Understanding the mechanism of microbial control on carbon cycling in soil

Dissertation

To fulfill the requirements for the Degree of

"doctor rerum naturalium" (Dr. rer. nat.)

Submitted to the Council of the Faculty of Biology and Pharmacy

of the Friedrich Schiller University Jena

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Date of defense: 03/07/2014

Acknowledgement

This is probably the most exciting and stimulating part of my thesis and for me no less than an Oscar acceptance speech. So I took all the efforts to literally pen down my thesis acknowledgement.

Foremost, I would express my deepest gratitude to Prof. Dr. Gerd Gleixner (or simply Gerd) for offering me a PhD position in his group at the Max Planck Institute for Biogeochemistry (MPI-BGC), Jena. He has not only been a captivating and inspiring supervisor but also a true mentor. I thank him for his encouragement, advice and insights that helped shape me into a "researcher". I also thank Dr. Robert Griffiths, my external supervisor for hosting me in his group at the Centre for Ecology and Hydrology (CEH), Oxford where I performed part of my doctoral research. Thanks also to Prof. Dr. Erika Kothe, my advisor at the Friedrich-Schiller-University of Jena for her timely inputs through the Thesis Advisory Committee meetings that streamlined my research direction. Likewise, I thank Prof. Dr. Susan Trumbore (MPI-BGC, Jena) and Dr. Evgenia (Jane) Blagodatskaya (University of Goettingen) for fruitful scientific discussions. My sincere appreciation also goes to Dr. P.A. Lokabharathi from the National Institute of Oceanography, Goa for giving me a strong foundation in academia.

I appreciate being part of the Molecular Biogeochemistry group at the MPI-BGC and thank all its members who helped me quickly integrate into the team. Special thanks go to Vanessa-Nina Roth, Andrea Scheibe and Julia Baumert for their help and support. I thank Helena Dannert and Melanie Armbruster (Master students involved in the project) for their contribution and help in running the experiments and analyses. I thank my fellow researchers Roman Witt, Saadat Malghani, Anne-Gret Seifert, Perla Mellado, Ronny Thoms, Stefan Karlowsky, Carolin Thoms, Markus Lange, Franziska Guenther and Andrej Thiele for scientific and non-scientific exchanges; not to forget our pub crawls, international dinners and the annual Thann retreat. I also thank Emily Solly, Jakob Zscheischler, Martin Nowak and Theresa Kloetzing for friendly conversations over lunch or otherwise.

Many thanks to the technical and administrative support staff at the MPI-BGC for simplifying my life as a researcher. Steffen Ruehlow was the one person I ran to almost every day for help with the HPLC or other things in the lab. I thank him for being so supportive (I still wonder if he has a magic wand!). I also appreciate the help I received from Iris Kuhlman and Uta Gerighausen. My research would not have materialized without support from Agnes Fastnacht, Karl Kuebler, Heike Geilmann and Willi Brand from the central facilities. I thank them for their support in establishing the experimental setup or with isotopic analyses.

During my research stay in Oxford, I was happy to be part of the small Molecular Microbial Ecology group at CEH consisting of Robert Griffiths, Bruce Thomson and Daniel Read. I thank them for welcoming me with open arms. The Friday evening pub jaunt was fun but also the best time for scientific discussions over some English ale.

I thank Katrin Krause, coordinator of my graduate school DFG Graduiertenkolleg (GRK) for her support through my PhD journey. I also thank Anna Goerner of the International Max Planck Research School for Global Biogeochemical Cycles (IMPRS-gBGC) and Carsten Thoms of Jena School for Microbial Communication (JSMC), coordinators of the other graduate schools I was associated with. Thanks to them and

the others involved in organizing a variety of scientific and transferable skill courses that helped improve my skills and foster interdisciplinary exchanges.

My research was funded by the Max Planck Society (MPG) and the German Research Foundation (DFG). I thank DFG for the fellowship in the research training group GRK 1257 'Alteration and element mobility at microbe-mineral interface'. I also thank the MPG for covering part of the stipend.

When life is cheerful, it improves productivity at work. I thank all my friends and family for being always there, in good times and in bad. My special thanks go to Zhipeng Liu (Jena), Richard Falk (Oxford) and distant Shishir Kotkar who helped me through the struggles and frustrations as well as the celebration of my PhD life.

Finally many thanks to my parents (Anita Malik and Anil Malik) and siblings (Apurva Malik and Amit Malik) for their continuous unconditional love and support in all my endeavors. My achievements, including this PhD, were made possible only due to their sincere efforts. I would, therefore like to dedicate this thesis to my parents.

The outcome of this journey is the person who came out of it.

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List of abbreviations

^{13}**C**: ^{13}carbon

AMF: arbuscular mycorrhizal fungi

C: carbon

CFE: chloroform fumigation extraction

DIC: dissolved inorganic carbon

DNA: deoxyribonucleic acid

DOC: dissolved organic carbon

DOM: dissolved organic matter

EA: elemental analyzer

GC: gas chromatography

HMW: high molecular weight

HPLC: high-performance liquid chromatography

IRMS: isotope ratio mass spectrometry

LC: liquid chromatography

LMW: low molecular weight

MCL: microbial carbon loop

MGE: microbial growth efficiency

NLFA: neutral lipid fatty acid

PLFA: phospholipid fatty acid

RNA: ribonucleic acid

rRNA: ribosomal RNA

SEC: size exclusion chromatography

SIP: stable isotope probing

SOC: soil organic carbon

SOM: soil organic matter

SMB: soil microbial biomass

TMB: total microbial biomass

vHMW: very high molecular weight

Chapter 1

General Introduction

Overview

Carbon is the most important element of life and forms the building block of all living forms. Its dynamics in the environment and flow through the different global pools maintains a balanced carbon cycle. However in the last century man-made changes have led to shifts in this equilibrium. Anthropogenic increase in the atmospheric concentration of carbon dioxide (CO_2) has led to warming of the earth through the greenhouse effect. Organic carbon in soil forms a large reservoir and globally this is more than the amount of carbon in both the atmosphere and the terrestrial biosphere put together (Amundson, 2001). From the global climate change perspective the terrestrial carbon cycle gains prominence due to the fast response of the soil organic carbon (SOC) pool to environmental changes (Lal, 2010). Plant fixed carbon forms the main source of soil organic matter (SOM) whereas autotrophic and heterotrophic soil respiration drives loss of carbon. The terrestrial feedback due to global warming is hard to predict partly due to lack of inherent knowledge regarding the physiological response of soil microorganisms to environmental change (Schimel, 2013). Soil organic carbon is also a significant driver of agricultural productivity (Lal, 2010). Human interferences have led to decreased soil fertility, desertification and soil erosion in addition to the effects of extreme climate events. The relatively large size and fast response time of the soil organic carbon pool and its importance in maintaining agricultural productivity and mitigating atmospheric CO_2 levels highlights the need for in-depth research on soil organic carbon dynamics and its response to environmental changes.

Soil microorganisms come into focus because microbial growth and activity largely controls soil carbon cycling (Schimel and Schaeffer, 2012). Microorganisms act as gatekeepers for soil-atmosphere carbon exchange by regulating the release of carbon from soils to the atmosphere. This regulation is a result of a balance between the rate of microbial decomposition and stabilization of organic carbon in soil. The balance can shift under altered environmental conditions as a result of global climate change (Davidson and Janssens, 2006). Therefore, predicting the soil carbon response to climate change is critical in projecting future global warming. Such projections are based on Earth system models which often do not explicitly consider key biogeochemical mechanisms like microbial physiological responses (Allison and Martiny, 2008; Wieder et al., 2013; Zhou et al., 2012). The reason for the exclusion of microbial physiology in climate-carbon models is largely the lack of mechanistic understanding of the feedback responses of soil microbial communities (Bardgett et al., 2008). It is therefore essential to understand the intrinsic mechanisms of organic carbon decomposition, transformation and stabilization in soil.

