleavage of bindings is typically required during sample preparation to release and investigate dicarboxylic acids (Mendez-Millan et al., 2010a). In free lipid extracts obtained after extraction with low polar solvents, dicarboxylic acids have been scarcely described and therefore, their potential diagnostic character has not been identified. Hence, the relative contribution of dicarboxylic acids to total FA was used for the first time in the current study to identify their diagnostic potential.

The relative content (C_{rel}) of unsaturated FA and dicarboxylic acids was calculated as the ratio of total unsaturated FA (∑ unsaturated FA) or dicarboxylic acids (∑ dicarboxylic acids) to total FA (∑ FA)

$$C_{rel} = \frac{\sum \text{unsaturated FA}}{\sum \text{FA}} \cdot 100 \quad (3)$$

2.2.2.4 Statistical evaluation

In the figures and tables all results are presented as means of the three replicate plots and standard errors of the means. A factorial analysis of variance (ANOVA) was conducted to test if C_{org}, total lipid extract contents (TLE), ACL, as well as the relative amounts of unsaturated FA and dicarboxylic acids, representing the dependent variables, were significantly different between the soil compartments and the depth intervals and if there were interactions between these. If there was no interaction effect, a posthoc Tukey HSD test was conducted. It was a 3x4 factorial design using soil compartment (3 levels) and depth interval (4 levels) as factors. Normal distribution of the residuals was tested using the Shapiro-Wilk normality test. Levene's test was conducted to test for homogeneity of variances using the R package car (Fox and Weisberg, 2011). The residuals of the ANOVA model for C_{org} and CPI were not normally distributed. Therefore, the 3x4 factorial ANOVA was calculated using log transformed C_{org} and CPI data that revealed a normal distribution of the residuals and improved homoscedasticity.

A linear discriminant analysis was conducted to test whether FA can be used to separate drilosphere, rhizosphere and bulk soil OM. Unsaturated and dicarboxylic acid contents were

used as predicting variables and the soil compartment was used as categorical variable. To conduct linear discriminant analysis the R package MASS was used (Venables and Ripley, 2002). Unsaturated and dicarboxylic acids that were found in less than 10% of all samples were excluded from the analysis to eliminate co-linearity among variables. To assess multivariate normality within the groups, a QQ plot was used. One-way ANOVA followed by a Tukey HSD post-hoc test was conducted to test if the discriminant scores of the linear discriminant function 1 and the linear discriminant function 2 differ significantly between the soil compartments. ANOVA and linear discriminant analysis were calculated using R version 3.0.2 (R Core Team, 2013).

2.2.3 Results

2.2.3.1 Organic carbon and total lipid extract contents

The average organic carbon (C_{org}) content differed significantly between the soil compartments. It was highest in drilosphere, intermediate in rhizosphere and lowest in bulk soil (Figure 2.2-2 a, Table 2.2-1). C_{org} of all soil compartments was highest in the uppermost soil horizon and decreased significantly with increasing soil depth (Table 2.2-1). Interaction effects were absent, meaning that in every soil depth the C_{org} content was highest in drilosphere, intermediate in rhizosphere and lowest in bulk soil. The distribution of C_{org} with depth was described using a power function in bulk soil ($47 \times ^{-0.57}$), rhizosphere soil ($49 \times ^{-0.49}$) and drilosphere soil ($42 \times ^{-0.40}$) (Figure 2.2-2 a). The total lipid extract contents normalized to C_{org} (TLE_{Corg}) did neither differ between bulk soil, rhizosphere and drilosphere OM nor were affected by soil depth (Figure 2.2-2 b) (Table 2.2-1). TLE_{Corg} of roots was three orders of magnitude higher than that of soil compartments, but did not change with depth (Figure 2.2-2 b).

2.2.3.2 Free extractable fatty acid molecular proxies

The fatty acid distribution patterns of all soil samples including bulk soil, rhizosphere and drilosphere in general seemed to be similar, but differed from root samples. A range from

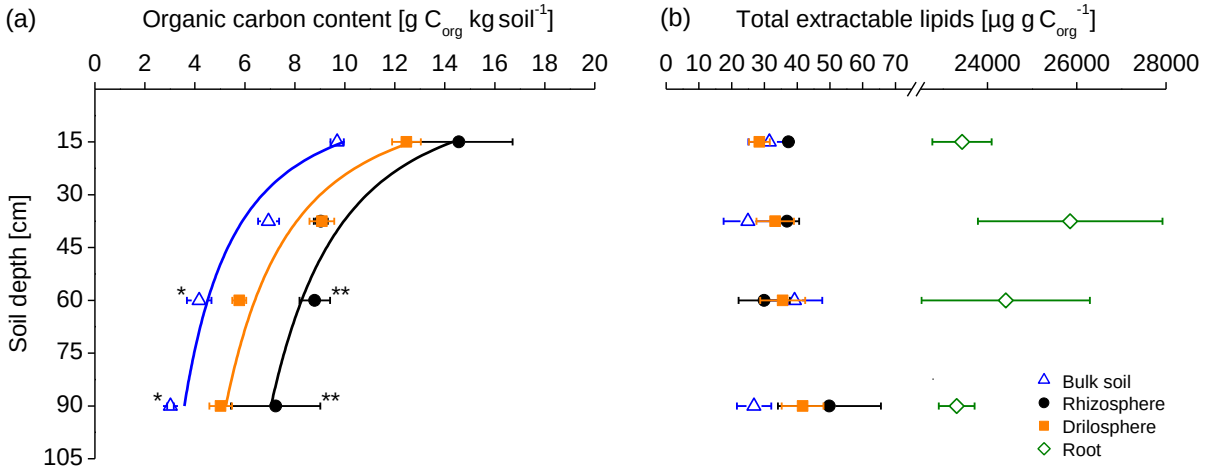

Figure 2.2-2: Organic carbon content (a) and total lipid extract contents normalized to C_{org} (b) (± SEM) in bulk soil, rhizosphere and drilosphere. A power function (C_{org}) or linear regression (total lipid extract contents normalized to C_{org}) using the least squares fitting method was used to describe its distribution with depth. * Asterisk indicate significant differences of C_{org} between the soil compartments in particular soil depths (3x4 ANOVA followed by TukeyHSD post-hoc test, $p<0.05$).

$C_{12:0}$ to $C_{34:0}$ saturated FA could be determined in the whole sample set. Dicarboxylic acids appeared in the sample set between $C_{9:0}$ and $C_{24:0}$. Branched FA were observed between C_{12} and C_{24} and unsaturated FA between $C_{16:1}$ and $C_{24:1}$. A clear even over odd predominance was observed for all types of FA in the whole sample set. The most abundant FA was $C_{18:1}$ for root samples and $C_{16:0}$ for all other samples. The most abundant long-chain FA was $C_{22:0}$ in almost every sample. In some samples also $C_{24:0}$ and $C_{20:0}$ were similar in abundance compared to $C_{22:0}$ or even slightly enriched. Differences in the distribution pattern of FA between soil compartments and with soil depth were evaluated using various molecular proxies.

The ACL of the bulk soil FA was identical in both upper soil horizons (Figure 2.2-3 a). Below these, the ACL value dropped below 18 and remained constant down to 90 cm depth. Similar trends were observed for rhizosphere and drilosphere samples. Thus, the ACL did not differ between the soil compartments and between the depth intervals (Table 2.2-1). Significantly lower values compared to most soil samples were observed for roots (17.3-17.8), which tended to increase with depth.

The CPI of the bulk soil FA ranged between 4 and 5 and did not significantly vary with soil depth or between soil compartments (Figure 2.2-3 b, Table 2.2-1). The CPI of the drilosphere FA was slightly higher compared to bulk soil maximizing at 5.3 ± 0.5 at a depth of 45-75 cm.

Table 2.2-1: Results of the 3x4 analysis of variance showing the main and interaction effects of the factors soil compartment and soil depth on the dependent variables organic carbon content (C_{org}), total lipid extract contents normalized to C_{org} (TLE_{Corg}), the average chain length (ACL) and the relative amount of unsaturated FA and diacids. Degrees of freedom (df), F-values (F) and the significance level (p) are shown for soil compartment (compartment), soil depth (depth) and the interaction between the factor levels of soil compartment and soil depth (compartment:depth).

factor	log(C_{org})			TLE_{Corg}			ACL			log(CPI)			unsaturated FA			Diacids		
	df	F	p	df	F	p	df	F	p	df	F	p	df	F	p	df	F	P
compartment	2	31.48	***	2	1.20	n.s.	2	0.17	n.s.	2	2.36	n.s.	2	15.14	***	2	2.04	n.s.
depth	3	50.17	***	3	0.56	n.s.	3	1.50	n.s.	3	0.09	n.s.	3	1.72	n.s.	3	0.33	n.s.
compartment:depth	6	2.03	n.s.	6	1.02	n.s.	6	0.20	n.s.	6	0.69	n.s.	6	1.69	n.s.	6	0.28	n.s.
Residuals	24			23			21			20			21			21		

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

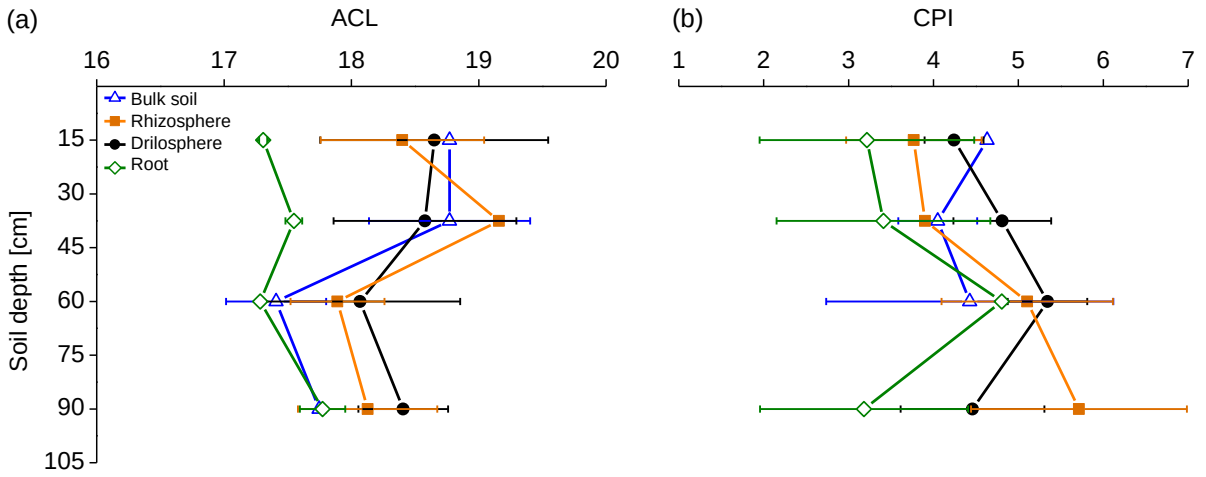

Figure 2.2-3: a) Average chain length (ACL) and b) carbon preference index of long-chain fatty acids (CPI) (± SEM).

The rhizosphere FA were characterized by CPI below 4 in the depth interval of 0-45 cm and increased up to 5.7 ± 1.3 in 75-105 cm depth. The CPI of roots were lower compared to rhizosphere and drilosphere FA and ranged between 3.2 and 3.4 from top- to subsoil except for an increase in depth interval 45-75 cm (4.8).

The average relative contribution of dicarboxylic acids to total FA was highest in bulk soil, intermediate in rhizosphere and lowest in drilosphere, but did not differ significantly between the soil compartments (Figure 2.2-4 a). With increasing soil depth the proportions of dicarboxylic acids did not change significantly (Figure 2.2-4 a, Table 2.2-1). The contribution of dicarboxylic acids in roots were significantly lower compared to most soil compartments,

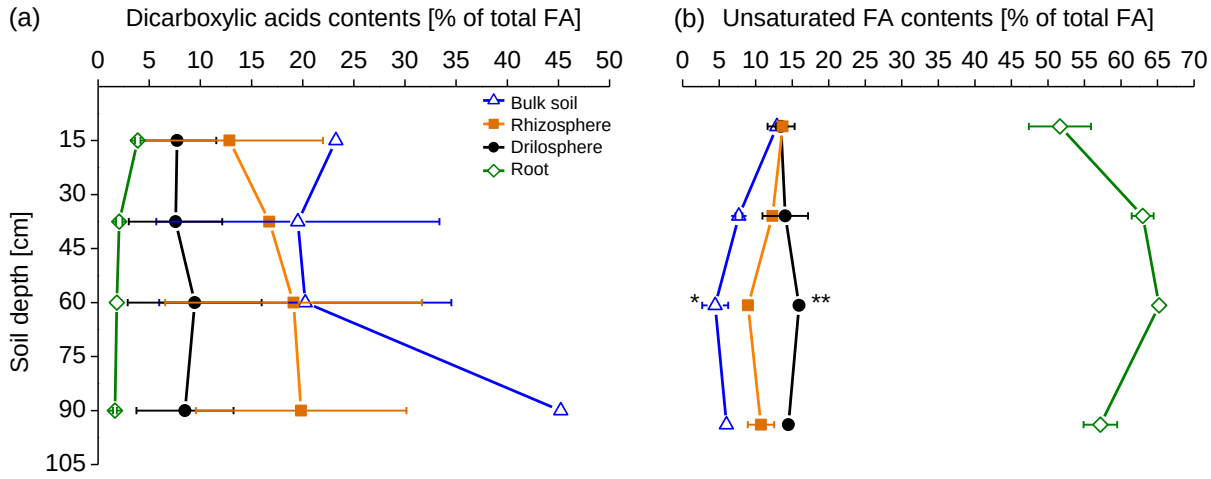

Figure 2.2-4: Relative contribution of a) unsaturated and b) dicarboxylic acids to total free fatty acids (FA) (± SEM). * Asterisk indicate significant differences of the relative unsaturated fatty acid contents between the soil compartments in particular soil depths (3x4 ANOVA followed by TukeyHSD post-hoc test, $p<0.05$).

but did not change with depth.

The average contribution of unsaturated FA to total FA differed significantly between the soil compartments (Figure 2.2-4 b, Table 2.2-1). Highest contents of unsaturated FA were found in drilosphere, intermediate in rhizosphere and lowest in bulk soil. The relative amounts of unsaturated FA decreased from 12.9 % in the topsoil to less than 6 % in the subsoil horizons in bulk soil. The relative amounts of unsaturated FA in rhizosphere and drilosphere were in general identical to bulk soil in the topsoil. Nevertheless, their relative amount was significantly higher in drilosphere compared to bulk soil at 45-75 cm depth. Significantly higher amounts of unsaturated FA were observed for root samples compared to the soil compartments. The contribution of unsaturated FA to total FA in roots was highest in the depth interval between 30 cm and 75 cm (63 ± 1.5 % and 65 %, respectively) and lowest in the topsoil (52 ± 4%).

2.2.3.3 Separation of drilosphere, rhizosphere and bulk soil organic matter using linear discriminant analysis

Discriminant function analysis was conducted to test if bulk soil, rhizosphere and drilosphere OM differ significantly on a linear combination of the measured relative di- and unsaturated FA contents (Figure 2.2-5). To separate between the 3 soil compartments, 2 linear discriminant functions were calculated. For every function an Eigenvalue was calculated that represents a relative measure on the separating power of a discriminant function (Table 2.2-2). It represents the ratio of the discriminant score variance between the soil compartments to the discriminant score variance within the soil compartments. The Eigenvalue for function 1 (11.8) was higher compared to function 2 (8.0) indicating that function 1 was more effective in separating between the soil compartments compared to function 2 (Table 2.2-2). 68.6 % of

Table 2.2-2: Eigenvalue of each linear discriminant function and the percentage of between-group variance explained by the first and second linear discriminant function

Function	Eigenvalue	Explained between-group variance (%)
1	11.8	68.6
2	8.0	31.4

Table 2.2-3: Wilks' Lambda and Chi square significance test for all linear discriminant functions and for the linear discriminant function 2. Degrees of freedom (df) and significance level (p) are shown.

Function	Wilks' Lambda	Chi-square	d.f.	p value
1 and 2	0.019	83.78	38	0.0003
2	0.190	34.83	18	0.01

the total variance was explained by function 1 compared to 31.4 % that confirmed the higher importance of function 1 in separating between the soil compartments (Table 2.2-2). However, function 2 improved the discrimination between bulk soil and the other two soil compartments (Figure 2.2-5). Wilks' Lambda is used to determine if the mean discriminant scores differ between the soil compartments, as it is defined as the ratio of variance within the soil compartments to the total variance. The lower Wilk's Lambda the higher are the differences between the mean discriminant scores for bulk soil, rhizosphere and drilosphere OM. Wilks' Lambda was low, if both functions were considered and if only function 2 was considered (Table 2.2-3). The corresponding overall Chi-square test was significant. This indicates that the mean discriminant scores that result, if both linear discriminant functions are considered, differ between the soil compartments (Table 2.2-3). As the Chi-square test for the second function was significant too, both functions have to be used to separate between the soil compartments (Table 2.2-3). The mean discriminant scores of function 1 differed significantly between all soil compartments (Table 2.2-4 supplementary material). The mean scores obtained by function 2 significantly differed between bulk soil and the other 2 soil compartments. Therefore, the hypothesis that a linear combination of relative di- and unsaturated fatty acids content can separate bulk soil, rhizosphere and drilosphere OM was confirmed.

For the linear discriminant analysis, relative di- and unsaturated FA measured in every depth of the soil profile were used. The discriminant scores of the two linear discriminant functions were calculated for every depth interval indicating that the linear combinations of the dicarboxylic and unsaturated FA were depth independent (Figure 2.2-6).