The degree of microbial contribution to the stable soil carbon pool still remains a major uncertainty in the terrestrial carbon budget. It is a readily accepted understanding that majority of plant carbon passes through the soil microorganisms which either respire it as CO_2 or stabilize it as SOM (Figure 1.1) (Kögel-Knabner, 2002; Mambelli et al., 2011). New climate-carbon models that explicitly consider the microbial control over soil carbon dynamics on a global scale use microbial growth efficiency as a parameter to determine the microbial feedback effect (Wieder et al., 2013). Microbial growth efficiency (MGE) is the proportion of substrate that microorganisms assimilate as against that lost in respiration. In the microbial carbon models MGE is highlighted as critical in controlling soil carbon dynamics, the degree of soil carbon loss or gain being dependent on the sensitivity of MGE to warming. However results from various climate change experiments are either inconclusive or contradictory (Conant et al., 2011). Thus it is

imperative to better understand the microbial physiology in soil and its control over soil carbon fluxes.

A major bottleneck in analyzing soil microbial communities has been technological limitations (Simpson et al., 2007). The challenges include complexity of soil as a system and the presence of a huge diversity of microorganisms in soil—a gram of soil houses more than 10,000 individual microbial taxa (Schimel, 2013). Moreover, the differences in physiology of soil microorganisms and hence the variable response to environmental change makes it very difficult to represent these dynamics into global models (Schimel and Schaeffer, 2012; Zhou et al., 2012). Thus it is necessary to first shed more light into the physiology of microorganisms in soil and study its adaptation to environmental change (Gleixner, 2013; Schimel, 2013). Insights into the mechanisms of soil carbon formation and destabilization, quantification of microbial inputs to soil organic matter, turnover of microbial biomass and community dynamics to link identity to substrate incorporation are major themes in soil biogeochemistry that need urgent attention. This thesis aims at tackling some of these questions using new tools and techniques to better understand the mechanism of key microbial processes in soil carbon cycling.

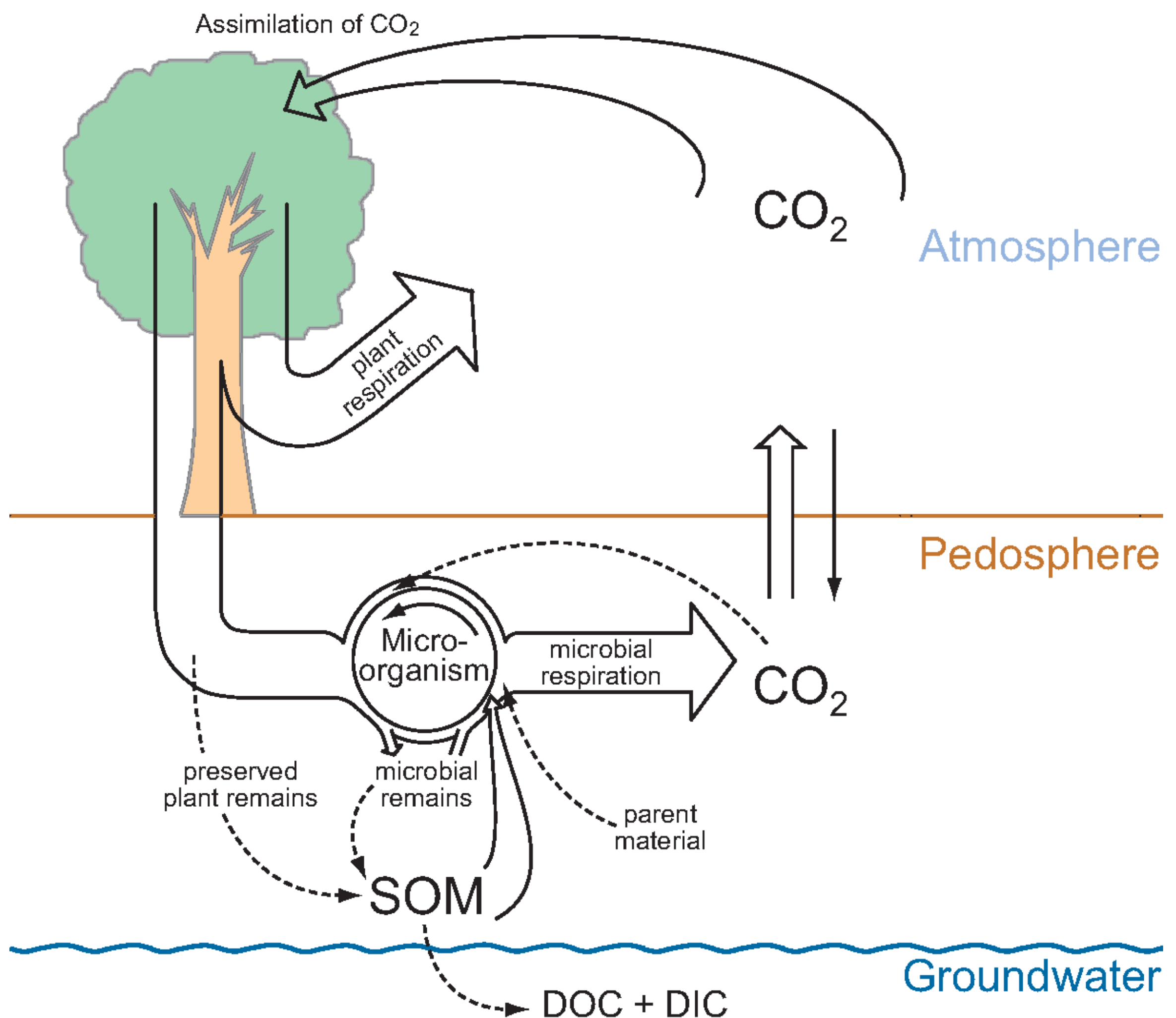

Figure 1.1: A schematic view of the terrestrial carbon cycle showing flow of plant carbon into soil and formation of soil organic matter. Source: Gleixner, 2013.

Soil microbial carbon cycling

Soil microorganisms play a central role in belowground soil carbon cycling (Figure 1.1). It is well established that soil microorganisms play an important role in respiration of newly fixed plant carbon. Recent results show that they also contribute significantly to formation and decomposition of soil organic matter (Kindler et al., 2006; Kögel-Knabner, 2002; Mambelli et al., 2011; Simpson et al., 2007). Its exact mechanism is not yet fully understood but it is hypothesized that soil microorganisms use part of the plant derived carbon for cellular energy needs and the rest for biomass buildup; and after cell death part of this microbial biomass carbon forms SOM (Miltner et al., 2012). Besides, abiotic factors like chemical recalcitrance of certain compounds as well as inaccessibility due to occlusion in aggregates or sorption to mineral surfaces also control SOM dynamics (Krull et al., 2003; Schmidt et al., 2011; Torn et al., 1997; von Lutzow et al., 2006). These factors also play an important role in production and decomposition of dissolved organic matter (DOM), a small but reactive fraction of the soil organic matter (Kalbitz et al., 2000).

Plant organic matter is the main source of carbon to soil microorganisms. Photosynthetically fixed carbon and its transfer to soil decomposer community is the major link between aboveground and belowground processes. Root associated carbon transfer occurs in the form of root exudation, fine root turnover, tissue sloughing off, etc. Aboveground litterfall also accounts for this transfer from plants to soil microbial biomass; however it has been widely accepted that its contribution is smaller relative to the root-derived inputs (Kramer et al., 2012). Root associated or rhizosphere soil is known for its high microbial diversity as well as activity. A plethora of microorganisms inhabit the rhizosphere and these may have different ecological statuses; pathogenic, saprophytic or mutualistic (Philippot et al., 2013). Plants provide organic carbon and other resources to the rhizosphere-associated community and the microorganisms decompose the organic carbon, mobilize nutrients, produce toxins and/or infect plant tissues and thus regulate plant growth. A variety of pathogenic or parasitic bacteria and fungi are known to suppress plant growth and these have been characterized widely. Among the root symbionts, the arbuscular mycorrhizal fungi (AMF) are well known. The AMF are quite widespread and are known to colonize about 80% of the land plants. They have been demonstrated to be an important functional group involved in belowground transfer of plant fixed carbon. Recent observations suggest that plant carbon is rapidly translocated to AMF that is followed by an indirect release to other fungi and bacteria in the rhizosphere interface (Drigo et al., 2010).

While a significant proportion of the plant derived carbon is respired both autotrophically and heterotrophically, a considerable fraction ends up in the soil organic matter. Microbial turnover in soil is a significant source for the maintenance of the SOM (Miltner et al., 2012). Microbial cell generation, multiplication and death are continuous processes which lead to soil carbon addition. Soil microorganisms almost completely decompose incoming plant carbon and form structurally new carbon which makes up most of the soil organic matter (Gleixner, 2013; Mambelli et al., 2011). Although the contribution of living microbial biomass to maintenance and accumulation of soil organic matter formation could be tiny, the turnover of microbial non-living biomass is estimated to be as high as 80% of organic carbon in soil (Liang and Balser, 2011). The stability of microbial compounds in soil has been attributed to its molecular structure and biochemical composition as well as ecosystem specific effects (Simpson et al., 2007; Throckmorton et al., 2012).

Soil dissolved organic carbon dynamics

Soil dissolved organic matter (DOM) is a small fraction of the total organic matter in soil. However, its high reactivity and dynamic flux highlights its significance in various biogeochemical processes (Battin et al., 2009; Kindler et al., 2011). DOM is the organic matter that is dissolved in the free flowing water present between soil pores and is operationally defined as the filtrate from a 0.45 μm filter. The movement of DOM through soil pores and its interaction with solid organic matter makes it a highly dynamic carbon pool. It is thus subject to constant physical, chemical and (micro)biological alteration. Most DOM is rapidly mineralized or is a product of decomposition both affected by microbial activity. DOM sources can vary from recent plant biomass in the form of aboveground litter, root litter and/or root exudates to relatively old soil organic matter (SOM) (Kalbitz et al., 2000). Recent results suggest that DOM in mineral soil is dominated by compounds derived from native soil organic matter, and not from newly introduced plant organic matter (Froeberg et al., 2007; Steinbeiss et al., 2008). Furthermore, other studies demonstrate that plant residues or residues of microbial biomass feeding on the former are short-lived and beyond this transient pulse most of the DOM compounds are derived from SOM (Gregorich et al., 2000; De Troyer et al., 2011). The evidence thus far suggests that DOC in mineral soil originates primarily from highly altered SOM and not merely from the fresh plant leachates transported downward in the soil solution (Karltun et al., 2004; Sanderman et al., 2008).

DOM concentration is soil solution is not only determined by its production and degradation which are largely controlled by biotic factors, but also by immobilization and leaching which are influenced by abiotic factors like hydrologic variability and temperature (McDowell, 2003). The significance of abiotic processes like exchange, sorption or dissolution reactions in DOC production have been sufficiently demonstrated in several studies (Guggenberger and Kaiser, 2003; Toosi et al., 2012). Various mechanisms describing the vertical movement of DOC in soil have been put forth. The earlier view assumes that organic compounds originating primarily from litter decomposition leach downward and some compounds get selectively stripped by the reactive mineral phases in soil (McDowell and Likens, 1988). A recent conceptual model of DOC transport by Kaiser and Kalbitz (2012) describes that organic compounds in soil water get precipitated or sorbed followed by its microbial processing and re-release by desorption or dissolution. Thus, the intimate linkages between the DOC pool and the SOC pool which is influenced greatly by soil microbial activity underpins the importance of experimental investigations to discern the role of microorganism in belowground carbon cycling and track the carbon flow through the different pools.

Techniques to study soil microbial carbon cycling

Microorganisms drive the biogeochemical cycling of elements on earth. Linking the identity of microorganisms to their metabolic function in natural environments has gained interest in the last decade. In soils it is crucial to understand how microorganisms govern the functioning of ecosystem processes, particularly belowground carbon cycling. However studying terrestrial microbial ecology is a major challenge due to the physiochemical complexity of soils and the overwhelming (micro)biological diversity. Despite the advent of tools and techniques for robust measurements of element fluxes, understanding the physiology of microorganisms in environment and characterizing their functional traits is still cumbersome and often technologically limited. Culture independent molecular biology tools and stable isotope probing are powerful approaches to link the composition of microbial communities to their functioning

(Boschker and Middelburg, 2002). However we still do not fully understand the functional role and the physiological mechanism of microbial contribution to soil organic matter formation. It is thus essential to develop new tools and applications that provide insights into soil microbial processes and their consequences on carbon cycling. Interdisciplinary approaches help integrate the effects of biotic and abiotic factors in environmental processes. Therefore, I tried to study soil carbon dynamics with a combination of stable isotope chemistry, carbon flux measurements and molecular ecology tools.

Stable carbon isotope technologies in combination with compound specific isotope measurements of biomarkers help track the flow of carbon in natural environments. Stable isotope probing (SIP) helps linking the identity of uncultivated microorganisms to isotope labelled substrate utilization detected using a particular biomarker (Murrell and S. Whiteley, 2011). Biomarkers are compounds which are produced by a limited group of organisms and thus represent this sub-population due to its specificity. Many different biomarkers have been tried and tested; each has its own advantages and disadvantages. Phospholipid fatty acids (PLFA) can be measured for stable carbon isotopes on a gas chromatography- isotope ratio mass spectrometry (GC-IRMS) and isotope incorporation in specific groups can be measured even at normal abundance levels (Boschker et al., 1998). However, the phylogenetic resolution is very limited and the PLFA patters are often linked to the cultivated representatives only thus being very speculative with little information about unculturable majority.

The use of labelled nucleic acids offers higher phylogenetic resolution and the potential to link identity to function with a greater confidence using high throughput sequencing of the isolated labeled DNA or RNA (Manefield et al., 2002; Radajewski et al., 2000). An RNA based method is more advantageous compared to DNA SIP since it uses the rRNA, an active pool which is a reflection of *in situ* metabolic activity independent of cell replication. Moreover, RNA is labelled more quickly than DNA and hence better suited in SIP experiments. However, efficient density separation of labeled and unlabeled nucleic acids through isopycnic centrifugation requires high amount of isotope enrichment in the biomarker which is difficult to obtain. A major shortcoming of nucleic acid SIP over PLFA SIP is that it only allows investigation of communities and does not give quantitative information on substrate incorporation. Therefore it is necessary to measure the stable isotope content in DNA and RNA. Still better is to isolate rRNA from a specific microbial group using probes and magnetic bead capture hybridization followed by its stable carbon isotope analysis using liquid chromatography- isotope ratio mass spectrometry (LC-IRMS) (MacGregor et al., 2002; Pearson et al., 2004). This allows linking identity of microorganisms to function, quantitatively. However, large amount of total RNA extract is required to obtain enough captured rRNA of a particular group for IRMS analysis. It is often beneficial to carefully choose the right technique or combination of techniques depending on the hypothesis to be tested.

The measurement system employed for stable isotope analysis of an analyte depends on its chemical nature. While GC-IRMS has been widely used in the past, availability of the commercial LC-IRMS interface has opened up possibilities to measure nonvolatile compounds in complex aqueous mixtures more efficiently. It has also allowed development of applications depending on the chromatographic potential for LC-based separation of compounds in a mixture followed by online stable carbon isotope analysis. The LC-IRMS has been used successfully to measure stable carbon isotope ratios in amino acids, carbohydrates, cellular metabolites (Godin and McCullagh, 2011) as well as environmental samples like dissolved organic carbon in soils and streams

(Scheibe et al., 2012). An improved LC-IRMS method was used to measure the stable isotope content in soil microbial DNA, RNA and chloroform fumigation extraction (CFE) derived microbial biomass. A new LC-IRMS application was also developed for online stable carbon isotope analysis of molecular size classes of soil dissolved organic carbon and microbial biomass.

Objectives

The overarching goal of the thesis was to understand the mechanism of microbial control over carbon cycling in soil. A more specific aim was to realize whether cellular biochemistry influences stabilization of microbial carbon in soil. To this end, photosynthetically fixed plant carbon was traced into soil microbial compounds differing in biochemical properties. It was also hypothesized that microbial molecular size influences its carbon turnover rate and thus controls its persistence in soil. Varying persistence of microbial fractions could mean differential contribution to soil carbon storage. Carbon flow was also monitored into different microbial functional groups in order to reappraise carbon flow paths through the soil microbial food web and to highlight the trophic interactions. Soil microbial carbon dynamics are closely linked to the large soil organic matter pool and the small but reactive dissolved organic carbon pool. Turnover of soil microbial biomass actively contributes to SOM maintenance while DOC compounds are an end result of microbial processes. The aim was to dissect these linkages to better understand the mechanism of microbial processing of organic matter in belowground carbon cycling.