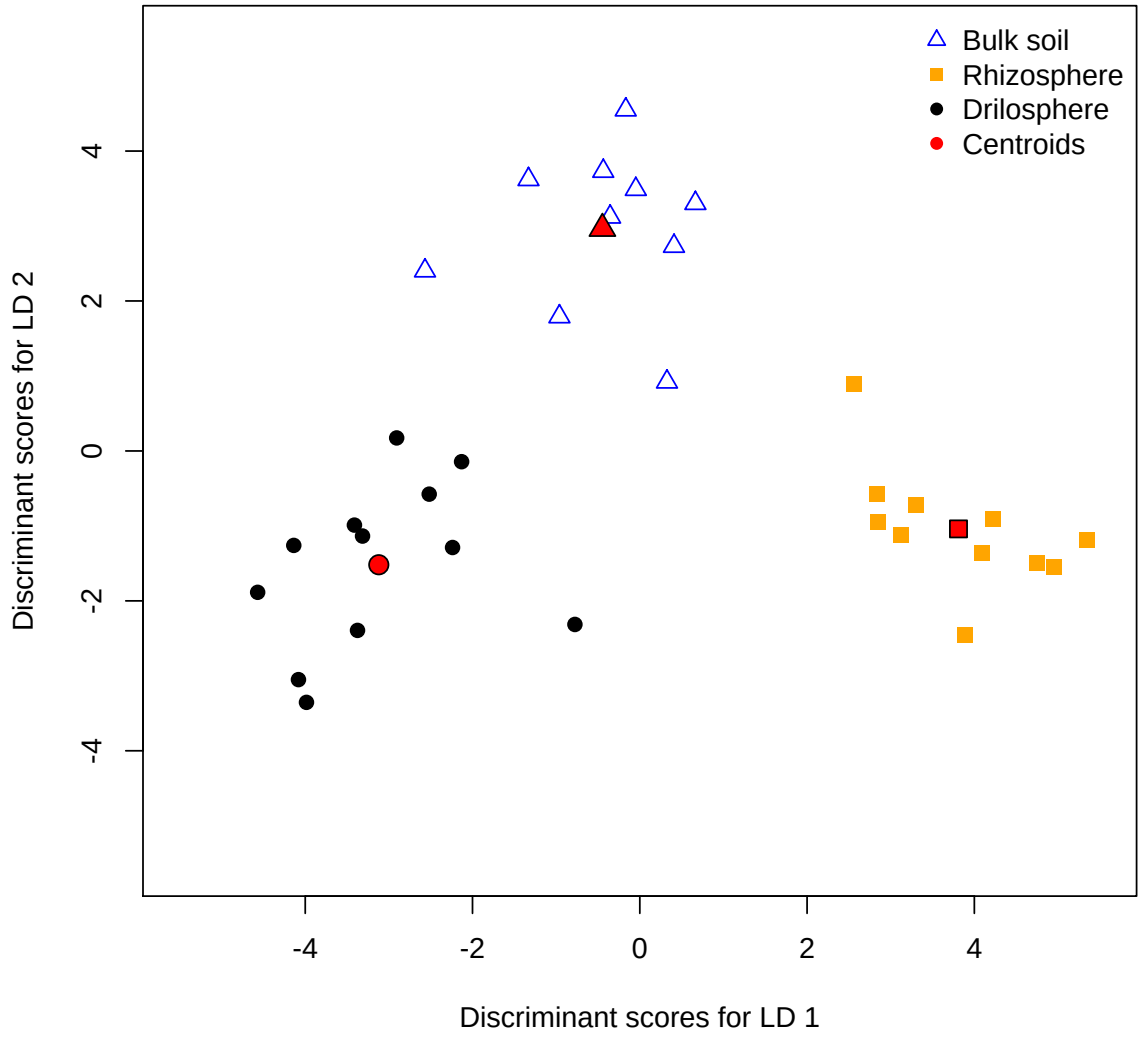

Figure 2.2-5: Scatterplot of the discriminant scores for the soil compartments obtained by the linear discriminant function 1 (LD1) and 2 (LD2). The mean values of the discriminant scores for every soil compartment (Centroids) are shown.

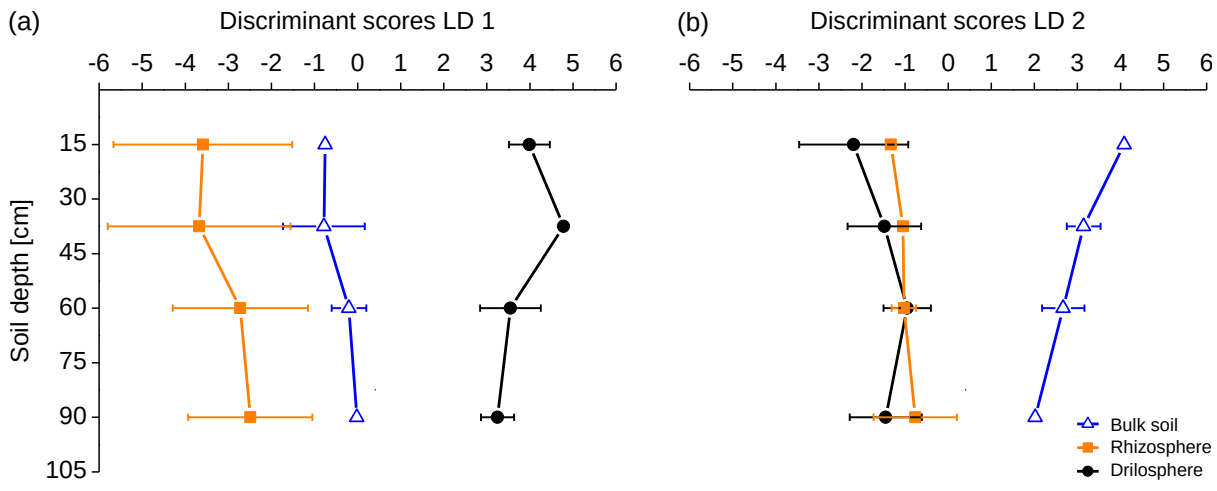

Figure 2.2-6: Discriminant scores (± SEM) for bulk soil, rhizosphere and drilosphere obtained by the linear discriminant function 1 (a) and 2 (b) for the soil depth intervals.

2.2.4 Discussion

2.2.4.1 Differences of organic carbon and total lipid extract contents in drilosphere, rhizosphere and bulk soil

The C_{org} and TLE content of bulk soil determined for the site Klein-Altendorf (Figure 2.2-2) were in the range of agricultural soils of temperate climate (Wiesenberg et al., 2006) and confirmed previously observed C_{org} values for that site (Gaiser et al., 2012). The decrease of C_{org} and TLE contents with increasing soil depth is common and was also described before (Wiesenberg et al., 2006).

The C_{org} and TLE contents changes with increasing soil depth was significantly higher in the rhizosphere and even higher in drilosphere compared to bulk soil. This can be attributed to an input of organic matter at the pore wall with a higher spatial variability due to root- and earthworm-derived organic matter. The enrichment of the drilosphere in C_{org} by 20% compared to bulk soil indicated the input of earthworm-derived OM into the burrow. The enrichment in OM in the rhizosphere originates from the input of root litter, rhizodeposits and their stimulation of microbial growth (Jones 2009). Consequently, the enrichment of C_{org} in the drilosphere and rhizosphere compared to bulk soil is related to the incorporation of organic matter released by the producer of the biopore. This is confirmed for TLE contents by the very high TLE contents observed for roots in the current study. Thus, even if no macroscopic root remains were observed, root- and rhizodeposit-derived lipids must have contributed to the enriched Corg and TLE contents in rhizosphere samples compared to bulk soil. Further, microorganisms feeding on this freshly incorporated organic matter in pore walls likely contribute to this enrichment in bulk organic matter and also likely to higher TLE contents (Uksa et al., 2014). An enrichment of C_{org} in earthworm casts has been previously reported (Marhan et al., 2007). However, as the material from the pore wall has not processed the earthworm gut like the casts, it remains unclear whether the stabilization of organic matter is similar in casts and the drilosphere. Our results point at least to a short-term enrichment of organic matter in the pore wall and potentially also stabilization in the long-term, which needs to be investigated in further studies.

Despite significant differences in C_{org} contents between drilosphere, rhizosphere and bulk soil, C_{org} content is not a diagnostic criteria to reliably distinguish between drilosphere, rhizosphere and bulk soil OM, due to the high spatial variability of C_{org} contents between field replicates

and integrating among various compound classes (Marschner et al., 2008). Similar to C_{org}, also the TLE contents could not be used for a clear differentiation between pores of different origin, although they were slightly higher in drilosphere and rhizosphere compared to bulk soil OM. As soil lipids can derive from several sources like microorganisms, plants, fungi, insects and others, a low specificity of the TLE contents could be expected. Thus both, C_{org} and TLE contents indicated an increased incorporation of OM into rhizosphere and drilosphere compared to bulk soil. A higher specificity can be expected from molecular proxies, which specifically point to the characteristic chemical composition of their organic sources.

2.2.4.2 Source assessment of biopores via molecular proxies

ACL and CPI values have been frequently applied in biogeochemical studies for source apportionment and degradation of organic matter. The ACL values are related to the specific sources of organic matter differentiated by chain length and together with the CPI values can give insight into the degradation of organic matter.

The high ACL values (> 18.5) down to 45 cm indicated the contribution of fresh aboveground biomass to FA as aboveground plant tissues are typically enriched in long-chain FA (> 19 carbons) (Harwood and Russell, 1984) due to the transport and formation of long-chain fatty acids in plant cuticles (Post-Beittenmiller, 1996). In contrast to these, the investigated alfalfa roots were always <18 and thus characterized by the lowest ACL values in the sample set. These low values compared to aboveground biomass are typical for roots (Wiesenberg et al., 2012) as the lipid biosynthesis is different in roots and leaves, presumably leading to lower amounts of elongated FA in roots compared to leaves. As roots and bulk soil showed identical values below 45 cm depth, the source of FA in bulk soil in this depth can be attributed mainly to root origin. Further, microorganisms like bacteria and fungi could be other sources of FA than only roots at the large depth as they are also characterized by ACL values around 17. Rhizosphere ACL values did not differ significantly from bulk soil, and did not show considerable influence of roots compared to bulk soil. Thus, the microorganism related overprint in the rhizosphere, leading to selective degradation of short chain FA and thus selective enrichment of longer chain homologues might have caused these unexpected results. However, as the results are not significantly different, these tendencies should not be overinterpreted. The ACL values of drilosphere samples revealed the lowest changes with soil depth compared to other soil samples, which likely indicated the translocation of OM from

topsoil towards greater depth within the biopores by earthworms, which is already a well known feature of vertical earthworm burrows (Lee, 1985).

The CPI values of bulk soil samples ranged between 4 and 5, which is common in soil samples (Matsumoto et al., 2007; Wiesenberg et al., 2012). As all root, drilosphere and rhizosphere samples also had CPI values between 3 and 6, and thus did not differ significantly from bulk soil, no conclusion could be made related to specific origin of FA in the different samples. Hence, these established molecular proxies have little potential to allow for a differentiation between different samples, which can be related to the ubiquitous microorganisms that prevail in bulk soil, drilosphere and rhizosphere.

2.2.4.3 Source assessment of biopores via the portions of dicarboxylic acids and unsaturated FA

Especially dicarboxylic acids and unsaturated FA have been described to predominantly derive in soils from specific sources (Harwood and Russell, 1984; Kolattukudy, 1981; Mendez-Millan et al., 2012). Unsaturated FA are related to plant biomass that has not been strongly degraded, but can be also of bacterial, insect or other origin. However, as a major part of FA in soils are degradation products, they are less specific in terms of location of side chains and double bonds within the molecule than phospholipid fatty acids, that allow tracing of living microbial and plant biomass (Frostegard and Baath, 1996). Dicarboxylic acids in soils have been determined to be mainly attributed to root-derived origin, as the suberin polymer present in roots and not in aboveground tissues contains large amounts of dicarboxylic acids (Kolattukudy, 1981).

Roots revealed a strong enrichment of unsaturated FA compared to saturated and dicarboxylic acids (Figure 2.2-4), which can be explained by the typical enrichment of mono- and poly-unsaturated FA in plant tissues as main components of cell membranes (Ohlrogge and Browse, 1995). The highest portions of unsaturated FA in bulk soil OM were determined in topsoil (0-30 cm), whereas in all other depth intervals less than 10 % unsaturated FA were determined in bulk soil OM (Figure 2.2-4 b). The relative enrichment of unsaturated FA in the topsoil compared to subsoil layers can be related to the continuous input of fresh biomass via litterfall, root biomass and microorganism-derived organic matter. In subsoil horizons, the input of fresh biomass is distributed more heterogeneously. Therefore, the degradation of bulk soil OM that affects itself the composition of FA is stronger in subsoil compared to topsoil.

As biopores are known to incorporate fresh biomass into subsoils (Kautz et al., 2013) the relative content of unsaturated FA should be higher in biopores, due to lower degradation of the freshly incorporated OM. Drilosphere and rhizosphere samples revealed similar portions of unsaturated FA like bulk soil OM in the topsoil. In subsoil horizons the portions of unsaturated FA were always higher than those of the bulk soil. However, as the relative portions of unsaturated FA remained always below 20 %, the contribution of fresh, undegraded microorganism- or plant-derived organic matter can be assumed to be relatively low in the different pore walls. This is supported by the very high values of unsaturated FA in roots (> 50%). Thus, degradation of OM in biopores leads to a significant alteration of the FA composition and not a preservation of the plant-derived FA signal in terms of unsaturated FA.

The largest portions of dicarboxylic acids were observed in bulk soil, followed by rhizosphere, and drilosphere. Surprisingly, the dicarboxylic acids were very low in root samples although they were frequently described to be biomarkers for root-derived organic matter (Mendez-Millan et al., 2010a). In living root biomass the dicarboxylic acids occur as part of the polymer suberin, but only extractable monomers were investigated in the current study and not bound lipids and their monomers. This different sample preparation, which leaves the bound lipids not extracted, can explain why the alfalfa roots do not reveal considerable amounts of dicarboxylic acids. During degradation of suberin, dicarboxylic acids can then be released as monomers into the soil. They can be assumed to be intermediate degradation products until further degradation of the functional groups, which could explain the large abundance (> 20 %) of dicarboxylic acids in bulk soil. The increase of the portion of dicarboxylic acids below 45 cm indicated stronger contribution of root-derived organic matter, which was already determined based on bound dicarboxylic acids, elsewhere (Mendez-Millan et al., 2012). It could be possible that the rhizosphere samples contained a considerable portion of bound dicarboxylic acids, which was not investigated in the current study.

The formation of drilosphere biopores by earthworms could not be proven by the chosen FA proxies. In principle, various FA proxies should enable the differentiation of biopores of known origin, whereas ultimately a significant differentiation of different sources of OM in biopores in soil was difficult. A limitation could arise, if the organic matter in biopores is strongly degraded, which makes a comparison to the bulk soil difficult. For future applications the implication of biopores of known origin and the bulk soils is always

recommended. Furthermore, the analyses of bound lipids might support the identification of root-derived biopores amongst other sources.

2.2.4.4 Separation of drilosphere, rhizosphere and bulk soil OM using linear discriminant analysis

The proportions of unsaturated and dicarboxylic FA differed between drilosphere, rhizosphere and bulk soil OM. Therefore, a combination of unsaturated FA and dicarboxylic acids was assumed to improve the separation of OM between these soil compartments. A linear discriminant analysis that was applied to the FA fingerprint of drilosphere, rhizosphere and bulk soil OM revealed a clear separation of the soil compartments.

The separation of bulk soil, rhizosphere and drilosphere OM by the linear combination of relative contents of unsaturated FA and dicarboxylic acids was not affected by soil depth (Figure 2.2-6). Despite differences in the relative contents of unsaturated FA increased with depth, they were lowest in bulk soil, intermediate in rhizosphere and highest in drilosphere in every soil depth (Figure 2.2-4). The same applied to the relative contents of dicarboxylic acids, but the soil compartments differed in reverse order. The lack of a depth effect enabled the utilization of the relative contents of unsaturated and dicarboxylic FA of every soil depth for the discriminant analysis. This indicated depth independency of these FA proxies for OM separation.

The back-tracing of the separation to specific processes could determine, if the combination of unsaturated and dicarboxylic FA yields appropriate molecular markers to differentiate between bulk soil, rhizosphere and drilosphere OM. The effective separation between rhizosphere and drilosphere OM by function 1 is assumed to result from the higher dicarboxylic acid content in the rhizosphere and the lower content of unsaturated FA in the rhizosphere compared to the drilosphere. In arable soil, dicarboxylic acids can trace root-derived organic matter (Mendez-Millan et al., 2010b). Dicarboxylic acids are constituents of suberin that mainly occurs in roots in herbaceous plants (Kolattukudy, 1981; Mendez-Millan et al., 2010b). Degradation of suberin releases dicarboxylic acids into soil. Therefore, higher dicarboxylic acid contents in the rhizosphere compared to the drilosphere could have been caused by the higher amount of root litter in the rhizosphere (Figure 2.2-4). In contrast to dicarboxylic acids, the relative amount of unsaturated FA was lower in the rhizosphere compared to the drilosphere (Figure 2.2-4). It was shown that the relative contribution of

unsaturated compounds to plant biomass was much higher than to soil organic matter (Mendez-Millan et al., 2010b; Nierop et al., 2003). Due to the double bond, unsaturated compounds are preferentially degraded in soil compared to saturated compounds (Mendez-Millan et al., 2010b; Nierop et al., 2003). Organic material that passed the gut of an earthworm was shown to be already partially stabilized (Marhan et al., 2007). Consequently, the higher relative amount of unsaturated FA in drilosphere was assumed to result from a higher input of fresh OM and an increased protection against microbial degradation compared to the rhizosphere. The improved separation of bulk soil OM from rhizosphere and drilosphere OM due to linear discriminant function 2 is assumed to reflect the longer degradation process that reduced the relative unsaturated FA content and increased the content of more recalcitrant dicarboxylic acids (Mendez-Millan et al., 2010b).

2.2.5 Conclusions

Currently, the identification of potential places of carbon allocation in soils is one of the major tasks in soil organic matter research. As biopores were identified as one potential pathway to sequester carbon in subsoil horizons, the source apportionment and fate of biopore carbon might be a key to improve our understanding on carbon cycling and carbon sequestration in subsoils. Thus, the aim of the current study was to determine the carbon concentrations in walls of biopores of known origin, i.e. earthworm- or root-derived to trace carbon incorporation via pore systems and another aim was to identify the potential of FA as biomarkers for source apportionment of organic matter in biopores. Carbon significantly accumulated in the biopore walls compared with bulk soil in different soil horizons with higher values for earthworm- than for root-derived pores. This highlights the potential of pore systems to contribute to carbon incorporation especially in carbon-depleted subsoils. However, further research is required to determine the long-term fate of this incorporated carbon for tracing the sequestration potential. To study pore systems in soils in the long term it is also a prerequisite to know the biogenic origin of the carbon in pore walls, which might be traced by biomarker approaches. Here, the combination of unsaturated and dicarboxylic acids enabled the separation of bulk soil, rhizosphere and drilosphere OM. We could show for the first time that the relative amounts of unsaturated FA and dicarboxylic acids differed between bulk soil, rhizosphere and drilosphere OM but did not change with depth. Therefore, separation of bulk soil, rhizosphere and drilosphere OM in other ecosystems can be possible. A universal application of the linear combination of relative contents of unsaturated FA and dicarboxylic acids to categorize OM of unknown origin into bulk soil, rhizosphere and drilosphere organic matter requests the evaluation of the discriminant model with samples from other sites and ecosystems as well as a validation using e.g. biopore OM of different origin (including mixed sources) produced under controlled conditions.

2.2.6 Acknowledgements

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2.2.7 Author contributions

YK and GW initiated the study. SH collected samples and organized laboratory analyses. Laboratory work was conducted by G.W., S.H. and student helpers. GW conducted GC measurements and lipid data evaluation. SH performed statistical analyses. GW wrote the manuscript, while SH prepared figures, wrote the manuscript parts connected to linear discriminant analysis and significantly contributed to manuscript improvement. YK provided comments on the manuscript.

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2.2.9 Supplementary material

Supplementary material for

Molecular differentiation between root- and earthworm-derived biopores in soil based on free extractable fatty acids

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Table 2.2-4: One-way analysis of variance showing the effect of soil compartment on the discriminant scores for the linear discriminant function 1 (LD 1) and the linear discriminant function 2 (LD2). TukeyHSD post-hoc test was applied to reveal significant differences between each soil compartment. Degrees of freedom (df), F-values and significance level (p) are shown for soil compartment.

factor	Discriminant scores LD 1			Discriminant scores LD 2		
	df	F	p	df	F	p
compartment	2	139.30	***	2	63.79	***
Residuals	30			30		

Tukey HSD	diff	p	Diff	p
BU - DS	-2.67	***	-4.49	***
BU - RS	4.26	***	-4.01	***
RS - DS	6.93	***	0.48	

*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

Study 3

2.3 Spatial distribution and turnover of root-derived carbon in alfalfa rhizosphere depending on top- and subsoil properties and mycorrhization

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Abstract

Aims: This study analyzed the extent to which root exudates diffuse from the root surface towards the soil depending on topsoil and subsoil properties and the effect of arbuscular mycorrhizal fungal hyphae on root-derived C distribution in the rhizosphere.

Methods: Alfalfa was grown in three-compartment pots. Nylon gauze prevented either roots alone or roots and arbuscular mycorrhizal fungal hyphae from penetrating into the rhizosphere compartments. $^{14}CO_2$ pulse labeling enabled the measurement of ^{14}C-labeled exudates in dissolved (DOC) and total organic carbon (TOC) in the rhizosphere, distributed either by diffusion alone or by diffusion, root hair and hyphal transport.

Results: Root exudation and microbial decomposition of exudates was higher in the rhizosphere with topsoil compared to subsoil properties. Exudates extended over 28 mm (DOC) and 20 mm (TOC). Different soil properties and mycorrhization, likely caused by the low arbuscular mycorrhizal colonization of roots (13 ± 4% (topsoil properties) and 18 ± 5% (subsoil properties)), had no effect.

Conclusions: Higher microbial decomposition compensated for higher root exudation into the rhizosphere with topsoil properties, which resulted in equal exudate extent when compared to the rhizosphere with subsoil properties. Higher ^{14}C activity used for labeling compared with previous studies enabled the detection of low exudate concentrations at longer distances from the root surface.

Keywords: Plant-soil-microorganism interactions; $^{14}CO_2$ pulse labeling; C partitioning; subsoil; topsoil; *Medicago sativa*

2.3.1 Introduction

Soils are characterized by complex physical, chemical and biological properties. Various resources such as nutrients or water are heterogeneously distributed in soil, resulting in hot spots, i.e. in microsites with increased resource availability (Beare et al. 1995). Higher microbial abundance and activity in these hot spots compared to bulk soil increase organic matter turnover and nutrient mineralization (Cheng 2009; Kuzyakov 2010). One of these hot spots in soil is the rhizosphere, which was first mentioned by Hiltner in 1904, and is defined as the soil volume surrounding the root that is affected by root activity (Darrah 1993).

The soil volume affected by plant roots, i.e. the extent of the rhizosphere, depends on the processes and parameters considered (Gregory 2006). Most previous studies have determined the extent of various parameters in the rhizosphere of topsoil A_p or A_h horizon (WRB IUSS-ISRIC-FAO 2006; Kuzyakov et al. 2003; Schenck zu Schweinsberg-Mickan et al. 2012). However, the spatial distribution of e.g. rhizodeposits and therefore the soil volume affected by rhizodeposition can be assumed to change with increasing soil depth, because of changing pedological, environmental, physicochemical (Salomé et al. 2010) and biological features.

Radial gradients that develop in the vicinity of the root can be used to describe the extent of the rhizosphere (Uren 2007). The size of the gradients ranges from less than one millimeter for microbial populations up to tens of millimeters for volatile compounds (Gregory 2006). Gradients develop due to nutrient uptake, which causes their depletion from bulk soil to root surface (Jungk 2002) or due to the release of rhizodeposits from the root into the soil (Sauer et al. 2006). Rhizodeposits comprise a wide range of organic compounds (Rovira 1956), which can be divided into water-soluble exudates such as e.g. sugars, amino acids and low molecular organic acids and water-insoluble components such as decaying fine-roots, root hairs, cell walls, sloughed cells and mucilage (Wichern et al. 2008). Root exudates have been reported to diffuse up to 12 mm from the root surface (Sauer et al. 2006), indicating that a relatively large soil volume is affected. Root exudates are released from the root into the soil due to: 1) passive diffusion, which is mainly controlled by concentration differences of individual solutes in the cytoplasm and in the soil and the permeability of cell membranes of the solute; and 2) possible additional active release due to the opening of membrane pores (Jones et al. 2004). In turn, this affects the distribution of the respective compound from the root surface into bulk soil. Once exuded into the soil, the spatial distribution of the solute depends on the diffusivity of the solute in water (Watt et al. 2006), the soil water content (Olesen et al. 2000;

Watt et al. 2006), re-uptake by roots (Jones and Darrah 1993), uptake by microorganisms (Hill et al. 2008; Fischer et al. 2010) and adsorption to the soil matrix (Jones and Edwards 1998).

The properties that affect the distribution of root exudates from the root surface to bulk soil change with increasing soil depth. C, N, C/N ratios (Salomé et al. 2010) and nutrient availability decrease with soil depth (Jobbagy and Jackson 2001). As an increase in the exudation of organic compounds by roots was observed under low N or P supply (Paterson and Sim 1999; Neumann and Römheld 1999), the same can be expected in subsoil. Due to a decrease in microbial biomass with soil depth (Fierer et al. 2003), the decomposition of the released root exudates will be slower. Changes in soil texture and the amount and distribution of soil organic matter with depth affect the sorption of root exudates to the solid phase. In turn, the distribution of root exudates in the rhizosphere is affected by differences in sorption as it reduces their bioavailability and biodegradation (Jones and Edwards 1998).

Arbuscular mycorrhizal (AM) symbioses are formed between the majorities of land plants and AM fungi (Smith and Smith 2011). The symbiosis is based on the exchange of C and nutrients between the host plant and the AM fungi (Smith and Smith 2011). AM symbioses affect rhizosphere extent by C translocation from the host plant to the AM fungi and exudation by external AM fungal hyphae that extend the rhizosphere into the mycorrhizosphere (Jones et al. 2004). The extent of the mycorrhizosphere depends on the spatial distribution of the AM fungal hyphae. Hyphal density and the spread of external hyphae from the root surface into soil differ between AM fungi and depend on the time of existing symbiosis (Jakobsen et al. 1992). Moreover, root exudation is changed in quality and quantity, as the colonization of roots by AM fungi changes the permeability of root membranes (Ratnayaker et al. 1978; Mada and Bagyaraj 1993).

Generally, soil composition, moisture, temperature, pH, nutrient availability and anthropogenic stressors affect the formation and function of AM symbiosis (Entry et al. 2002). With increasing soil depth, the mentioned soil conditions change, spore abundance decreases and AM fungi species composition changes (Oehl et al. 2005).

We hypothesize that: 1) root exudation into the soil with subsoil properties is higher per unit of root mass due to lower nutrient content compared to the soil with topsoil properties; 2) the diffusion distance of root exudates is longer in the soil with subsoil properties, because of lower microbial biomass content and consequently lower decomposition of root exudates; 3)

mycorrhization further extends the rhizosphere due to the transport and exudation of assimilated C by AM fungal hyphae; 4) the effect of mycorrhization on rhizosphere extent differs between the soil with top- and subsoil properties, because soil properties affect the formation and function of AM symbiosis.

To test these hypotheses, alfalfa was grown in three-compartment pots filled with either homogenized top- or subsoil (Figure 2.3-1). Two months old plants were pulse labeled with $^{14}CO_2$ to distinguish root-derived C (^{14}C) in dissolved (DOC) and total organic carbon (TOC) from all other C sources. Due to the installation of nylon gauze root exudates were distributed in the rhizosphere either by diffusion alone (1 µm gauze) or by diffusion, root hair and hyphal transport (30 µm gauze). To identify AM symbiosis, the colonization of roots by AM fungi was measured.

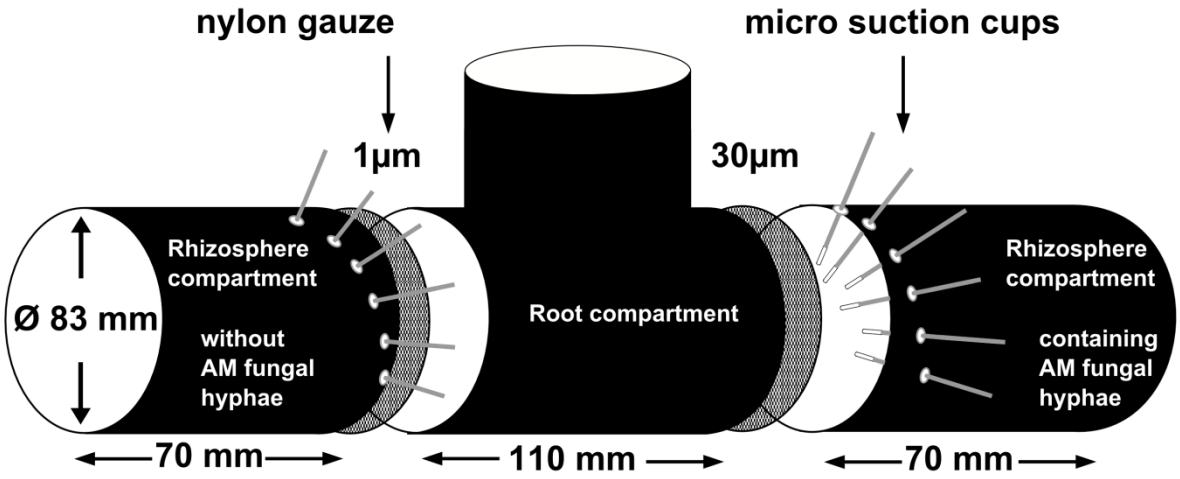

Figure 2.3-1: T-shaped three-compartment pots, containing a central root compartment and two side compartments that represent the rhizosphere of alfalfa. The rhizosphere compartments are separated from the root compartment by nylon gauze to prevent either roots alone or roots and arbuscular mycorrhizal (AM) fungal hyphae from penetrating into the rhizosphere compartments. One rhizosphere compartment was separated by 30 µm gauze from the root compartment to allow AM fungal hyphae and root hairs to penetrate into the rhizosphere compartment. The other rhizosphere compartment was separated from the root compartment by nylon gauze with a mesh size of 1 µm in order to exclude AM fungal hyphae, roots and root hairs. Micro suction cups were installed at a distance of 3, 6, 9, 13, 19 and 28 mm from the nylon gauze, i.e. the root surface.

2.3.2 Material and Methods

2.3.2.1 Experimental setup

Alfalfa (*Medicago sativa*) was grown in three-compartment pots (Sauer et al. 2006) (Figure 2.3-1). The pots consisted of T-shaped tubes with an inner diameter of 83 mm. The three-compartment pots were filled with either homogenized topsoil (sampled from 0-30 cm, A_p horizon) or subsoil (45-75 cm, B_t horizon) of a Haplic Luvisol (WRB IUSS-ISRIC-FAO 2006) collected from the field trial of the DFG research group 1320 in Klein Altendorf (06° 59‘ 29‘‘ E, 50°37‘21‘‘ N) (Gaiser et al. 2012) (Table 2.3-1). Both topsoil and subsoil samples were taken from three replicate plots of alfalfa cultivated in the second year. Replicate samples were combined and sieved to 2 mm for homogenization prior to setting up the three-

Table 2.3-1: Properties of the homogenized soil sampled from top- (A_p) and subsoil (B_t) horizon of a Haplic Luvisol, including soil organic carbon (SOC) content, C/N ratio and texture, before the cultivation of alfalfa. Shoot and root dry weight (DW) of alfalfa grown in top- or subsoil pots two months after sowing are presented. Comparison of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) content and C/N ratio of the soil solution and average pH between the rhizosphere with top- or subsoil properties two months after sowing [a].

		Topsoil			Subsoil		
Soil properties							
SOC	(g kg^{-1})	9.5	± 0.1	a*	5.1	± 0.1	b
C/N		8.8	± 0.1	a	7.7	± 0.1	b
	Sand (%)	16			12		
Texture [b]	Silt (%)	67			59		
	Clay (%)	17			29		
Plant biomass two months after sowing							
Shoot	(g DW pot^{-1})	18.1	± 0.8	a	18.7	± 0.9	a
Root	(g DW pot^{-1})	4.4	± 0.5	a	2.7	± 0.2	b
Rhizosphere compartment [c]							
DOC	(mg C l^{-1})	98.1	± 2.1	a	88.2	± 1.6	b
TDN	(mg N l^{-1})	23.2	± 3.9	a	4.6	± 0.1	b
C/N		11.1	± 1.5	a	20.0	± 0.5	b
pH (H_2O)		7.5	± 0.0	a	7.7	± 0.0	a

[a] Values are given as means and standard errors of the means.

[b] Texture values originate from Uteau Puschmann et al. (*Oxygen and redox potential gradients in the rhizosphere of alfalfa grown on a loamy soil.* Under Review).

[c] DOC, TDN, C/N ratio and pH values are means of the rhizosphere compartments with top- or subsoil properties.

* Different letters indicate significant differences between top- and subsoil properties (Mann-Whitney U test; $p < 0.05$).

compartment experiment.

The side rhizosphere compartments were filled by adding the required amount of soil needed to adjust a bulk density of 1.2 g cm^{-3}. To avoid heterogeneities related to filling, soil was pressed into the rhizosphere compartment by applying pressure once from one side. After filling, the nylon gauze was first fixed to the opposite side of the rhizosphere compartment using an elastic band and then the rhizosphere compartments were connected to the root compartment. The three-compartments were put into a vise, which was built specifically for the pots, to fix the connection. The soil was filled into the root compartment after fixing both rhizosphere compartments to ensure connectivity. A total of 0.5 g of alfalfa seeds per pot was sown directly into the soil of the root compartment.

The plant roots grew in the root compartment, but could not penetrate into the rhizosphere compartments due to nylon gauze (Kuchenbuch and Jungk 1982). Within one pot, two mesh sizes were used. As root hairs are approximately 10 µm in diameter (Gahoonia et al. 1997; Grierson and Schiefelbein 2002) and the diameter of AM fungal hyphae ranges from 2-20 µm (Smith and Smith 2011), one side was covered with 30 µm gauze to allow AM fungal hyphae and root hairs to penetrate into the rhizosphere compartment. However, roots were not able to penetrate this gauze. The second rhizosphere compartment was divided from the root compartment by 1 µm nylon gauze to exclude AM fungal hyphae and root hairs.

The experiment was conducted under controlled conditions. Water content was daily adjusted to 80% of the water holding capacity and was checked gravimetrically. Plants were watered from the top of the root compartment. The photoperiod was 14 hours light to 10 hours dark. Light intensity was 300 µmol $m^{-2}s^{-1}$ and the room temperature was 23°C during light and 20°C during dark periods.

To gain insight into changes in C partitioning over time, pots were harvested destructively one, three and six days after ^{14}C labeling (see below). In total, the experiment consisted of 24 experimental units (homogenized soil sampled from A_h or B_t horizon, three sampling dates and four replicates, each).