Approach

Different tracer experiments were performed to track the flow of carbon from plants into the soil microorganisms at different time scales. A greenhouse-based, pulse chase $^{13}CO_2$ plant labeling experiment was used to monitor the flux of plant carbon over a short time scale and a C3-C4 vegetation change field experiment was employed to study longer term C turnover dynamics. In the former experiment, the flow of pulsed plant ^{13}C was chased into different microbial biochemical and size fractions. The isotope dilution pattern with new unlabeled post-pulse plant carbon was used to estimate the turnover time of different microbial fractions. In the latter experiment, relative contribution of new C4 plant carbon and > 5 y old C3 SOM carbon was used to estimate the turnover time of microbial biomass compounds. With the same experiment, the sources of dissolved organic carbon size classes were discerned over an annual seasonal cycle and it was linked to microbial processes.

An inter-disciplinary analytical approach with a combination of molecular biology and stable isotope chemistry tools was then employed. The main constraint in microbial ecology and biogeochemistry studies to trace carbon sources in the environment using isotopes is techniques and instrumentation. Soil is a complex physiochemical media which makes it hard to study the physiology of microorganisms and the consequences of their processes. The situation is aggravated by the immense microbiological diversity and heterogeneity in soils. This led to the initial focus of the thesis work on developing new tools to study microbial carbon cycling; specifically to investigate the sources of microbial carbon and to track their carbon footprints in soil. A new application was developed with online coupling of liquid chromatography- isotope ratio mass spectrometry (LC-IRMS) to size exclusion chromatography (SEC). This allowed online measurement of carbon stable isotope ratios of compounds in an aqueous

mixture differing in molecular size. This technique was later applied to measure stable carbon isotope ratios of size classes of soil microbial biomass and soil dissolved organic carbon. The size dependent separation of compounds before online isotope analysis also gets rid of the contaminants inadvertently remaining in the final exact of microbial biomarker of interest like DNA/RNA which could affect the isotope values of the biomarker.

To summarize, following were the key specific objectives of the thesis:

1. To develop and standardize an LC-IRMS application for online measurement of stable carbon isotope ratios of compounds in complex aqueous mixtures differing in molecular size.
2. To elucidate the relationship between microbial molecular size and carbon turnover at short and long time scales.
3. To substantiate that microbial cellular biochemistry by affecting the turnover time plays an important role in stabilization of microbial carbon in soil.
4. To reappraise soil microbial trophic interactions in terms of carbon flow in the plant-rhizosphere-soil continuum.
5. To discern the microbial control over soil dissolved organic carbon production and decomposition.

Thesis structure

Chapter 2 explains the novel application of the LC-IRMS interface that was used for analytical measurements of soil water samples from the C3-C4 vegetation change experiment. The SEC-LC-IRMS measurement technique to measure stable carbon isotope content of molecular size separated compounds in aqueous mixtures was calibrated with standard compounds and thoroughly tested with soil DOC samples. Freshwater and marine water samples were also tested for dissolved organic carbon size fractionation coupled online to isotope analysis. Brief description of the technique to measure $\delta^{13}C$ values of soil microbial biomass size classes is also presented in Chapter 3 which elucidates the relationship between microbial carbon turnover and molecular size. Results from an improved LC-IRMS based method for ^{13}C analysis of RNA and DNA from soil microorganism is presented in Chapter 5. SEC-LC-IRMS allows separation of nucleic acids from soil co-extracts like humic acids as well as carbon contaminants from protocol reagents thus improving the measurement potential for stable carbon isotope analysis of nucleic acids.

Chapter 3 focuses on elucidating the relationship of microbial carbon turnover to its molecular size. This was realized using the long term C3-C4 vegetation change experiment performed on two soil types. Stable carbon isotope analysis of CFE derived soil microbial biomass and its molecular size classes using the new SEC-LC-IRMS application allowed relative quantification of new C4 plant derived and old C3 SOM derived carbon in microbial biomass.

In **Chapter 4**, soil dissolved organic carbon sources and its seasonal dynamics are elucidated through analysis of soil DOC size fractions. Here, DOC collected from the C3-C4 vegetation change experiment was analyzed for natural abundance level changes in stable carbon isotope content. The main aim of this study was to understand the microbial linkages to DOC formation and decomposition. The section finally discusses the probable mechanisms of vertical transport of DOC in the soil profile and the role of biotic and abiotic factors that decide the fate of DOC.

Chapter 5 addresses objective 2, 3 & 4; and is based on the results from the pulse $^{13}CO_2$ plant labeling experiment. The analyses focused on stable isotope measurement of different microbial biomarkers like DNA, RNA, fatty acids, chloroform fumigation extraction derived microbial biomass and its size classes. The pulsed plant ^{13}C was temporally monitored into these markers. Plant carbon was also monitored into different microbial functional groups using GC-IRMS based ^{13}C measurement of different signature phospholipid and neutral lipid fatty acids. The chapter then discusses the implications of differential carbon turnover of microbial biochemical fractions and the trophic interaction in the microbial food web.

The results from the different studies are summarized, discussed and evaluated with potential implications and future prospects in **Chapter 6.**

Chapter 2

Carbon stable isotope analysis of size separated organic compounds in aqueous mixtures by coupling size exclusion chromatography to LC-IRMS

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Accepted: August 2012 in *Environmental Science and Technology*

ABSTRACT:

Stable isotopic content of dissolved organic carbon ($\delta^{13}C$-DOC) provides valuable information on its origin and fate. In an attempt to get additional insights into DOC cycling, we developed a method for $\delta^{13}C$ measurement of DOC size classes by coupling high-performance liquid chromatography (HPLC) – size exclusion chromatography (SEC) to online isotope ratio mass spectrometry (IRMS). This represents a significant methodological contribution to DOC research. The interface was evaluated using various organic compounds, thoroughly tested with soil water from a C3-C4 vegetation change experiment and also applied to riverine and marine DOC. $\delta^{13}C$ analysis of standard compounds result in excellent analytical precision ($\leq$0.3‰). Chromatography resolved soil DOC into 3 fractions: high molecular weight- HMW (0.4-10 kDa), low molecular weight- LMW (50-400 Da) and fully retained fraction- FR. Sample reproducibility for measurement of $\delta^{13}C$-DOC size classes was ±0.25‰ for HMW fraction, ±0.54‰ for LMW fraction and ±1.3‰ for FR fraction. The greater variance in $\delta^{13}C$ values of the latter fractions was due their lower concentrations. The limit of quantification (SD $\leq$0.6‰) for each size fraction measured as a peak is 200 ngC (2 mgC/L). $\delta^{13}C$-DOC values obtained in SEC mode correlated significantly with those obtained without column in the μEA mode ($p<0.001$, intercept 0.17‰), which rules out SEC-associated isotopic effects or DOC loss. In the vegetation change experiment, fractions revealed a clear trend in plant contribution to DOC; those in deeper soils and smaller size fractions had less plant material. It was also demonstrated that the technique can be successfully applied to marine and riverine DOC without further sample pretreatment.

INTRODUCTION:

Dissolved organic carbon (DOC) in soil and aquatic systems is an important carbon pool that plays a central role in many biogeochemical processes (Hansell et al., 2009; Hedges and Oades, 1997; van Hees et al., 2005). DOC is a complex heterogeneous mixture of compounds ranging from low molecular weight (LMW) aliphatic and aromatic compounds to macromolecules sometimes larger than 100 kDa (McDowell and Likens, 1988; Strobel et al., 2001). More recent investigations using high resolution mass spectrometry or other molecular-level analysis, however, suggest that majority of compounds in DOC are ~1 kDa size range (Hertkorn et al., 2006; Kujawinski et al., 2002; Ouellet et al., 2008). These results are in agreement with studies of molecular size distribution of DOC using size exclusion chromatography (SEC) (Her et al., 2002a; Müller et al., 2000; Römkens and Dolfing, 1998). It is, however, unknown whether molecular weight size fractions show differential chemical reactivity and bioavailability. Stable carbon isotopic content ($\delta^{13}C$ values) of DOC gives valuable information on its origin and cycling (Fröberg et al., 2007a; Steinbeiss et al., 2008). A combination of isotope analysis and DOC size fractionation would thus be valuable in elucidating the origin and fate of individual molecular size classes of DOC.