2.3.2.2 $^{14}CO_2$ pulse labeling

Two months after sowing, plants were pulse-labeled with $^{14}CO_2$ to trace root exudates in the rhizosphere compartments. The pulse-labeling procedure was previously described by e.g. Cheng et al. (1993) and Gocke et al. (2011). For the ^{14}C pulse labeling, four pots were placed

into a sealed transparent acrylic glass chamber (length x width x height; 0.5 x 0.5 x 0.7 m). Two replicate pots containing homogenized soil from A_h horizon and two replicate pots containing homogenized soil from B_t horizon of the same sampling date were labeled simultaneously. The $^{14}CO_2$ pulse was applied by adding 5 M sulfuric acid (H_2SO_4) to the labeling solution containing $Na_2{}^{14}CO_3$ diluted in 10 ml of deionized water. Evolving $^{14}CO_2$ was pumped into the labeling chamber and a 12 V fan enabled the uniform distribution of the labeled CO_2. The added ^{14}C activity of the labeling solution was adjusted to 1.85 MBq per three-compartment pot. Before the $^{14}CO_2$ pulse, the chamber was closed and plants assimilated the label for three hours. The unassimilated $^{14}CO_2$ in the chamber air prior to opening was trapped in 15 ml of 1 M sodium hydroxide solution (NaOH).

2.3.2.3 Soil solution sampling

Soil solution was sampled in the rhizosphere compartments at various distances to the gauze, i.e. to the root surface, to detect root exudates in the DOC pool ($DO^{14}C$) (Figure 2.3-1). For this purpose, micro suction cups (PI Ceramic, Lederhose, Germany) (Göttlein et al. 1996) were installed at distances of 3, 6, 9, 13, 19 and 28 mm from the root surface two weeks before labeling to minimize disturbance. After the ^{14}C labeling, the micro suction cups were directly connected to a vacuum collection device using polytetrafluoroethylene (PTFE) tubes (Göttlein et al. 1996). The micro suction cups were set to a suction of 400 hPa for 3.5 h and soil solution was collected once in 2 ml reaction vials.

2.3.2.4 Destructive sampling of the three-compartment pots

After soil solution sampling, the three-compartment pots were carefully opened. Shoots were cut at the soil surface. Roots of the root compartment were removed and put into a beaker containing deionized water to separate soil from roots. To improve separation, the beaker was put into an ultrasonic bath for five minutes. After removing the roots, the soil of the root compartment, roots and shoots were dried at 60°C, weighed and ball milled (ball mill, Retsch MM2).

The rhizosphere compartments were cut at room temperature into slices at distances of 2, 4, 6, 8, 10, 12, 14, 16, and 20 mm from the previous root surface (i.e. the nylon gauze) using a microtome. Each obtained soil slice was mixed for homogenization, dried at 60°C and milled for the measurement of ^{14}C activity in total organic carbon (TOC).

2.3.2.5 ^{14}C Analysis

^{14}C activity was measured in soil solution from micro suction cups, in TOC of every soil slice as well as in shoots, roots and TOC of the root compartment by Liquid Scintillation Counting (LS 6500 Multi-154 Purpose Scintillation Counter, 217 Beckman, USA). Before measuring, milled plant biomass and soil samples were combusted at 600°C and evolving CO_2 was trapped in 10 ml of 1M NaOH. An aliquot of 2 ml was transferred to scintillation vials and mixed with 4 ml of the scintillation cocktail (Rothiszint eco plus, Carl Roth GmbH & Co. KG, Germany). Samples were measured 24 hours after mixing with the scintillation cocktail, enabling the decay of chemoluminescence. Soil solution samples were mixed with the scintillation cocktail at a ratio of 1:5 and measured directly after collection.

2.3.2.6 Colonization of roots by arbuscular mycorrhizal fungi

In order to identify the colonization of roots by AM fungi, AM fungi structures in root tissue were first stained using ink and vinegar (Vierheilig et al. 1998). Second, the proportion of root length containing arbuscles, the arbuscular colonization, was determined (McGonigle et al. 1990).

2.3.2.7 Calculation of ^{14}C partitioning

Measured ^{14}C activity of the samples was multiplied by the correspondent pool size (shoot and root dry weight, soil dry weight of the root compartment or of the considered soil slice of the rhizosphere compartment). The resulting ^{14}C activity of the C pools is presented as percentage of the total ^{14}C activity (the sum of ^{14}C activity in plant biomass, soil and DOC) in the three-compartment pots harvested after one day. For the pots harvested after one day, total ^{14}C activity was calculated for each pot.

Due to the destructive sampling, the ^{14}C activity in a C pool of the pots harvested three or six days after labeling was normalized to the average total ^{14}C activity of the replicate topsoil and subsoil pots after one day.

The recovered ^{14}C in TOC of the soil slices (% of recovered ^{14}C) was related to a 1 mm distance as the thickness of the soil slices varied in the experimental setup. The recovered ^{14}C in DOC was related to 1 mL of soil solution and a 1 mm distance, because the soil solution volume obtained and the distance between the suction cups was different. The volume for

each suction cup was defined as the distance between the middle to the previous adjacent and to the next adjacent suction cups.

2.3.2.8 Spatial distribution of root-derived C in the rhizosphere

The spatial distribution of root-derived C in the rhizosphere compartments in DOC and TOC was described on the basis of ^{14}C recovery in DOC (% of recovered ^{14}C ml^{-1} mm^{-1}) and TOC (% of recovered ^{14}C mm^{-1}). An exponential decay function (one phase decay) or linear regression using the least squares fitting method was used to obtain the best fitting result for ^{14}C in DOC and TOC. Functions were fitted to the means of ^{14}C recovery in TOC of associated soil slices and in DOC of associated micro suction cups of the four replicate rhizosphere compartments.

The distance from the root surface ($x_{1/2}$), at which the ^{14}C recovery in DOC and TOC was half that of the initial ^{14}C recovery at x = 0 cm, was calculated using the obtained rate constants (k) after the exponential fitting:

$$x_{\frac{1}{2}} = \frac{ln\ 2}{k} \quad (1)$$

2.3.2.9 Dynamics of root-derived C in DOC and TOC of the rhizosphere

Changes in ^{14}C recovery over the six days lasting chase period in DOC and TOC within the whole rhizosphere compartments were used to describe the dynamics of C exuded and translocated to the AM fungi. The functions fitted to the distribution of $DO^{14}C$ and $TO^{14}C$ in the rhizosphere compartments were integrated. Limits of integration were 0 to 28 mm for $DO^{14}C$ and 0 to 20 mm for $TO^{14}C$. The integrals, R, (the areas under the curves) obtained one, three, and six days after labeling were compared between the rhizosphere compartments filled with either homogenized top- or subsoil. For comparison, the integrals determined one or three days after labeling were set to 100%, to calculate the relative changes of the integral between the time steps.

The decrease –R (% d^{-1}) or increase rates +R (% d^{-1}) of the integral were calculated for the periods between one and three and one and six or between three and six days after labeling. Changes between the time steps were assumed to decrease or increase exponentially:

$$A(t) = A(0) \cdot e^{kt} \quad (2)$$

$$R = (e^{k} - 1) \cdot 100 \quad (3)$$

where, A (t) (%) is the percentage of the integral after three or six days t (d) related to the integrals after one or three days A (0) after labeling, respectively. k is the rate constant of the exponential decrease (-k) or increase (+k) between the individual time steps.

2.3.2.10 Statistics

To determine if the fitted parameters of the exponential model, the rate constant (k) and the y-intercept (Y_0) differ between the rhizosphere with top- or subsoil properties as well as between with and without AM fungi, the extra-sum-of-squares F test ($p < 0.05$) was used. The non-parametric Mann–Whitney U test was applied to reveal significant differences of 1) soil organic carbon (SOC) content and C/N ratio ($n = 5$, $p < 0.05$) between the top- and subsoil used for the experiment before the cultivation of alfalfa; 2) root ($n = 7$, $p < 0.05$) and shoot biomass ($n = 12$, $p < 0.05$) between the topsoil and subsoil pots after two months of growth; 3) DOC and total dissolved nitrogen (TDN) content and C/N ratio ($n = 40$, $p < 0.05$) of the soil solution of the rhizosphere compartments; and 4) ^{14}C allocation in shoots, roots, soil of the root compartment and the rhizosphere compartments with 1 µm and 30 µm gauze ($n = 4$, $p < 0.05$) between topsoil and subsoil pots at one, three and six days after labeling and between the time steps. Means and standard errors of the means are presented in the figures and tables. Statistical analyses of significant differences were carried out using STATISTICA for Windows (version 10.0; StatSoft Inc., Tulsa, OK, USA). Fitting of exponential decay functions and comparison of fits as well as the integration of the functions were carried out using Graph Pad Prism (version 6; GraphPad Software, Inc., La Jolla, CA, USA).

2.3.3 Results

2.3.3.1 Bulk elemental analyses

Before the experimental cultivation of alfalfa, SOC content and C/N ratio of the homogenized soil sampled from the A_p horizon were significantly higher compared to the homogenized soil sampled from the B_t horizon of a Haplic Luvisol (WRB IUSS-ISRIC-FAO 2006) (Table 2.3-1). The textural differences were mainly expressed by the clay content that was almost twice as high in the B_t compared to the A_p horizon (Table 2.3-1).

After two months of alfalfa growth under controlled conditions, all nylon gauzes were completely covered by roots. The average shoot biomass per pot did not differ between the topsoil and subsoil pots after two months (Table 2.3-1). However, root biomass was significantly lower in the subsoil compared to the topsoil pots. The average DOC and TDN contents were significantly higher in the soil solution of the rhizosphere with topsoil properties (TP) compared to the rhizosphere with subsoil properties (SP). However, the average C/N ratio of dissolved organic matter in the TP rhizosphere was much lower compared to the SP rhizosphere (Table 2.3-1). The average pH in the rhizosphere compartments did not differ between the top- and subsoil pots.

2.3.3.2 ^{14}C partitioning

The average total ^{14}C activity per pot was 1.4 ± 0.1 MBq in topsoil pots and 1.3 ± 0.2 MBq in subsoil pots one day after labeling. Comparison of ^{14}C recovery in C pools of the root compartment (shoots, roots and TOC) did not reveal any significant differences between the top- and subsoil pots one, three or six days after labeling (Table 2.3-2). Likewise, total ^{14}C recovery in TOC did not differ significantly for any sampling dates between the TP and SP rhizosphere (Table 2.3-2).

After six days, ^{14}C recovery in shoots in top- and subsoil pots was higher compared to all other C pools. In root biomass, only 2.5 ± 0.7 and 2.7 ± 0.2% of ^{14}C were recovered after six days in top- and subsoil pots, respectively. In TOC of the root compartment, 16.3 ± 0.6% of

Table 2.3-2: Partitioning of assimilated ^{14}C between shoots, roots and total organic carbon (TOC) in the root compartment and TOC and dissolved organic carbon (DOC) in the rhizosphere compartments one, three and six days after $^{14}CO_2$ pulse labeling of alfalfa plants [a].

	Pool	Days after labeling	Topsoil pot		Subsoil pot	
			^{14}C recovery (% of recovered ^{14}C)			
Root compartment	Shoot	1	80.6 ± 3.2	a[*]	86.0 ± 1.0	a
		3	49.8 ± 8.1	b	49.1 ± 4.5	b
		6	53.1 ± 9.9	ab	46.7 ± 6.7	b
	Root	1	8.6 ± 2.7	a	3.6 ± 1.2	a
		3	6.2 ± 0.9	a	2.6 ± 0.5	a
		6	2.5 ± 0.7	a	2.7 ± 0.2	a
	TOC	1	10.7 ± 0.9	a	10.3 ± 0.9	a
		3	15.5 ± 1.3	ab	13.6 ± 1.5	ab
		6	16.3 ± 0.6	b	15.0 ± 0.4	b
Rhizosphere - AM fungal hyphae	TOC	1	0.07 ± 0.03	a	0.05 ± 0.02	a
		3	0.06 ± 0.03	a	0.06 ± 0.03	a
		6	0.05 ± 0.02	a	0.09 ± 0.05	a
	DOC	1	0.0030		0.0005 ± 0.0001	a
		3	0.0009		0.0013 ± 0.0005	a
		6			0.0005 ± 0.0001	a
Rhizosphere + AM fungal hyphae	TOC	1	0.06 ± 0.02	a	0.04 ± 0.01	a
		3	0.03 ± 0.01	a	0.06 ± 0.01	a
		6	0.08 ± 0.05	a	0.06 ± 0.02	a
	DOC	1	0.0020		0.0007 ± 0.0001	a
		3	0.0010		0.0016 ± 0.0006	a
		6			0.0005 ± 0.0001	a

[a] Values are given as means and standard errors of the means.

[*] Significant differences between the time steps are indicated by different letters (Mann-Whitney U test, $p < 0.05$, $n = 4$). Not any significant differences were found between the topsoil and subsoil pots for any sampling date.

^{14}C was recovered in topsoil and 15.0 ± 0.4% in subsoil pots six days after labeling. ^{14}C recovery was lowest in the C pools of the rhizosphere compartments compared to all other C pools after six days (Table 2.3-2).

In shoots and roots, the ^{14}C recovery decreased during the experiment, whereas ^{14}C recovery in TOC of the root compartment increased (Table 2.3-2). No significant changes of total ^{14}C recovery in the rhizosphere compartments were determined between the first and the sixth day after labeling (Table 2.3-2).

2.3.3.3 Effect of top- and subsoil properties on spatial distribution of root exudates

The ^{14}C distribution in DOC in all rhizosphere compartments decreased exponentially with increasing distance to the root surface (Figure 2.3-2). In the SP rhizosphere, ^{14}C-labeled root exudates in DOC were lacking at a distance of 28 mm after one and six days, because ^{14}C recovery was insignificant. However, after three days, the spatial extent of ^{14}C-labeled root exudates in DOC exceeded the experimental sampling distance, because ^{14}C was recovered even at a distance of 28 mm (Figure 2.3-2). Likewise, the spatial extent of ^{14}C in DOC exceeded the experimental sampling distance of 28 mm in the TP rhizosphere one and three days after labeling. Therefore, no maximal spatial extent of root exudate C (^{14}C) in DOC was obtained. DO^{14}C gradients from the root surface to bulk soil were compared between the TP and SP rhizosphere based on fitted rate constants (k) (Figure 2.3-2). No significant differences between the TP and SP rhizosphere were observed. However, after one day DO^{14}C gradients from the root surface to bulk soil were steeper in the TP compared to SP rhizosphere, whereas these gradients were uniform for the TP and SP rhizosphere after three days (Figure 2.3-2).

^{14}C recovery in DOC at the root surface (Figure 2.3-2, y-intercept) was three times higher in the TP compared to the SP rhizosphere after one day. In contrast, after three days, the ^{14}C recovery at the root surface was higher in the SP compared to the TP rhizosphere (Figure 2.3-2).

TO^{14}C distribution in the rhizosphere commonly decreased exponentially with increasing distance to the root surface (Figure 2.3-3). Exceptions were the TO^{14}C distribution after three and six days in the TP rhizosphere with AM fungal hyphae and after six days in the TP rhizosphere with and without AM fungal hyphae. TO^{14}C distribution in the SP rhizosphere without AM fungal hyphae did not decrease exponentially one and three days after labeling; hence, a linear regression was used instead.

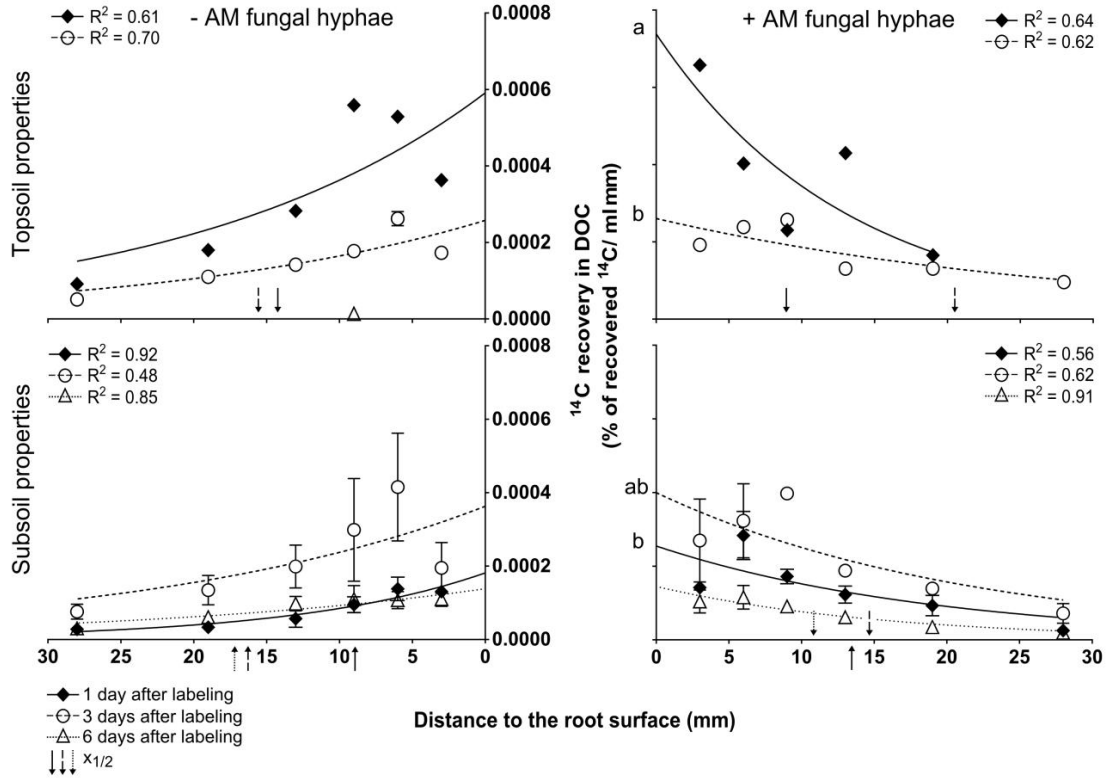

Figure 2.3-2: Spatial distribution and dynamics of exudates (^{14}C) in DOC (% of recovered ^{14}C ml^{-1} mm^{-1}) in the rhizosphere with topsoil and subsoil properties. Both the DO^{14}C distribution in the rhizosphere with (+) and without (-) arbuscular mycorrhizal (AM) fungal hyphae are shown. Arrows indicate the distance from the root surface at which the ^{14}C recovery is half that of the initial ^{14}C recovery at the distance x = 0 cm. Different letters indicate significant differences at $p < 0.05$ in ^{14}C recovery at the root surface.