In the terrestrial context, the natural isotopic differences between C3 ($\delta^{13}C \approx -27$ ‰) and C4 plants ($\delta^{13}C \approx -14$ ‰) can be used to differentiate the sources of carbon in soil DOC. In soils undergoing a vegetation change from C3 to C4 plants, soil organic matter (SOM) is naturally labeled with C3 plant signature and the new plant carbon introduced into the soil in the form of litter or root exudates is labeled with C4 plant signature. Thus using stable isotope measurements it is possible to distinguish between the old carbon derived from C3 vegetation and the new C4-derived carbon (Fröberg et al., 2007a; Kramer and Gleixner, 2008). In the aquatic realm, $\delta^{13}C$ of freshwater DOC is generally more depleted (≈ -27 ‰) compared to that of marine systems which tends to

be more enriched (≈ −21 ‰) (Boschker and Middelburg, 2002; Fry et al., 1998). In estuaries and coasts which are mixing zones of fresh and marine water, $\delta^{13}C$ of DOC can vary between the two and this depends on various factors (Cifuentes and Eldridge, 1998). Stable isotope analysis of aquatic DOC can thus provide valuable information on biogeochemical cycling as well as to trace the origins of organic compounds in the environment.

Several techniques have been reported to measure the stable isotope composition of DOC (De Troyer et al., 2010; Panetta et al., 2008; St-Jean, 2003). The commercially available high-performance liquid chromatography (HPLC) interfaced with isotope ratio mass spectrometry (IRMS) has been a technological leap in carbon stable isotope analysis of aqueous mixtures (Krummen et al., 2004). This system allows efficient chromatographic separation of injected mixtures which then are sequentially oxidized under acidic conditions and the resulting CO_2 after separation from the liquid phase is passed into the mass spectrometer for isotope measurements. The system has undergone subsequent improvements (Hettmann et al., 2007) and a number of applications with amino acids, carbohydrates and cellular metabolites have been successfully demonstrated (Boschker et al., 2008; Heuer et al., 2006; McCullagh et al., 2006). Recently, it has been successfully demonstrated that the interface can be used to measure $\delta^{13}C$ values of DOC in soils and streams (Albéric, 2011; Scheibe et al., 2012). However, almost no results have been reported for on-line DOC size fractionation coupled to carbon stable isotope analysis. Some authors have successfully measured stable isotope ratios of chromatographically separated fractions in natural samples like landfill leachates but it involved preparative fraction collection followed by off-line analysis using a total carbon analyzer interfaced with IRMS (Marschner et al., 2005; Mohammadzadeh et al., 2005). Others have demonstrated the use of solid phase extraction for elution of adsorbed organics prior to compound specific isotope analysis (Benbow et al., 2008). More recently, $\delta^{13}C$ analysis of organic samples was performed using wet oxidation followed by headspace sampling of evolved CO_2 using a GasBench preparation device coupled to an IRMS (Lang et al., 2012). Thus a new operational on-line interface with HPLC-SEC coupled to an IRMS represents a significant analytical contribution to DOC research.

Here we validate the measurement of stable isotope ratio of DOC size classes by employing size exclusion chromatography for molecular size-dependent separation of DOC compounds. This system interfaced with HPLC-IRMS allowed direct on-line isotope analysis of the eluted size fractions. The technique was first evaluated using various organic compounds and later applied to soil water samples from a C3-C4 vegetation change experiment. We also tested riverine, coastal and marine water samples to demonstrate the feasibility of the method to measure $\delta^{13}C$ values of aquatic DOC. Sample analysis is simple and fast as no sample pre-processing is required except the removal of inorganic carbon. Furthermore, an enhanced detection limit allows measurement of low concentration natural samples including marine DOC without pre-concentration.

EXPERIMENTAL:

Instrumentation

DOC size fractionation and isotope analysis was carried out using an HPLC system coupled to a Delta^{+} XP IRMS through an LC IsoLink interface (Thermo Fisher Scientific, Germany). Details of the LC-IRMS system and modifications included are given elsewhere (Hettmann et al., 2007; Scheibe et al., 2012). A chromatographic column was introduced into the interface through a six port valve. We used a mixed bed analytical column (TSK-

GEL GMPW$_{XL}$- 7.8 mm × 30 cm; Tosoh Bioscience, Germany) with a guard column (TSKgel PW$_{XL}$); both maintained at a temperature of 25°C using a column oven. Analytes eluting from the column were quantitatively oxidized using peroxodisulphate at an acidic pH in a heated oxidation reactor. CO_2 produced was subsequently separated from the cooled liquid phase in a membrane separation unit and then entrained into a stream of He. CO_2 from individual eluting peaks were dried using two online Nafion gas dryer units and the excess O_2 produced during the oxidation step was removed using a copper filled reduction reactor (Hettmann et al., 2007). The gas was then directly admitted into the IRMS via an open split.

Sample injection was performed using an autosampler (Surveyor autosampler, Thermo Fisher Scientific). 100 µL of analyte was injected into the mobile phase maintained at a constant flow rate of 500 µL/min using a Surveyor MS pump. Various eluents were tested for efficient separation of DOC; these included double distilled water, KH_2PO_4 solution 20 mM (pH 3.5) and phosphate buffer 20 mM (pH 6.2). Eluents and reagents were degassed under vacuum (20 mbar) in an ultrasonic bath for 30 min and to prevent regassing a constant helium stream was maintained in solutions during analysis. Chromatographic runs were made for 45 min.

Standardization

The HPLC system was first evaluated for chromatographic resolution of the column using molecular sizes standards: sodium bicarbonate - 84 Da (Carl Roth, Germany); polyethylene glycol (PEG) 200 Da and 600 Da (Alfa Aesar, Germany); and polyethylene oxide (PEO) 24 kDa, 107 kDa and 930 kDa (Tosoh, Japan). Linearity of the coupled system was ascertained using varying concentrations (5-40 mgC/L) of different organic compounds that included a sugar (sucrose, $\delta^{13}C$ = –25.95‰), an organic acid (citrate, –18.58‰), a glycol (PEG, –30.45‰) and an amino acid (arginine, –13.5‰). Standards were supplied by Carl Roth, Germany and Merk, Germany. The LC-IRMS was thus calibrated against these standards that were in turn calibrated against the international reference standard VPDB (Werner and Brand, 2001). Pulses of CO_2 reference gas ($\delta^{13}C_{CO2}$ = −38.16 ‰) were also used for calibration of the LC-IRMS system during every chromatographic run.

Samples

Soil water samples were collected from a long term field experiment at the Max Planck Institute for Biogeochemistry, Jena, Germany. Two soil plots (48 m^2 each) were established in 2006 one with forest A-horizon soil; "Sandy" (50 % sand, 44 % silt and 6 % clay; pH 6.9) and the other with former B-horizon of a calcareous soil; "Clayey" (9 % sand, 75 % silt and 16 % clay; pH 7.8). Both soils previously carried continuous C3 vegetation. Each of the two plots was subdivided into C3 and C4 patches. The latter subplot was continuously cultivated with C4 plants since 2006 and the former was maintained in C3 cultivation for comparison of $\delta^{13}C$ values. The average $\delta^{13}C$ of C3 and C4 vegetation was −28.3 ‰ and −13.4 ‰, respectively. More details about the experimental site are provided in Appendix 1 (Table A-1.1).

Soil water was extracted using borosilicate glass suction plates (thickness- 9 mm, diameter- 120 mm, pore size- 1 µm; UMS, Germany) at 10, 20 and 30 cm depth in triplicates from all four plots (n=3x3x4=36). A vacuum of 200 mbar was applied to suck soil solution into 2 L borosilicate flasks that was collected on the subsequent day. Sampling was carried out in March 2012. Samples were immediately frozen (−20°C) until

analysis. All water samples were acidified and purged in order to remove the dissolved inorganic carbon (DIC). 1 mL of sample was placed in 1.5 mL brown glass vials (silanized), 20 µL of 8.5 % phosphoric acid (Merk, Germany) was added and vortexed for a minute. Samples were purged with a gentle stream of nitrogen for 10 min using stainless steel syringe needles fitted to an automated 12-port-chamber (VisiprepTM, VisidryTM, Supelco, Sigma-Aldrich, USA). Both acidified (DOC) and non-acidified (total dissolved carbon- TDC) samples were analyzed by LC-IRMS in the column mode (HPLC mode) as well as the bulk mode (µ-EA mode). In the µEA mode, the HPLC column is excluded from the interface and thus it measures the isotopic composition of bulk DOC (Scheibe et al., 2012). System linearity for peak height/area as well as isotope ratios for environmental samples was tested using a dilution series of two soil water samples with an original concentration of ~20 mgC/L. Sample analysis was always performed in triplicates.

We also tested DOC from aquatic environments. Freshwater samples were obtained from River Saale in Jena, Germany in May 2012. Marine water samples were collected onboard ORV Sindhu Sankalp in June 2011 along the west coast of India (off Mangalore, eastern Arabian Sea). We analysed water samples obtained from the mixed layer depth at a coastal station and an offshore station. These samples were treated and prepared in the same way as the soil solutions.

Data acquisition and analysis

Isodat 2.0 SP 2.67 software (Thermo Fischer Scientific, Germany) was used to control the HPLC-IRMS system. The basic algorithms of the software were used to perform data processing and optimization of the chromatograms. The algorithm also considers the background signal and subtracts it from the peak calculations. Wherever necessary, peak and baseline corrections were performed manually. To verify the reliability of results obtained by SEC coupled to LC-IRMS, bulk DOC $\delta^{13}C$ values calculated using the $\delta^{13}C$ of separated size fractions were compared with the measured $\delta^{13}C$ of bulk DOC (by µEA mode). Bulk values were calculated as the weighted mean from $\delta^{13}C$ values of individual DOC fractions analyzed by column mode.

RESULTS AND DISCUSSION:

Optimization of SEC-HPLC-IRMS

The HPLC-IRMS system has been successfully adapted for fractionation of DOC and online stable isotope analysis of resolved fractions by interfacing SEC into the system. The mixed bed polymer column with hydroxylated polymethacrylate as the base material (pore size 100Å-1000Å) achieved a simple chromatographic separation of DOC components based on their hydrodynamic size. This column was chosen as it allows separation of aqueous mixtures with a broad molecular weight distribution. Molecules with larger hydrodynamic radii that do not fit into the pores elute out of the column first, whereas smaller molecules enter the pores and are thus retained. A fully retained component will elute last and will have the highest retention time. Thus the molecular weight of the separated compounds is inversely proportional to the retention time in the SEC column.

Mobile phase composition is an important parameter that affects chromatographic separation of components in a mixture and hence it is essential to choose an eluent that provides the best resolution and sensitivity. A major analytical constraint of an HPLC-IRMS interface is that only inorganic buffers can be used (Krummen et al., 2004). Among the different eluents tested for optimal separation of

DOC, phosphate buffer 20 mM at pH 6.8 gave the best results. The high background (~ 0.4 V) associated with phosphate buffer can be reduced significantly by slight acidification of the eluent with orthophosphoric acid (this changes the pH of the eluent to 6.2) without having any effect on DOC resolution. Eluent flow rate was optimized at 500μL/min.

Standardization

After achieving optimal separation of DOC, the SEC column was calibrated with PEG and PEO size standards. This yielded a significant logarithmic relationship between retention time and molecular weight ($p < 0.001$, $R^2 = 0.99$, n=6) (Appendix 1: Figure A-1.1A). This equation was used to calculate the approximate molecular size of individual fractions in the DOC. Molecular weight determination of DOC size classes with these calibration curves gives only an apparent value; since the matrices of test substances used match only moderately to that of the DOC compounds. A highly linear relationship was also observed between concentration of standards and area of eluted peaks ($p < 0.001$, $R^2 > 0.99$, n=5) which was used to calculate concentration of the resolved DOC size fractions (Appendix 1: Figure A-1.1B). For the different standards tested the slope of the regression line was very similar, only for arginine we observed a slight deviation at concentrations higher than 20 mgC/L.

Stability and linearity of the IRMS was measured with CO_2 reference gas and standards. Multiple injections of CO_2 reference gas at a constant pressure into the IRMS inlet gave very precise isotope ratios (mean standard deviation, SD- 0.03 ‰, n=10). CO_2 reference gas pulses with increasing pressure were also admitted into the IRMS, the resulting isotope values being highly reproducible (SD- 0.15 ‰, n=7) (Appendix 1: Figure A-1.1C). The linearity test for isotope measurements with organic compounds as standards at different concentrations also gave results within the acceptable limits with a negligible slopes (SDs: Citrate- 0.19 ‰, n=10; Arginine- 0.15 ‰, n=8; Sucrose- 0.28 ‰, n=8; PEG- 0.3 ‰, n=5) (Appendix 1: Figure A-1.1D). These values were verified with the standard EA-IRMS with acceptable accuracy (Scheibe et al., 2012). The precision and accuracy of HPLC-IRMS and reliable data interpretation has also been evaluated in other publications (Boschker et al., 2008; Godin et al., 2005; McCullagh, 2010). There was no isotopic fractionation during HPLC separation in the SEC column as the well-known "isotope swing" was not observed in the isotope ratio trace values of standard materials (Appendix 1: Figure A-1.2).

DOC size fractionation

HPLC of soil DOC samples resulted in chromatograms with 2 or 3 discrete peaks (Figure 2.1). After assessing the chromatograms, different size fractions as recurring peaks were assigned to 2 or 3 retention time intervals. The first peak eluting from 27 to 31 min was assigned as the "high molecular weight"- HMW (0.4-10 kDa) size class. The second peak with retention time between 31 and 34 min was assigned as the "low molecular weight"- LMW (50-400 Da) fraction. The third fraction eluted after 35 min outside the void volume of the column and corresponds to organic compounds that interact with the column material and are thus retained. This fraction was named the "fully retained"- FR fraction and no molecular size range were assigned to it. Such fractionation of DOC has been demonstrated in other studies that estimated the molecular weights of organic matter fractions in water by combining SEC with total carbon analyzers; two to four fractions of DOC have been reported (Her et al., 2002a;

Her et al., 2002b; Römkens and Dolfing, 1998). The DOC detector of the HPLC-IRMS interface also allows accurate estimation of carbon concentration of the resolved size fractions. The average concentration of the three fractions viz. HMW, LMW and FR fraction across all sampling plots and depths (n=36) was 6.8 ± 3.6 mgC/L, 2.1 ± 0.8 mgC/L and 1.1 ± 0.2 mgC/L, respectively.