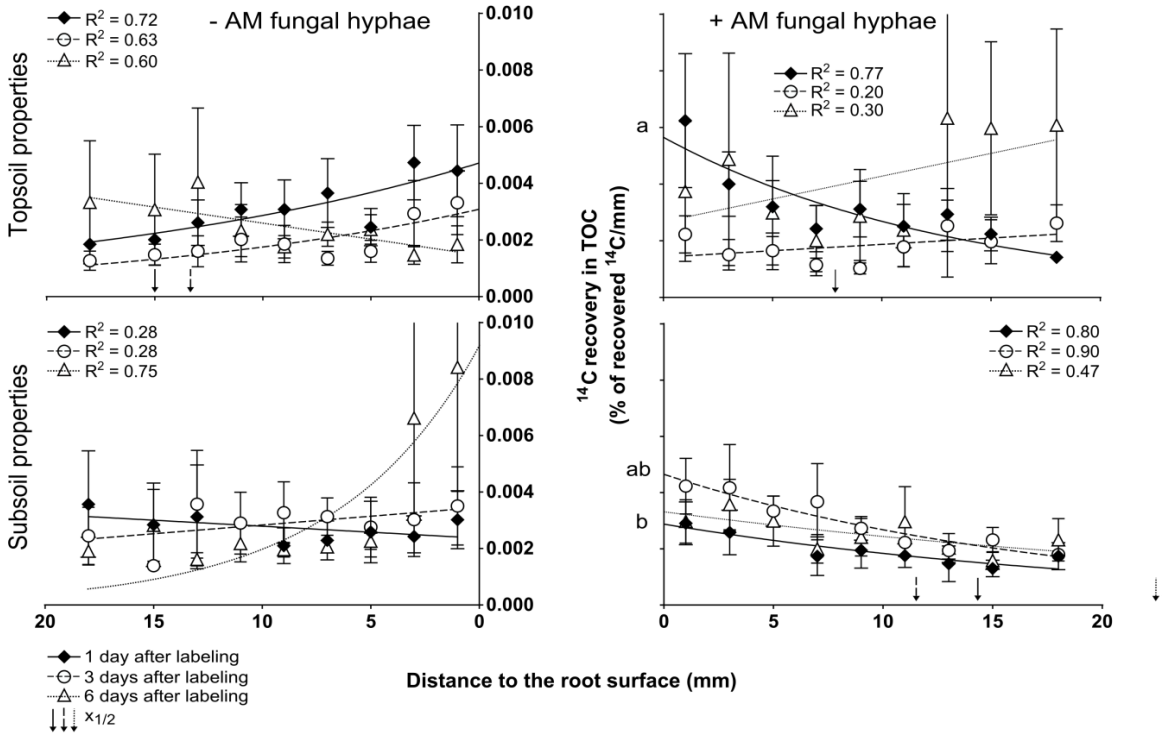

Figure 2.3-3: Spatial distribution and dynamics of root-derived C (^{14}C) in TOC (% of recovered ^{14}C mm^{-1}) in the rhizosphere with topsoil and subsoil properties. Both the TO^{14}C distribution in the rhizosphere with (+) and without (-) arbuscular mycorrhizal (AM) fungal hyphae are shown. Arrows indicate the distance from the root surface at which the ^{14}C recovery is half that of the initial ^{14}C recovery at the distance x = 0 cm. Different letters indicate significant differences at $p < 0.05$ in ^{14}C recovery at the root surface.

Within 0–20 mm distance from the root surface, ^{14}C recovery in TOC was significant for all treatments and time steps (Figure 2.3-3). Consequently, spatial extent of ^{14}C-labeled root exudates in TOC exceeded 20 mm in TP and SP rhizosphere. $TO^{14}C$ gradients (with exponential decrease) from the root surface to bulk soil did not differ significantly in rate constant (k). The fitted value of ^{14}C recovery at the root surface was three times higher in TP compared to SP rhizosphere after one day. However, the differences disappeared after three days.

2.3.3.4 Effect of mycorrhization on spatial distribution of root-derived C in the rhizosphere

Roots in all pots were colonized by AM fungi (Table 2.3-3). Arbuscular and hyphal colonization of the roots growing in subsoil were higher compared to that of the roots growing in topsoil. 18 ± 5% of root length was colonized by arbuscles in the roots growing in subsoil and 13 ± 4% in the roots growing in topsoil.

Comparing the TP rhizosphere containing AM fungal hyphae to that without AM fungal hyphae, no significant differences between ^{14}C recoveries at the root surface in DOC were obtained after one day (Figure 2.3-2). Likewise, ^{14}C recovery at the root surface in TOC did not differ between the TP rhizosphere with and without AM fungal hyphae (Figure 2.3-3). Equal results were obtained for the effect of AM on ^{14}C recovery at the root surface in SP rhizosphere. Rate constants (k) of $DO^{14}C$ and $TO^{14}C$ gradients from the root surface into bulk soil did not differ in any treatment or sampling time due to the presence of AM fungal hyphae. An exception to this general pattern was the distribution of ^{14}C in TOC in the TP rhizosphere with AM fungal hyphae, which was no longer exponential after three days (Figure 2.3-3).

2.3.3.5 Dynamics of ^{14}C in DOC and TOC of the rhizosphere

Table 2.3-3: Proportion of alfalfa root length colonization by arbuscular mycorrhizal fungi. Arbuscular, Vesicular and Hyphal colonization of roots are presented.

	Arbuscular colonization	Vesicular colonization	Hyphal colonization
Topsoil pots	0.13 ± 0.04	0.00	0.30 ±0.08
Subsoil pots	0.18 ± 0.05	0.01 ±0.004	0.50 ± 0.08

The integrals of the DO^{14}C and TO^{14}C distribution from the root surface into bulk soil were used to compare the dynamic of root-derived C between the TP and SP rhizosphere (Table 2.3-4). Total ^{14}C recovery in DOC in both TP rhizosphere compartments was approximately four times higher compared to the SP rhizosphere after one day. In contrast, after three days, total ^{14}C recovery in DOC in both SP rhizosphere compartments was higher compared to the TP rhizosphere (Table 2.3-4). From the first to third day, total ^{14}C recovery in DOC decreased in the TP rhizosphere without AM fungal hyphae by 32.5% d^{-1}, whereas an increase of 68.3% d^{-1} was observed in the SP rhizosphere without AM fungal hyphae (Table 2.3-4). The decrease and increase rates in the rhizosphere were similar with and without AM fungal hyphae. From the third to sixth day, a further decrease in total ^{14}C recovery in DOC was observed in the SP rhizosphere.

Total ^{14}C recovery in TOC was higher in both TP rhizosphere compartments compared to the SP rhizosphere with AM fungal hyphae after one day. In contrast, after three days, total ^{14}C recovery was higher in the SP compared to the TP rhizosphere (Table 2.3-4). Total ^{14}C recovery in TOC of the TP rhizosphere decreased from the first to the third day by 21.5% d^{-1} with and 20.8% d^{-1} without AM fungal hyphae. In contrast, in the SP rhizosphere with AM fungal hyphae, an increase of 21.4% d^{-1} occurred (Table 2.3-4).

Table 2.3-4: Dynamic of root-derived C in DOC and TOC in the rhizosphere of alfalfa. Percentage change of the integral and decrease or increase rate (% d^{-1}) of total ^{14}C recovery in TOC and DOC between: (1) the first and third or the first and sixth day after the labeling and; (2) the third and sixth day after the labeling, depending on rhizosphere properties are presented.

	Rhizosphere properties	Days after labeling	Integral	Percentage change of the integral		[a] De- (-) or increase (+) rate	
				(1)	(2)	(1)	(2)
		(d)	(mm · % of recovered ^{14}C mm^{-1})	(%)	(%)	(% d^{-1})	(% d^{-1})
$TO^{14}C$	Topsoil properties						
	-AM fungal hyphae	1	0.060	100.0			
		3	0.037	61.7	100.0	-21.5	
		6	0.052	86.7	140.5	-2.8	12.0
	+AM fungal hyphae	1	0.059	100.0			
		3	0.037	62.7	100.0	-20.8	
		6	0.086	145.8	232.4	7.8	32.5
	Subsoil properties						
	-AM fungal hyphae	1	0.056	100.0			
		3	0.057	101.8	100.0	0.9	
		6	0.057	101.8	100.0	0.4	0.0
	+AM fungal hyphae	1	0.038	100.0			
		3	0.056	147.4	100.0	21.4	
		6	0.049	128.9	87.5	5.2	-4.4
			(mm · % of recovered ^{14}C mm^{-1} ml^{-1})				
$DO^{14}C$	Topsoil properties						
	-AM fungal hyphae	1	0.009	100.0			
		3	0.004	45.6		-32.5	
		6					
	+AM fungal hyphae	1	0.009	100.0			
		3	0.005	55.3		-25.6	
		6					
	Subsoil properties						
	-AM fungal hyphae	1	0.002	100.0			
		3	0.006	283.3	100.0	68.3	
		6	0.002	110.8	39.1	2.1	-26.9
	+AM fungal hyphae	1	0.004	100.0			
		3	0.006	165.6	100.0	28.7	
		6	0.002	50.0	30.2	-13.0	-32.9

[a] De- and increase rate of the integrals were calculated based on the assumption of exponential de- or increase of root-derived C in the rhizosphere between the time steps.

2.3.4 Discussion

2.3.4.1 Effect of top- and subsoil properties on spatial distribution and turnover of root exudates

The extent to which root exudates diffused from the root surface of alfalfa to bulk soil did not depend on top- or subsoil properties in our study, but exceeded previously reported distances. ^{14}C-labeled root exudates were found at a distance of 28 mm (DOC) and 20 mm (TOC) from the root surface after one, three and six days in both TP and SP rhizosphere (Figure 2.3-2 and Figure 2.3-3). Therefore, the extent of root exudates into bulk soil was larger than the considered distance in the experiment, which was contrary to previous findings (Kuzyakov et al. 2003; Sauer et al. 2006; Schenck zu Schweinsberg-Mickan et al. 2012). The concentration of root exudates was found to be highest within 3 mm of the root surface (Kuzyakov et al. 2003). However, root exudates were detected in previous studies from 6 mm (Schenck zu Schweinsberg-Mickan et al. 2012), and 10 mm (Kuzyakov et al. 2003), up to a distance of 12 mm from the root surface (Sauer et al. 2006). In our experiment, ^{14}C input was 1.85 MBq per pot and therefore higher compared to the previous studies that used either 0.46 MBq (Kuzyakov et al. 2003) or between 0.6 and 1.2 MBq per pot (Sauer et al. 2006). Coinciding with higher ^{14}C input, the detection of root exudates was not limited by low concentrations at larger distances from the root surface compared to previous studies.

Fischer et al. (2010) showed that microbial uptake and subsequent decomposition outcompeted the sorption of low molecular weight organic substances. Therefore, we assume that microbial utilization mainly determined the distribution of root exudates and the differences in sorption between the TP and SP rhizosphere can be ignored. Likewise, the re-uptake of root exudates is of minor importance for their distribution under soil conditions compared to fast microbial utilization (Biernath et al. 2008).

Microbial utilization of root exudates was higher in the TP compared to the SP rhizosphere after two months of alfalfa growth. The higher amount of exudates at the root surface in the TP rhizosphere after one day indicated higher root exudation compared to the SP rhizosphere. Steeper gradients of exudates in DOC from the root surface into bulk soil revealed that higher exudation was compensated by increased microbial utilization of root exudates in the TP rhizosphere (Figure 2.3-2). The increase of root exudates in DOC and TOC in the SP rhizosphere in comparison to a decrease in the TP rhizosphere between day one and three

(Table 2.3-4) further indicated the higher microbial utilization of root exudates, which could be related to a higher microbial biomass in the TP rhizosphere (Marschner and Kalbitz 2003). Higher microbial abundance and activity could be expected in the TP rhizosphere, due to the higher SOC content in the topsoil A_p horizon compared to the subsoil B_t horizon used in the experiment (Fierer et al. 2003; Gaiser et al. 2012) (Table 2.3-1). Significantly higher activities of leucin-amino-peptidase, ß-glucosidase and ß-N-acetylglucosaminidase in the TP compared to the SP rhizosphere further indicated higher microbial activity in the TP rhizosphere (Figure 2.3-4 supplementary material). De Nobili et al. (2001) showed that the input of trace amounts of low molecular weight organic substances caused an increase in metabolic activity of the microbial biomass. This effect was sustained and even more pronounced if a more substantial substrate for microbes like cellulose was present (De Nobili et al. 2001). Consequently, increased microbial utilization of root exudates in the TP rhizosphere was caused by higher substrate availability due to higher SOC content and higher input of root exudates compared to the SP rhizosphere.

Root exudation rates of ^{14}C-labeled organic compounds are highest within hours after the $^{14}CO_2$ pulse (Rattray et al. 1995; Dilkes et al. 2004). The utilization of exudates by microorganisms is fast in the rhizosphere, as the half-life of glucose in soil solution was reported to be several minutes (Hill et al. 2008; Fischer et al. 2010) and a similar half-life was obtained for acetate and alanine at concentrations that were relevant for the rhizosphere (Fischer et al. 2010). Due to the fast input of ^{14}C-labeled root exudates into the rhizosphere and their fast microbial utilization, a decrease of root exudates in DOC between day one and three should occur, which was true for the TP rhizosphere (Figure 2.3-2, Table 2.3-4). In contrast, an increase of root exudates in DOC of the SP rhizosphere between day one and three was observed. Lower microbial utilization of root exudates and ongoing root exudation could have caused the increase of root exudates in DOC between day one and three, despite the expected highest exudation within hours (Rattray et al. 1995; Dilkes et al. 2004).

The hypothesized higher exudation into the TP rhizosphere was not found. The nylon gauze was completely covered by roots in both treatments, but the amount of root exudates in DOC at the root surface one day after labeling was significantly lower in the SP rhizosphere (Figure 2.3-2). Exudate amounts in DOC at the root surface did not reflect total exudation, due to fast translocation of recent photosynthates and uptake by microorganisms (Rattray et al. 1995; Dilkes et al. 2004; Fischer et al. 2010). However, the lower exudate amount in DOC at the root surface in combination with the lower microbial utilization of root exudates (more gentle

gradients of ^{14}C in DOC), indicated lower root exudation into the SP rhizosphere. In conclusion, higher exudation into the TP rhizosphere could have been caused by the higher abundance of microbial biomass, because it was observed in other studies that microorganisms increase rhizodeposition (Meharg and Killham 1991; Schönwitz and Ziegler 1994). Consequently, the abundance of microorganisms rather than soil nutrient content (Paterson and Sim 1999; Neumann and Römheld 1999) affected root exudation.

Due to the fast decomposition of root exudates, their concentrations in soil solution are generally very low (µmol l^{-1} soil solution) (Fischer et al. 2007; Fischer and Kuzyakov 2010). Therefore, fast translocation of photosynthates and fast decomposition of root exudates (Dilkes et al. 2004; Hill et al. 2008) resulted in very low ^{14}C recovery in DOC one day after the $^{14}CO_2$ pulse (Figure 2.3-2). The half-life of root exudates taken-up by microorganisms ranges from hours to several days (Kuzyakov and Demin 1998; Rangel-Castro et al. 2005). Therefore, ^{14}C recovered in TOC of the rhizosphere, partly reflecting ^{14}C taken up by microorganisms, was 10 times higher compared to DOC in both treatments after one day (Figure 2.3-2 and Figure 2.3-3). As the obtained input of root exudates and the microbial biomass was higher in the TP rhizosphere, higher ^{14}C recovery in TOC resulted after one day in the TP compared to the SP rhizosphere.

2.3.4.2 Effect of mycorrhization on spatial distribution of root-derived C in alfalfa rhizosphere

Our study did not reveal any clear effects of mycorrhization on rhizosphere extent. Against initial expectations, the distribution and gradients of root-derived C in the rhizosphere with AM fungal hyphae were similar to those without hyphae (Figure 2.3-2 and Figure 2.3-3). After two months of alfalfa growth, the proportion of root length colonized by AM fungi was low (13 ± 4% and 18 ± 5% in TP and SP rhizosphere, respectively) compared to other studies (Table 2.3-3). Inoculation of pure cultures of AM fungi resulted in colonization between 44% and 95% of root length (Jakobsen and Rosendahl 1990; Li et al. 1991; Jakobsen et al. 1992).

Low AM colonization of alfalfa roots could have been caused by several factors. First, homogenization of the soil by mixing and sieving destroyed the existing hyphal network. Colonization of roots by AM can be caused by spores, infected root fragments and AM fungal hyphae, whereas the relative importance of every single inoculum for colonization potential is difficult to determine (Smith and Read 2008). However, it was shown that after the

destruction of the hyphal network in soil, AM colonization of roots was strongly reduced (Merryweather and Fitter 1998; Evans and Miller 1990). Second, two months could have been an insufficient period for the establishment of AM, as the colonization of *Medicago sativa* roots by *Glomus caledonius* increased from 35% after 6 weeks to 78% after 18 weeks of growth under low P supply (Nielsen and Jensen 1983). Third, low AM colonization of alfalfa roots could have been dependent on the AM fungi involved in the symbiosis. The development of root colonization by AM fungi follows a sigmoidal increase with time, but the time to reach maximum colonization differs between AM fungi (Sanders et al. 1977). Further environmental factors affecting colonization like temperature and light (Smith and Read 2008) are negligible, as they were comparable to field conditions within the growing season of alfalfa.