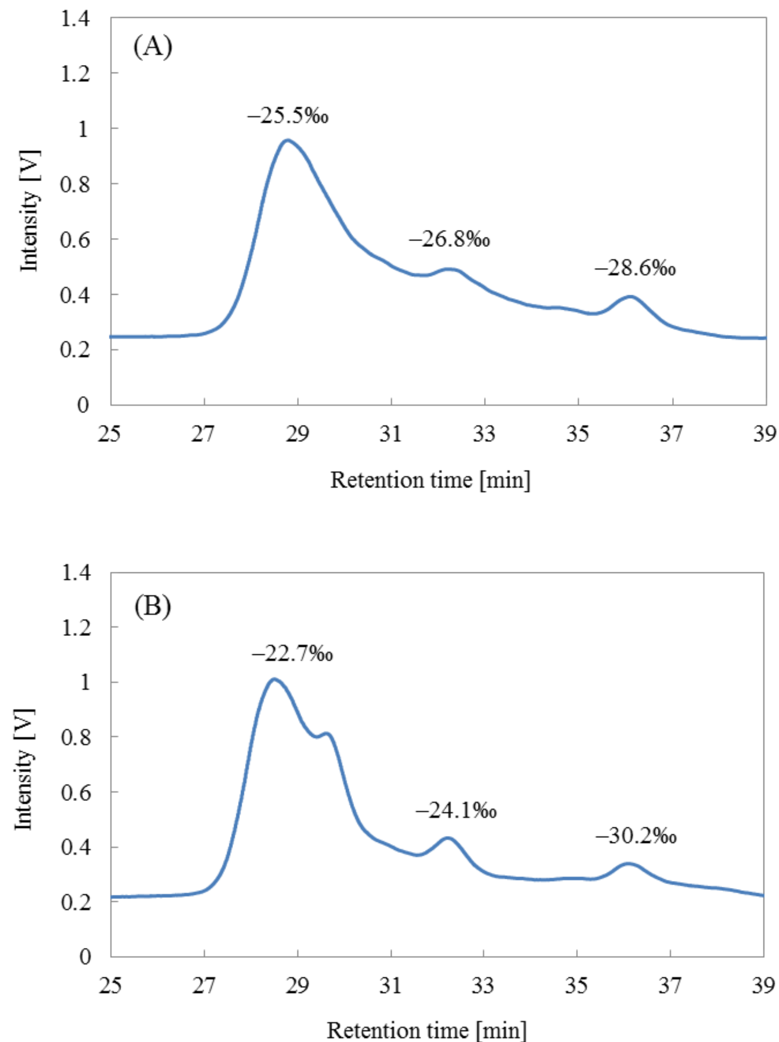

Figure 2.1: SEC-HPLC-IRMS chromatograms showing molecular size-based separation of DOC. Size fractionation of soil DOC from (A) "Sandy" and (B) "Clayey" soil both with vegetation change is presented here. Three fractions were observed: HMW fraction (0.4-10 kDa) elutes first from 27 to 31 min, LMW fraction (50-400 Da) elutes between 31 and 34 min and a retained (R) fraction elutes after 35 min. $\delta^{13}C$ values of fractions are presented next to the peaks.

Performance of SEC-HPLC-IRMS

The analytical performance of the system for measurement of $\delta^{13}C$ values, column retention time and peak area was thoroughly tested with soil DOC samples from a C3-C4 vegetation change experiment. Sample reproducibility for measurement of $\delta^{13}C$ of DOC size classes was excellent (±0.25‰) for HMW fraction, good (±0.54‰) for LMW fraction and acceptable (±1.3‰) for FR fraction. The greater variance in $\delta^{13}C$ values of the latter fractions is due their lower concentrations, the limit of quantification-LOQ (SD ≤0.6‰) for each size fraction measured as a peak was 200 ngC that corresponds to 2 mgC/L (Figure 2.2). In most samples the FR fraction was below the LOQ and hence the isotope values for this fraction are not satisfactory. The concentration values obtained for individual fractions using the peak area-calibration curve demonstrated excellent precision (SD ±0.16 mgC/L). Replicated analysis of the same sample resulted in chromatograms that were nearly identical with regard to retention time and peak height (data not shown).

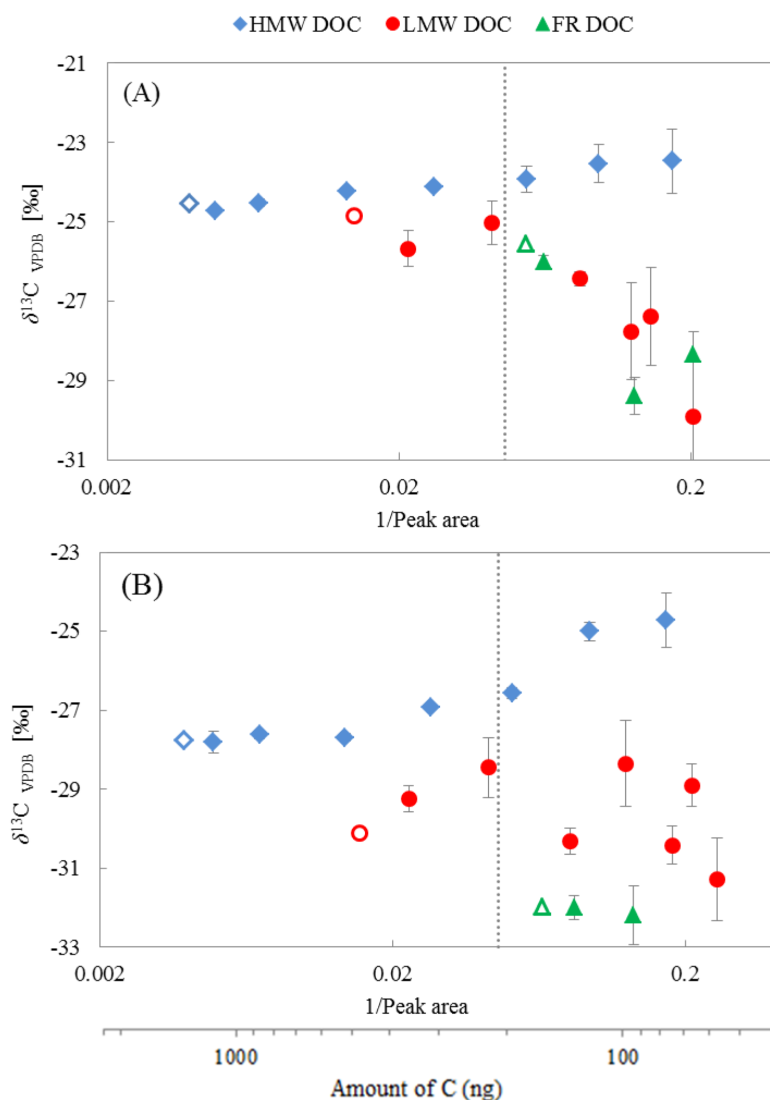

Figure 2.2: Dilution series of two soil water samples with an original concentration of ~20 mg C/L. Empty markers represent values for size fractions in the original sample. The dashed line at 200 ngC corresponds to the limit of quantification.

$\delta^{13}C$ measurements of bulk DOC using HPLC-IRMS have been validated with a standard EA-IRMS by analyzing freeze-dried samples (Scheibe et al., 2012). We compared the $\delta^{13}C$-DOC values obtained by column mode with the previously corroborated µEA mode on the same system. Using the $\delta^{13}C$ values of separated size fractions, bulk $\delta^{13}C$-DOC was calculated as a weighted mean and was compared with the measured $\delta^{13}C$ values of bulk DOC samples (by µEA mode). The measured bulk values correlated significantly with the calculated weighted mean ($p<0.001$, R^2 0.95, intercept 0.17 ‰) (Figure 2.3A). The total concentration of DOC samples obtained as the sum of individual size fractions also showed excellent correlation when compared to that measured by the µEA mode ($p<0.001$, R^2 0.99, intercept 1.2 mgC) (Figure 2.3B). Results obtained using SEC are in line with the bulk DOC values that rules out column associated isotopic effects or loss of DOC.

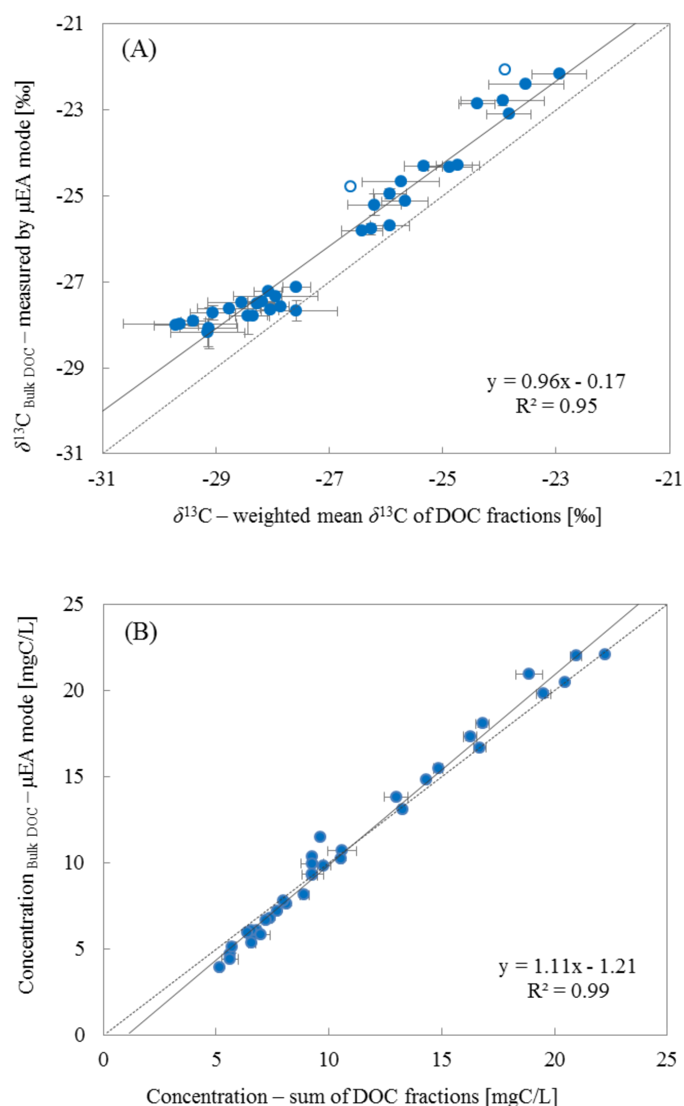

Figure 2.3: Comparison of $\delta^{13}C$-DOC measured by µEA/IRMS (bulk mode) with that obtained as the weighted mean of $\delta^{13}C$ values of separated DOC fractions (A). Also compared are DOC concentrations measured by µEA mode with that obtained as the sum of concentrations of resolved DOC size fractions (B). Note: outliers (empty markers) were excluded.