Even though AM colonization is not an indicator for the effect of the symbiosis on plant growth (Smith and Read 2008), low AM colonization did not affect the extent or the gradients of root-derived C from the root surface into bulk soil (Figure 2.3-3). The ^{14}C activity in the rhizosphere compartment was not determined in the AM fungal hyphae themselves after separation from soil, but in the soil slices containing hyphae. Although 0.7–0.8% of assimilated C can be incorporated into external hyphae and the total C usage of AM is much higher (Jakobsen and Rosendahl 1990; Pearson and Jakobsen 1993; Johnson et al. 2002), no effect on ^{14}C in TOC was obtained. The developed extraradical mycelium of AM fungi can be expected to be small due to low AM colonization. Therefore, ^{14}C activity in AM fungal hyphae and microorganisms due to hyphal exudation was too low to be detected in TOC.

The allocation of assimilated C into the extraradical mycelium of AM fungi was shown to be fast, as the maximum respiration of $^{13}CO_2$ by AM fungal mycelium was reached 9–14 hours after labeling of pasture plants (Johnson et al. 2002). In conclusion, small extraradical mycelium of AM fungi associated with fast allocation and turnover of assimilated C in AM fungal mycelium (Johnson et al. 2002) could have caused the absence of the effect of mycorrhization on the extent of root-derived C in alfalfa rhizosphere.

2.3.5 Conclusions

The extent and turnover of root-derived C from the root surface into soil was assessed in a laboratory experiment using three-compartment pots, grown with alfalfa, following $^{14}CO_2$ pulse labeling. Root exudates extended to a distance longer than 28 mm in DOC and 20 mm in TOC in the rhizosphere of alfalfa with topsoil and subsoil properties. The diffusion distance of root exudates observed here exceeded previously reported distances due to larger amounts of label (^{14}C) used in the current study. However, differing properties of the homogenized soil sampled from a top- (A_h) and subsoil (B_t) horizon of a Haplic Luvisol did not affect the diffusion distance of root exudates. Against initial expectations, root exudation per root mass was lower in the rhizosphere with subsoil compared to topsoil properties. Our results suggest that the diffusion distance of root exudates is independent from top- and subsoil properties, because higher root exudation into the rhizosphere with topsoil properties is compensated by the higher microbial utilization of root exudates.

Effects of mycorrhization on rhizosphere extent of alfalfa were not identified as the recovery of root-derived C in DOC and TOC was not affected by AM fungal hyphae. The absence of an effect was due to low root colonization by AM fungi, and the consequently expected low AM fungal hyphae biomass in the rhizosphere. To determine the effects of mycorrhization on the spatial distribution of root-derived C, ^{14}C activity needs to be measured in external AM fungal hyphae extracted from the soil.

2.3.6 Acknowledgements

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2.3.8 Supplementary material

Supplementary material for

Spatial distribution and turnover of root-derived carbon in alfalfa rhizosphere depending on top- and subsoil properties and mycorrhization

Plant and Soil

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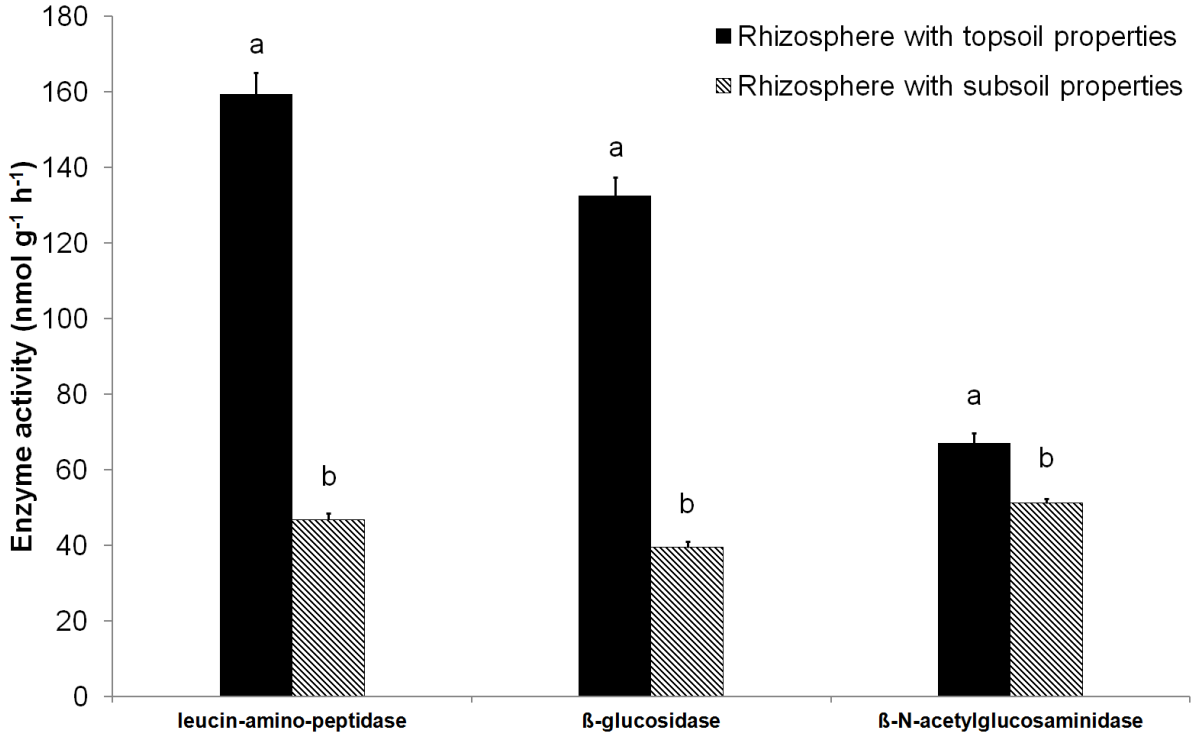

Figure 2.3-4: Average activity of leucin-amino-peptidase (EC 3.4.11.1), ß-glucosidase (EC 3.2.1.21) and ß-N-acetylglucosaminidase (EC 3.2.1.52) in the rhizosphere with top- and subsoil properties containing arbuscular mycorrhizal hyphae. Enzyme activities were measured using a microplate fluorimetric enzyme assay based on methylumbelliferone (MUB) substrates (Marx et al., 2001; German et al., 2011). Enzyme activities were measured in four replicate rhizosphere compartments with top- and subsoil properties, respectively. Therefore, the rhizosphere compartments were cut, using a microtome, into slices at a distance of 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 30, and 40 mm from the previous root surface. Means and standard errors of the means are presented in the figure. To identify significant differences of average enzyme activities between the rhizosphere with top- and subsoil properties the non-parametric Kruskal-Wallis ANOVA ($n = 48$; $p < 0.05$) was applied, as the data was not normally distributed (Kolmogorov-Smirnov-test, $p < 0.05$). Statistical analyses were carried out using STATISTICA for Windows (version 10.0; StatSoft Inc., Tulsa, OK, USA). Significant differences of enzyme activities between the rhizosphere with top- and subsoil properties are indicated by different letters.

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Study 4

2.4 Oxygen and redox potential gradients in the rhizosphere of alfalfa grown on a loamy soil

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Abstract

Oxygen (O_2) supply and the related redox potential (E_H) are important parameters for interactions between roots and microorganisms in the rhizosphere. Rhizosphere extension in terms of the spatial distribution of O_2 concentration and E_H is poorly documented under aerobic soil conditions. We investigated how far O_2 consumption of roots and microorganisms in the rhizosphere is replenished by O_2 diffusion as a function of water/air-filled porosity. Oxygen concentration and E_H in the rhizosphere were monitored at a millimeter scale by means of electroreductive Clark-type sensors and miniaturized E_H electrodes under various matric potential ranges. Respiratory activity of roots and microorganisms was calculated from O_2 profiles and diffusion coefficients. pH profiles were determined in thin soil layers sliced near the root surface.

Gradients of O_2 concentration and the extent of anoxic zones depended on the respiratory activity near the root surface. Matric potential, reflecting air-filled porosity, was found to be the most important factor affecting O_2 transport in the rhizosphere. Under water saturated conditions and near field capacity up to -200 hPa, O_2 transport was limited, causing a decline in oxygen partial pressures (pO_2) to values between 0 and 3 kPa at the root surface. Aerobic respiration increased by a factor of 100 when comparing the saturated with the driest status. At an air-filled porosity of 9% to 12%, diffusion of O_2 increased considerably. This was confirmed by E_H around 300 mV under aerated conditions, while E_H decreased to 100 mV on the root surface under near water saturated conditions. Gradients of pO_2 and pH from the root surface indicated an extent of the rhizosphere effect of 10-20 mm. In contrast, E_H gradients were observed from 0 to 2 mm from the root surface. We conclude that the rhizosphere extent differs for various parameters (pH, E_H, pO_2) and is strongly dependent on soil moisture.

Keywords: soil aeration, oxygen diffusion, air-filled porosity, rhizosphere, hotspots

2.4.1 Introduction

One of the most important factors influencing aerobic activity in soil is the availability of molecular oxygen (O_2). Spatial distribution of O_2 in soil depends strongly on the investigated scale. At the pedon scale, higher O_2 partial pressure (pO_2) are found in the topsoil and gradually decrease with depth (Gliński and Stępniewski 1985; Stępniewski and Stępniewska 2009) due to larger diffusion distance to the free atmosphere. At the aggregate scale, pO_2 decreases from the outside perimeter to the aggregate center, which can reach anoxic conditions (Sexstone et al. 1985; Zausig et al. 1993). At the rhizosphere scale, O_2 distribution from the root surface into bulk soil is driven by its consumption due to respiration processes and diffusive O_2 supply (Gliński and Stępniewski 1985). According to Raynaud (2010), a major part of the soil respiratory activity takes place in the rhizosphere, because of higher microbial activity compared to the bulk soil (Nunan et al. 2003) and root respiration (Kuzyakov 2002).

The rhizosphere, i.e. the soil surrounding roots, which is influenced by its activity (Darrah 1993), represents only about 1% of the total soil volume, but has an enormous ecological importance (Gregory 2006; Pausch and Kuzyakov 2011). It represents one of the hotspots in soil, where turnover of organic matter is increased compared to bulk soil due to higher microbial activity (Jones and Hinsinger 2008). To sustain this activity O_2 has to be sufficiently transported into the rhizosphere (Hinsinger et al. 2009). One of the main limitations in studying pO_2 and O_2 transport in the rhizosphere, are the temporal changes in air-filled porosity, microstructure formation and displacement of the root active zone (Flessa 1994). Concentration and transport of O_2 in soil are independently well documented but only few studies describe the spatial distribution of O_2 in the rhizosphere considering the interaction of respiration and transport at different matric potentials (Grabler 1966; Grable and Siemer 1968; Gliński and Stępniewski 1985).

By metabolizing soil organic matter (SOM), aerobic microorganisms transfer electrons to an end acceptor, in this case O_2. This process plays a fundamental role for the mineralization of SOM (Brzezińska et al. 1998; Jones et al. 2004; Allaire et al. 2008). If O_2 supply is low (e.g. because of high water contents blocking diffusion pathways in the pore network), microbes might switch from aerobic to anaerobic respiration. The descending order of alternative acceptors is NO_3^-, MnO_2, $Fe(OH)_3$, SO_4^{2-} and CO_2 (Ponnamperuma 1984; Fiedler et al. 2007). Under anaerobic conditions, mineralization of SOM decreases, hence nutrient availability for

plants (Drew et al. 1988). The redox potential (E_H) is therefore an important indicator determining the oxidation-reduction state in the soil (Mansfeldt 2004). Several authors have proposed critical ranges for E_H indicating lack of O_2 (e.g. Reddy et al 2000; Sposito 1989). Most of them agree that in soils with neutral pH a threshold can be set between 300 and 400 mV to separate oxic from anoxic conditions. A well known classification of the oxidation/reduction status of soils is the one proposed by Zhi-Guang (1985), where levels >400 mV represent an oxidizing status (O_2 as predominant electron acceptor), 400 to 200 mV are weakly reducing (O_2, NO_3^- and MnO_2), 200 to -100 mV are moderately reducing ($Fe(OH)_3$), and <-100 mV are strongly reducing (SO_4^{2-} and CO_2).

Hotspots of microbiological activity occurring in the rhizosphere may have great influence on E_H. Thus, E_H is highly variable in time and space. Furthermore, its variability also depends on matric potential changes resulting from precipitation events or ground water table changes (Flessa and Fischer 1992; Mansfeldt 2003; Fiedler et al. 2007; Hinsinger et al. 2009). Due to the well known triggering effect of O_2 on microbial activity and redox processes, gradients of E_H are expected to occur from root surface into bulk soil as a function of the air-filled porosity. Previous studies focused mainly on wetland cultivations or were conducted in sterile media (e.g. agar solutions) neglecting the highly variable properties of soils such as complex mineralogical composition, gas permeability, poise capacity, and microbial diversity amongst others (Fischer et al. 1989).

We hypothesize that: (1) increased respiratory activity at the root surface results in decreasing pO_2 from bulk soil to the root surface, (2) the difference in pO_2 depends on matric potential, as it controls O_2 supply from bulk soil to the root surface, (3) E_H dynamics in the rhizosphere are a function of the mentioned pO_2 gradients. To test these hypotheses, pO_2 and E_H gradients from the bulk soil to the root surface of alfalfa (*Medicago sativa* L.) depending on matric potential were measured in a jointed pot experiment. The aim of our study was to determine (1) the required air-filled porosity to sustain aerobic conditions in the rhizosphere and (2) the extent of the rhizosphere in terms of pO_2 and E_H.

2.4.2 Materials and Methods

2.4.2.1 Experimental setup

Alfalfa (*Medicago sativa* L.) was grown in a three-compartment pot (Figure 2.4-1) in a similar setup as used by Hafner et al. (2014). Soil material derived from a Haplic Luvisol (*IUSS Working Group WRB*, 2006) taken from the experimental station Klein Altendorf of the University of Bonn (50°37'21'' N, 6°59'29''E). Two pots were completely filled with homogenized topsoil (0–30 cm, silty loam) and two with subsoil (45–75 cm, silty clay loam) at bulk density of 1.2 g cm^{-3}. The central compartment contained roots, whereas the roots could not penetrate into both rhizosphere compartments (side parts) due to a nylon gauze with mesh sizes 1 µm on one side and 30 µm on the other (Kuchenbuch and Jungk 1982). The lateral compartments were sealed with plastic caps containing holes with a grid size of 1 x 1 cm where the O_2 and E_H microsensors could laterally be inserted.

Alfalfa was planted at a density of 0.5 g seeds per pot into the root compartment. The experiment was conducted under controlled conditions: Water content was daily adjusted to 80% of the water holding capacity and checked gravimetrically. Plants were watered from the top of the root compartment. The photoperiod was 14 hours light to 10 hours dark. Light intensity was 300 µmol $m^{-2}s^{-1}$ and the room temperature was 23°C during light and 20°C during dark periods. Two months after sowing, the whole surface of the nylon gauze was

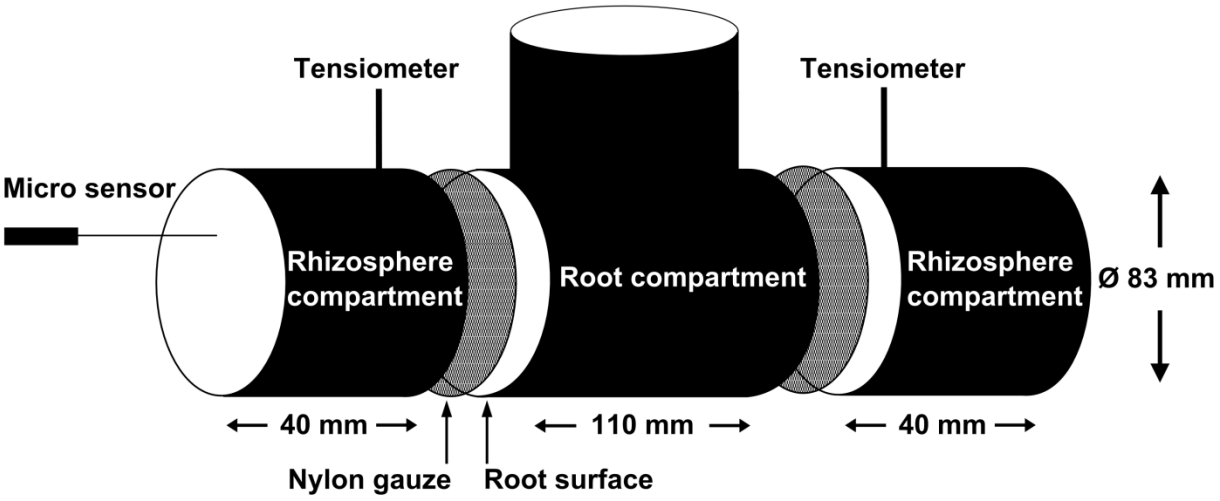

Figure 2.4-1: Setup of the experimental pots to study oxygen partial pressure and redox potential gradients in the rhizosphere of alfalfa. The three-compartment pots consisted of a central root compartment and two side compartments representing the rhizosphere of alfalfa. Nylon gauze prevented roots to penetrate into the rhizosphere compartments. The root surface of alfalfa is represented by the nylon gauze, after complete coverage by alfalfa roots.

covered with alfalfa roots and O_2 and E_H gradients were determined.

2.4.2.2 O_2 and E_H gradients and monitoring

An O_2 Clark-type microsensor (Unisense A/S, Aarhus, Denmark), with 100 µm diameter tip protected by a chirurgical needle, was used to determine the O_2 concentration. A two point calibration was made to convert the mV output of the O_2 sensors to the O_2 concentration. A linear interpolation was used between O_2 saturated water (pO_2 = 20.95 kPa) and a yeast-agar solution (pO_2 = 0 kPa). For the E_H measurements, miniaturized platinum electrodes (100 µm diameter tip, Unisense A/S, Aarhus, Denmark) were used. A two point calibration was made with two redox buffer solutions (Mettler Toledo Intl. Inc. Urdorf, Switzerland). No pH correction was made for the E_H measurements as the pH of the soil (pH = 6.8) was almost neutral (Bohn 1969; Mansfeldt 2003).

Three sets of measurements were made in the rhizosphere of the alfalfa planted pots to asses gradients of O_2 and E_H in space and time. The three sets were conducted sequentially, whereas the replication in each set was made in parallel to ensure equal conditions.

First, O_2 gradients from the bulk soil to the root surface were measured. The sensor was pushed gently into the lateral pot (from the side) by means of a micromanipulator. Measurements were done from the bulk soil in direction to the root surface in 0.5 mm steps (10 seconds each step). As the total length of the lateral pot was known (40 mm), inserting the microsensor 40 mm was necessary to get to the rooted nylon gauze. Each measurement took approximately 15 minutes. Measurements were made within four different matric potential ranges (-10 to -30, -50 to -100, -200 to -700 and < -900 hPa). After watering the plants, matric potential was monitored by a ceramic minitensiometer installed in the lateral pot at 10 mm distance from the root surface. When reaching the desired matric potential range, a pO_2 profile (insertion of the microsensor) was measured.

Second, gradients of O_2 and E_H were simultaneously measured inserting microsensors by the same procedure described before. Measurements were done at near saturation (matric potential range of -10 to -30 hPa) and near field capacity (-100 to -200 hPa) to limit respiration in the pots filled with topsoil.

Third, time series measurements were carried out in the pots filled with topsoil to assess temporal variations of O_2 concentration and E_H at the root surface. Microsensors were placed at a distance of 0–1 mm near the root surface and sealed from the outside to prevent O_2

diffusion along the sensor needle. The pots were watered until saturation after insertion of the microsensors to reduce air-filled porosity and to induce anoxic conditions. Levels of pO_2 and E_H were monitored at 1 second resolution for 20 hours. Three conditions were tested:

(1) Watering 24 hours after dry conditions (< -300 hPa) to simulate a heavy precipitation event: The soil was dried by evapotranspiration until a matric potential < -300 hPa was reached. After 24 hours, the soil was watered until saturation and the measurement started.

(2) Watering one hour after dry conditions to simulate drying/wetting cycles: After saturation, the soil was dried up to matric potential < -300 hPa by evapotranspiration. After one hour it was saturated again and the measurement started.

(3) Keeping 48 hours wet conditions to simulate longer wet periods where soil remains saturated for more days: The saturated condition of step 2 was maintained for two days at matric potential ranges of -10 to -30 hPa. Then the soil was fully saturated and the measurement started. The stress induced to the plants did not allow further measurements of time series on comparable conditions.

2.4.2.3 pH gradients

After pO_2 and E_H measurements were performed, the side compartments were air dried at 20°C for seven days. The soil was sliced parallel to the root surface at steps 2, 4, 7, 10, 15, 20, 25, 30, 40 mm from the root surface. The soil slices (e.g. from 0–2, 2–4, 4–7 mm etc.) were collected in individual cups and mixed with 0.01 M $CaCl_2$-solution for pH measurement.

2.4.2.4 Estimation of soil air-filled porosity

Air filled porosity was estimated from the water retention curve and matric potential measurements read from the minitensiometers. The texture of top and subsoil was analyzed by the method of wet sieving and precipitation (USDA 2011). Sand, silt and clay contents (Table 2.4-1) were used to fit the pedotransfer functions with the ROSETTA program (Schaap et al. 2001), which were used to calculate the van Genuchten (1980) soil water retention curve parameters (with restriction $m=1-n^{-1}$). The model number 3 was used, which considers textural percentages and bulk density as estimators. Although the uncertainty of the used pedotransfer functions may be elevated in some cases, our study was conducted with homogenized (unstructured) soil, thus the estimated parameters are greatly related to texture and bulk density. The calculation of the water retention curve parameters allowed the

Table 2.4-1: Texture analysis for topsoil and subsoil and hydraulic parameters estimated by pedotransfer functions

	Depth cm	Sand	Silt %	Clay	Class[a]	θr[a] $m^3\ m^{-3}$	θs[a] $m^3\ m^{-3}$	α[a]	n[a]
Topsoil (Ap)	0–30	16	67	17	SiL	0.071	0.460	0.0045	1.712
Subsoil (Bt)	45–75	12	59	29	SiCL	0.087	0.494	0.0070	1.565

[a]*USDA (2011)* texture classification. θr, θs, α and n are the van Genuchten (1980) water retention parameters.

estimation of the volumetric water content (θ, $m^3\ m^{-3}$) at each matric potential. Air-filled porosity (θ_a, $m^3\ m^{-3}$) was then calculated by the difference between total porosity and the corresponding water contents for each matric potential range.

2.4.2.5 Calculation of O_2 diffusivity and uptake

The O_2 relative diffusion coefficient (D_s/D_o) was calculated for the four matric potential ranges using the empirical equation [1] by (Moldrup et al. 1997):

$$\frac{D_s}{D_o} = 0.66 \times \grave{e}_a \left(\frac{\grave{e}_a}{\grave{E}}\right)^{\frac{12-m}{3}} \quad (1)$$

where D_s and D_o are the diffusion coefficients ($m^2\ s^{-1}$) of O_2 in soil and in free air, θ_a ($m^3\ m^{-3}$) is the air-filled porosity at a given matric potential, Θ ($m^3\ m^{-3}$) is the total soil porosity and "m" is an empirical parameter set equal to 6 for homogenized repacked soil (Moldrup et al. 1997).

Respiratory activity for each matric potential range was calculated by numerical modeling using a monolayer profile and constant diffusion coefficient. Assuming a constant O_2 consumption rate q at a distance x from the free atmosphere with a known diffusion coefficient in soil D, Gliński and Stępniewski (1985) calculated the O_2 concentration C by combining Fick's first law with the accumulated O_2 uptake (that is assumed to be steady state) using equation 2:

$$C = C_0 - \frac{q(2Lx - x^2)}{2D} \quad (2)$$

where C is the O_2 concentration (g m^{-3}) at a distance x (m) from a total layer of length L (m), C_0 (g m^{-3}) is the O_2 concentration at the upper boundary condition (free atmosphere), q is the O_2 consumption rate per unit soil (g m^{-3} s^{-1}) and D the gas diffusion coefficient of soil at a given matric potential (m^2 s^{-1}). Solving for q, as the concentration C and C_0 is known, we obtain:

$$q = \frac{2D(C_0 - C)}{2Lx - x^2} \quad (3)$$

Consumption of O_2 was compared between topsoil and subsoil pots to assess microbial activity in relation to the distance to the root surface and the transport of O_2 across the profile (40 mm).

2.4.3 Results

2.4.3.1 Gradients of O_2 from bulk soil to the root surface at different matric potentials

Generally, pO_2 increased with decreasing matric potential, however, differences between top- and subsoil were not observed (Figure 2.4-2). Under nearly saturated conditions (-10 to -30 hPa) the pO_2 was very low between 0 to 30 mm distance from the root surface. Under field capacity conditions (-50 to -100 hPa) pO_2 decreased strongly from bulk soil (13.9 kPa in top- and 11.6 kPa in subsoil) up to O_2 depletion (1.1 kPa and 1.8 kPa respectively) at the root surface. As the soil became dryer than field capacity (matric potential < -200 hPa), the air-filled porosity was high enough (>9% for top- and 12% for subsoil) to supply the root surface with O_2.

The low pO_2 for the conditions -10 to -30 and -50 to -70 hPa did not differ significantly, up to 9.5 (in topsoil) and 13.0 mm (in subsoil) distance to the root surface, respectively (Figure 2.4-2). Under drier conditions (- 200 to <-900 hPa) the pO_2 did not reach levels lower than 10 kPa but still decreased near to the root surface. At 28 mm for topsoil and 24.5 mm for subsoil, under field capacity similar pO_2 levels were observed compared to drier conditions. Consequently, with respect to pO_2, we could define the rhizosphere perimeter between 10 and 25 mm from the root surface.

Changes in E_H within the rhizosphere showed a strong interaction with the water saturation defined as matric potential. Under nearly saturated conditions (-10 to -30 hPa), lower E_H values were measured close to the root surface (2 mm) compared to the bulk soil, while under drier conditions (-100 to -200 hPa) only slight differences in E_H were determined (Figure 2.4-3). Under nearly saturated conditions, the E_H values changed from weakly to moderately reducing at the root surface according to Zhi-Guang (1985), while under drier conditions the levels were classified as weakly reducing.

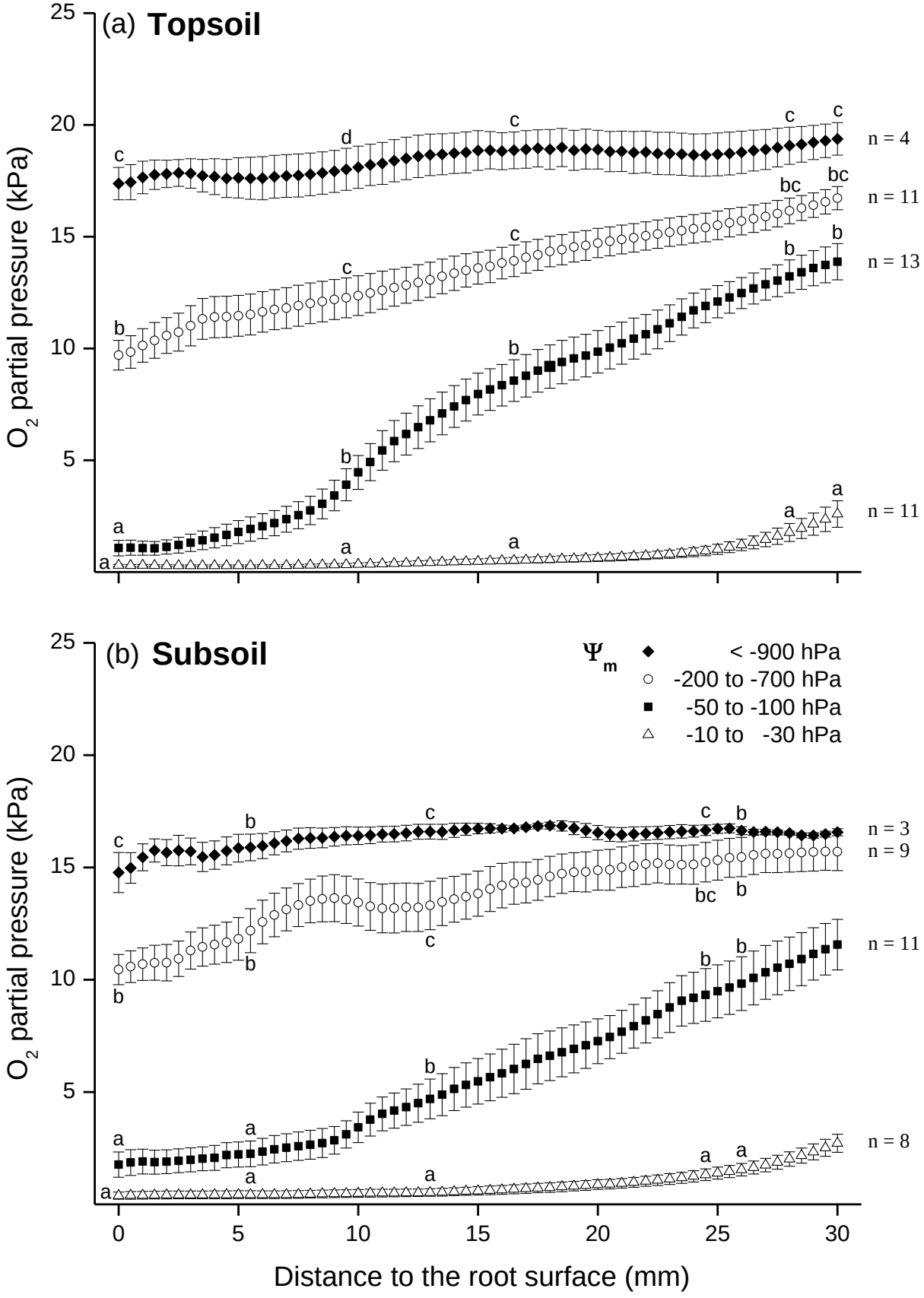

Figure 2.4-2: Oxygen partial pressure gradients from the bulk soil to the surface of alfalfa roots as a function of matric potential (Ψ_m) in top- (a) and subsoil (b). Error bars represent standard error of the mean. Different letters represent statistical differences between curves at same distance, after ANOVA test followed by Tukey HSD at $p<0.05$.

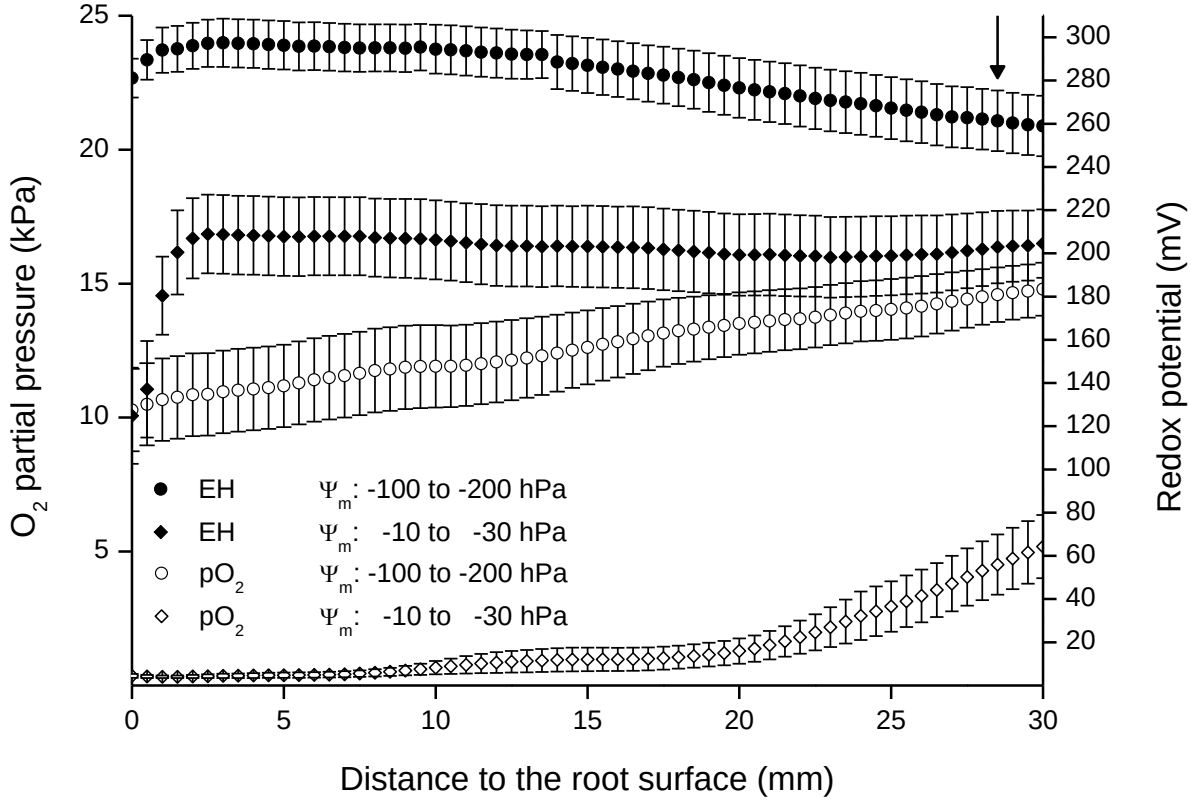

Figure 2.4-3: Oxygen partial pressure (pO_2) and associated redox potential (E_H) values in topsoil as affected by matric potential. The pO_2 is significantly different from 0 to 30 mm. Values of E_H show significant differences up to 28.5 mm (arrow). Error bars represent standard error of the mean. Statistical differences between curves at same distance were tested by ANOVA followed by Tukey HSD at $p<0.05$, n=15.

2.4.3.2 Dynamics of pO_2 and E_H at the root surface

A time delay of a few hours between changes of O_2 concentration and E_H occurred after saturation of the compartments filled with topsoil (Figure 2.4-4). Starting from matric potential < -300 hPa (Figure 2.4-4 a), a short period of 7 hours of low O_2 concentrations did not have an influence on E_H. According to the classification of Zhi-Guang (1985), during the

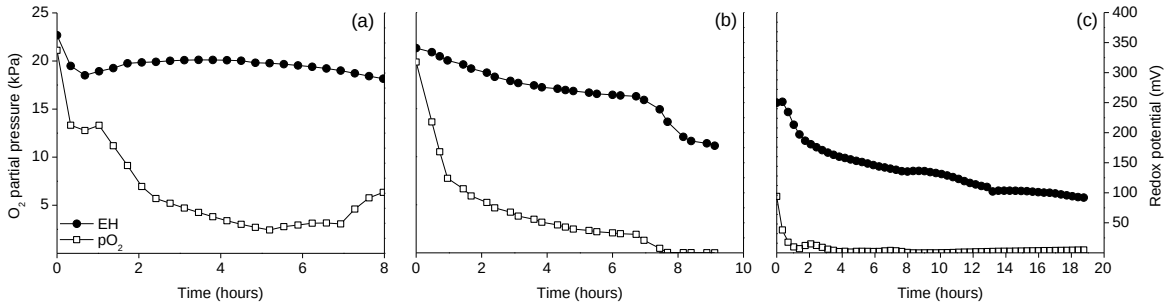

Figure 2.4-4: Dynamics of oxygen partial pressure (pO_2) and redox potential (E_H) at alfalfa root surface in topsoil. a: saturation of the soil after one day of dry conditions (< -300 hPa), b: saturation after one hour of dry conditions (< -300 hPa), c: after 2 days of wet conditions (-10 to -30 hPa).

complete measurement a weakly reducing status was observed (Figure 2.4-4 a). After short drying (-300 hPa for 1 hour) and rewetting (-10 hPa), a change in E_H was observed. This could be due to facultative microorganisms that might have changed to anoxic respiration after several hours of O_2 lack (Figure 2.4-4 b). Only under prolonged periods of water saturated conditions (Figure 2.4-4 c), E_H reached moderately reducing values, where reduction of other elements as Fe and Mn started.

2.4.3.3 pH gradients from bulk soil to the root surface

The pH of the studied bulk soil was neutral with values of 6.5 to 7.2. A gradient could be observed near to the root surface where more acidic conditions (down to 5.7) were found. The root induced acidification was stronger in the pots filled with subsoil compared to those filled with topsoil (Figure 2.4-5).

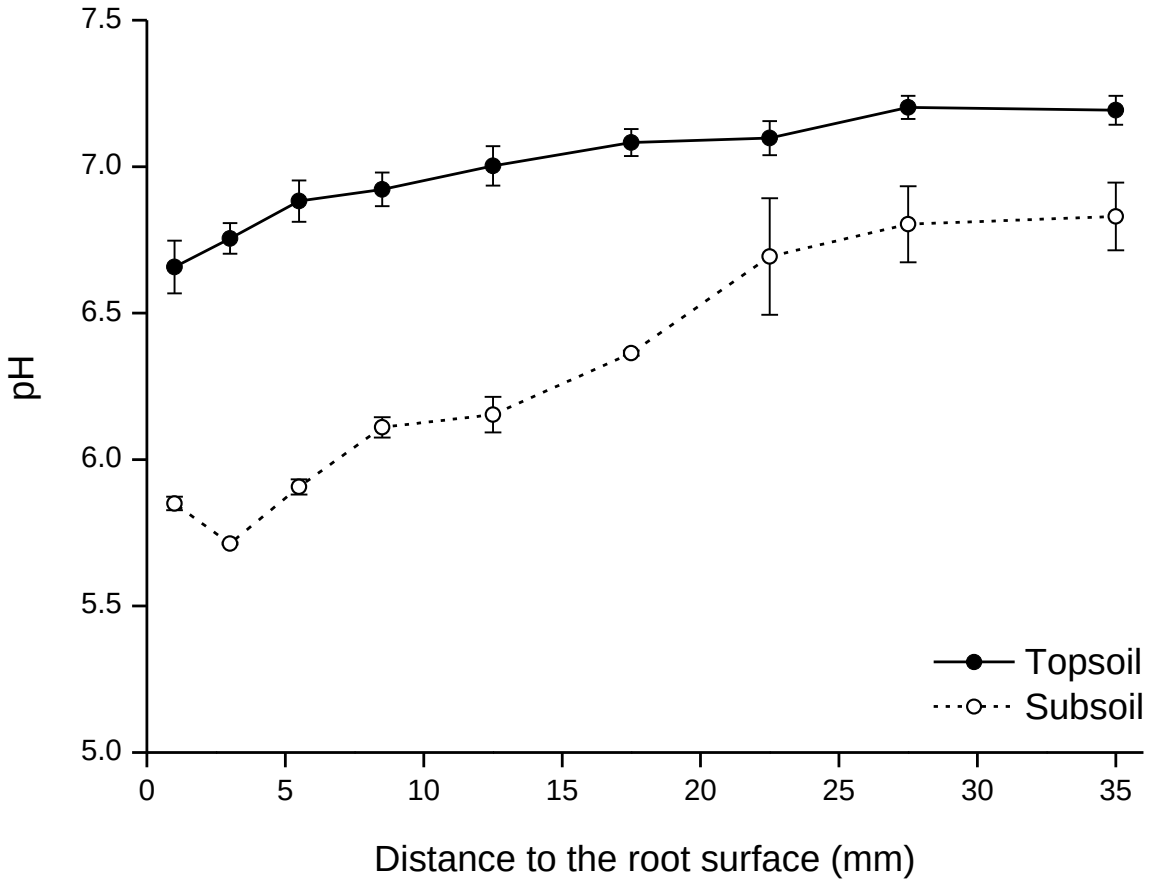

Figure 2.4-5: pH gradient from the bulk soil to the root surface for top and subsoil. Error bars represent standard error of the mean (n=4).

2.4.3.4 Consumption of O_2 in the rhizosphere as affected by matric potential

The pO_2 decreased linearly from bulk soil in direction to the root surface indicating that the O_2 decrease was determined by O_2 supply and constant O_2 consumption (Figure 2.4-2). On the contrary, an increase in slope steepness of the linear relation near to the root surface indicated increasing O_2 consumption due to higher biological activity (Figure 2.4-6). Below matric potential of -50 hPa, O_2 consumption was relatively even distributed, with a small increase near the root surface. Between -50 to -100 hPa, 100 times less O_2 was consumed in comparison to drier conditions. The air-filled porosity was found to be low (2% to 4% for topsoil and subsoil respectively) causing low diffusion of O_2 to the root surface. Under nearly saturated conditions (matric potential of -10 to -30 hPa) the air-filled porosity was too low to deliver enough O_2 to the rhizosphere and O_2 consumption decreased close to zero (Figure 2.4-6).

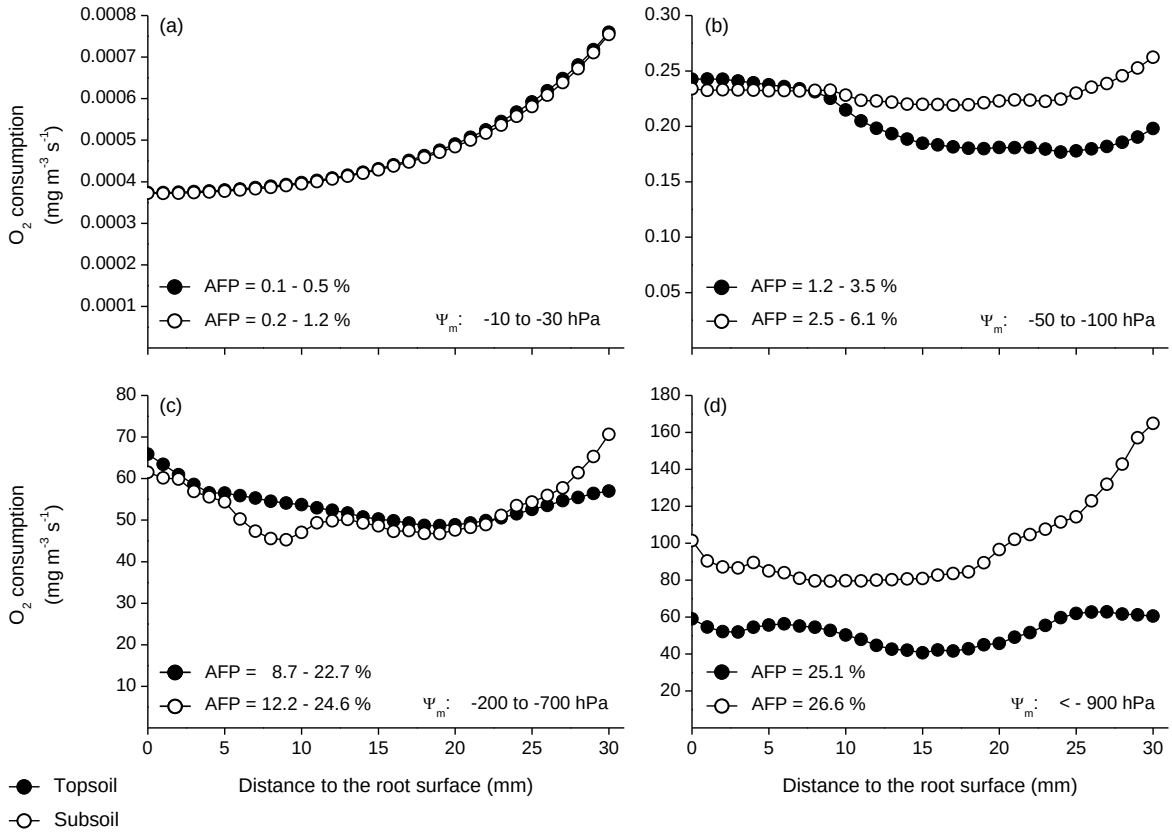

Figure 2.4-6: Calculated O_2 consumption (mg O_2 per m^3 soil per second) depending on matric potential (Ψ_m) ranges in top- (black dots) and subsoil (white dots). AFP = Air-filled porosity.

2.4.4 Discussion

2.4.4.1 Distribution of O_2 in the rhizosphere

It is well known that the distribution of O_2 depends on a variety of factors. Here we showed that, as hypothesized, the distribution of O_2 from bulk soil to the root surface is driven by rhizosphere respiration. At the same time, our second hypothesis was confirmed, as the ability to balance O_2 consumption near to the root is mainly controlled by matric potential (Figure 2.4-2). Biologically-mediated processes of O_2 consumption as root and microbial respiration also played a fundamental role. To which extent O_2 can be transported in the rhizosphere, depends on air-filled porosity. Thus, the matric potential is the driving parameter, because it controls the presence of water-blocked pores. A direct relation can be established between air-filled porosity and Fick's gas diffusion coefficient. Many authors suggest an exponential increase of the diffusion with higher air-filled porosity (Buckingham 1904; Millington and Quirk 1961; Ball 1981) while others suggest a linear relation (Penman 1940; Anderson et al. 2000) or combined relations (Deepagoda et al. 2011). Low water content will favor O_2 transport but will be a limiting factor for root water and nutrient uptake and for microbial respiration. Balogh et al. (2011) found higher respiration rates under water contents around 30–40% in a structured silty-clay-loam, which represented field capacity conditions (around -100 to -200 hPa). Under these conditions our homogenized soil was too saturated (Figure 2.4-2) to transport enough O_2 to the rhizosphere. A balance between air-filled porosity and water content should be optimal for plant growth. Our results showed a clear distinction beyond the threshold of -200 hPa matric potential, where air-filled porosity reached 9% and 12% for top- and subsoil, respectively. Over this critical values, enough O_2 could be transported to the rhizosphere and soil respiration increased exponentially (Figure 2.4-2 and Figure 2.4-6). This agrees with the general rule of 10% air-capacity established by Wesseling and van Wijk (1957) and confirmed by others (e.g. Grabler 1966; Robinson 1964). Textural differences between topsoil and subsoil were not sufficient to influence air-filled porosity, making the calculated respiration to be similar for both materials, which might be due to the fact that we established the same bulk density for both soil materials.

2.4.4.2 Dynamics of E_H in dependence of matric potential

We hypothesized that E_H dynamics are a function of root-influenced O_2 gradients. Hence, if the O_2 supply is not sufficient because of a high water saturation, anoxic conditions occur resulting in reduced (or almost none) microbial respiration (Hinsinger et al. 2006). In spite of its obviousness, most studies on E_H in dryland cultivations do not report root effects on its spatial variation (Richter et al. 2007). One of the few works describing rhizosphere gradients of E_H in dryland conditions and in natural soil was introduced by Fischer et al. (1989). They determined the root tip as the active part on E_H variation, and could observe its influence up to 3 mm from the root surface. A limitation of this study was the constant water content, fixed at -60 hPa throughout the experiment, which resulted in variations inside the aerobic respiration range (400 to 800 mV). Other studies report effects of water table fluctuations, temperature and SOM. Mansfeldt (2003 and 2004) has found annual fluctuations between -160 and 560 mV in a Typic Endoaquoll marsh induced by water table fluctuations. These ranges are typical for long periods of water saturation followed by dryer conditions during summertime. In our study, E_H values were also driven by matric potential (Figure 2.4-3) showing weakly reducing conditions with matric potential < -200 hPa and moderate reducing conditions when nearly saturated. We also found a critical spatial dependence of E_H within the first millimeters close to the root surface, which is in agreement with the findings of Fischer et al. (1989). According to the Nernst equation, low E_H in the rhizosphere could be an effect of pH changes: 59 mV per pH unit (Fiedler et al. 2007). We could observe a significant acidification of 0.5 to 1 pH unit near the root surface (Figure 2.4-5). However, the effect of up to 1 pH unit on E_H is negligible. Since the low E_H values of 130 to 200 mV were measured together with pO_2, we state O_2 lack (pO_2 < 1 kPa) as the primary effect on the rhizosphere E_H profile defining reducing conditions.

Saturation after a dry period impacts directly the pO_2 but does have little immediate influence on E_H. This is in accordance to the findings of Ewing et al. (1991) and Reddy and Patrick (1975) who reported a shift between a decrease in O_2 concentration and changes in E_H that can be as long as two days in soil samples. In our case of a rapid change between wet and dry conditions followed by re-wetting (Figure 2.4-4 b) E_H seems to respond faster than the cited shifts. This is because our study deals with a more densely colonized environment, where changes are much faster than in bulk soil experiments because of the higher microbial activity. Thus, the delay in response time of E_H following limited O_2 availability is reduced to a few hours. This process could represent intermittent rain showers in the field, which have a

great impact on denitrification, as N loss is reported to be greater under wetting/drying cycles (Patrick and Gotoh 1974). Although some authors see a benefit in low E_H, because Fe^{2+} and Mn^{2+} availability increases (Ponnamperuma 1984; Flessa and Fischer 1992; Stępniewski and Stępniewska 2009), most authors agree that a decrease in pO_2, and consequently in E_H, leads to a decrease in the nutrient uptake ability of most plants (Drew et al. 1988; Fiedler et al. 2007) together with other negative effects like lower mineralization of SOM, Fe and S phytotoxicity (Pezeshki et al. 1988), loss of mineral N (Stępniewski and Stępniewska 2009), or reduced root growth (Ponnamperuma 1984; Fiedler et al. 2007) among others. In our study, limiting conditions for aerobic respiration were found only directly at the root surface and under nearly saturated conditions, which in the field could represent longer humid periods.

2.4.4.3 pH gradients from the bulk soil to the root surface

The greatest differences between topsoil and subsoil were found in the rhizosphere effect on pH (Figure 2.4-5). Rhizosphere changes in pH are mostly driven by respiration activity and the CO_2 produced, especially in the region of the root tip (Flessa and Fischer 1992). The CO_2 concentration in the rhizosphere is known to be about ten to hundred times higher than that of the atmosphere (Pausch and Kuzyakov 2012). In addition to water it forms H_2CO_3, which as an acid decreases the pH. Rhizosphere pH levels can be up to 1–2 units below bulk soil pH (Hinsinger et al. 2009) which is confirmed by our results. As respiration rates were similarly distributed for the topsoil and the subsoil, similar pH gradients with respect to the root surface are expected in both materials. Nevertheless, in the subsoil a greater root induced acidification was observed, about one pH unit versus 0.5 pH units in the topsoil (Figure 2.4-5). This can be explained on the one hand by the higher C content in the pots with topsoil (0.99% versus 0.52% in the subsoil pots, data not shown). SOM is known to play a key role in the pH buffering capacity increasing it considerably in the pedon but also in the millimeter scale, which could be observed in the rhizosphere of our experiment. On the other hand the availability of soluble minerals is lower in deeper soil horizons, forcing plants to release more exudates and to larger distances to assess the nutrients (e.g. Gocke et al. 2014; Kautz et al. 2013). pH gradients were observed up to 20 mm from the root surface, which is a larger distance than reported based on short-term experiments by various authors of about 0.2 to 10 mm (Fischer et al. 1989; Kuzyakov et al. 2003; Sauer et al. 2006; Hinsinger et al. 2009). Our experiment (based on a root mat technique) gives information of the average effect of many roots, possibly resulting in an overestimation of processes in contrast to single root

measurements. This also explains the high respiration activity up to 20 mm as stated before. However, in the long-term, i.e. as a consequence of the whole lifetime of a root, even larger distances of rhizosphere effects of 5 cm or even more were described (Gocke et al. 2014). This clearly shows the high variability of rhizosphere effects at different distances depending on the experimental set-up and a certain need to investigate rhizosphere effects in the long-term to overcome effects of single experiments. There is also a need to improve our understanding on root and rhizosphere related processes, which are especially relevant for nutrient uptake and C storage in the subsoil (Rumpel and Kögel-Knabner 2011; Kautz et al. 2013).

2.4.5 Conclusions

This study dealt with O_2 availability and transport in alfalfa rhizosphere. This study confirmed our three hypotheses, as O_2 and E_H dynamics were clearly root driven. We found an extent of the rhizosphere for O_2 concentration up to 20 mm while the root influence over E_H was observed only up to about 2 mm. Matric potential played a predominant role in the O_2 transport, with a limiting threshold of -200 hPa below which O_2 supply was not limited. About 9–12% air-filled porosity was found to be sufficient to transport O_2 for rhizosphere aerobic respiration activity. Under more saturated conditions, the O_2 consumption rates decreased about 100 times and moderately reducing conditions were found. Although these results were produced under controlled conditions with homogenized soil, the determined thresholds allow a better assessment of optimal air-filled porosity in natural environments.

2.4.6 Acknowledgements

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2.4.7 References

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Declaration of originality and certificate of authorship

I, Silke Hafner, hereby declare that I am the sole author of this dissertation entitled “Origin and properties of microbial hotspots in top- and subsoil”. All references and data sources that were used in the dissertation have been appropriately acknowledged. I furthermore declare that this work has not been submitted elsewhere in any form as part of another dissertation procedure. I certify that the manuscripts presented in chapters 2.1, 2.2 (equal author contribution) and 2.3 have been written by me as first author and the manuscript presented in chapter 2.4 as second author.

Göttingen, March 2015 _____ (Silke Hafner)

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