Effect of DIC removal

Acidification of soil water releases the DIC and is necessary for reliable measurements of DOC (De Troyer et al., 2010; Panetta et al., 2008; St-Jean, 2003). Chromatogram overlaps of unprocessed (total dissolved carbon/TDC) and acidified (DOC) samples showed that in unacidified samples DIC interacts with the DOC compounds and significantly alters isotope ratios as well as retention time of fractions (Figure 2.4). DIC was mainly eluted in the FR fraction constituting more than 80% of the peak, but also affected other size fractions (contributing ≈ 20 % to the peaks). The latter effect could be due to chemical interaction of DIC with certain compounds in the DOC. This reiterates that reliable isotope analysis of DOC size fractions requires complete removal of DIC from soil water samples.

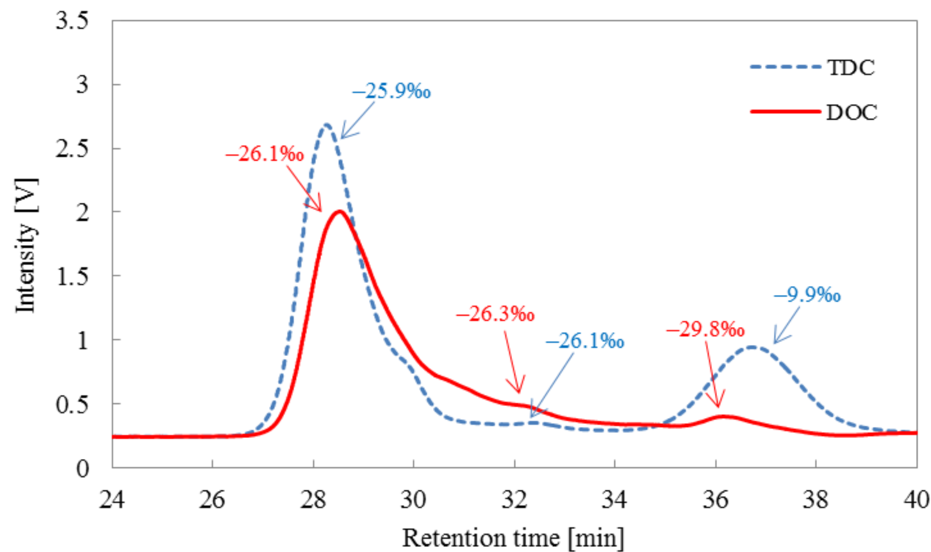

Figure 2.4: Chromatogram overlap of a representative soil DOC sample to illustrate the effect of decarbonization on isotope analysis; TDC (total dissolved carbon): unprocessed sample, DOC: acidified and purged. δ^{13}C values of fractions are presented next to the peaks.

δ^{13}C of soil DOC size classes

In the C3-C4 vegetation change experiment, δ^{13}C of DOC fractions showed distinct patterns based on soil and vegetation type, sampling depth and molecular size of fractions (Figure 2.5). Since substrates (plant and soil organic matter) as sources of DOC in C3 plots without vegetation change have the same isotopic signature of ~ −28.3 ‰, these plots can be used as a control to monitor the degree of isotopic fractionation in different DOC size classes. Here, the HMW DOC was isotopically very similar to the substrate ($\delta^{13}C_{HMW}$ = −27.9 ± 0.4 ‰), whereas the LMW and the FR fractions were isotopically depleted compared to the substrates available ($\delta^{13}C_{LMW}$ = –29.6 ± 0.03 ‰, $\delta^{13}C_{FR}$ = –30.4 ± 1.2 ‰). The contrasting isotopic signature in the DOC from C4 plots is clearly visible (Figure 2.5). Here, the isotope ratios of the HMW and the LMW fractions showed contribution of C4 plant carbon ($\delta^{13}C_{HMW}$ = –24.5 ± 1.1 ‰, $\delta^{13}C_{LMW}$ = – 25.8 ± 0.8 ‰). However, the FR fraction of DOC from C4 plots showed isotopic signatures of the old C3-devided soil organic matter ($\delta^{13}C_{FR}$ = –29.5 ± 0.6 ‰). ^{13}C tracer

experiments at natural abundance can thus help track the flow of carbon from differentially labeled plant biomass into different fractions of soil DOC.

Application to marine and freshwater DOC

We also applied the technique to DOC samples from aquatic environments and tested freshwater and marine samples. Freshwater DOC from river Saale resolved into 2 size classes very similar to the soil DOC fractions (Figure 2.6A). Both size fractions showed isotopic signatures of C3 plants and these values are very similar to those reported by other authors (Albéric, 2011; Ouellet et al., 2008; Panetta et al., 2008) as well as to soil DOC values that we have reported here (Figure 2.5). Marine DOC resolved into 5 discrete fractions, though some fractions showed very low abundance displaying tiny peaks in the chromatograms (Figure 2.6B-C). δ^{13}C values of all size fractions in the near shore DOC reflect the isotopic values of both phytoplankton (≈ −21 ‰) and terrestrial C3 plants (≈ −27 ‰) (Boschker and Middelburg, 2002; Fry et al., 1998; Panetta et al., 2008). The offshore/deep sea water sample showed a distinct pattern of size fractions with isotopic signatures of phytoplankton as well as terrestrial C3 plants in addition to very depleted values for the LMW fractions (Figure 2.6C).

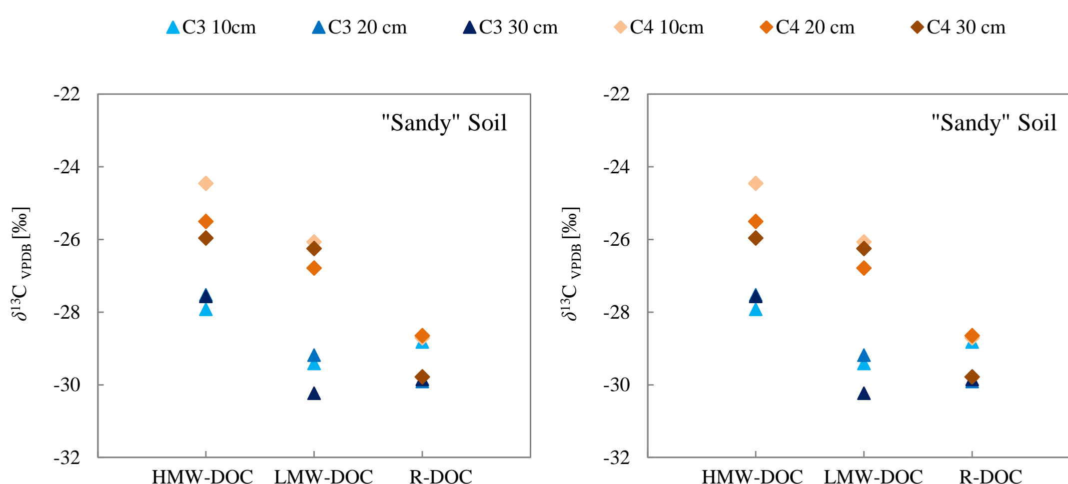

Figure 2.5: Absolute δ^{13}C values of individual DOC fractions from the two soils at 10, 20 and 30 cm depth. Note: error bars are not shown for better visual representation. Each point represents mean value of three samples measured in triplicates and average standard error of measurement for HMW, LMW and R fraction was ±0.25, ±0.54 and ±1.3‰, respectively.

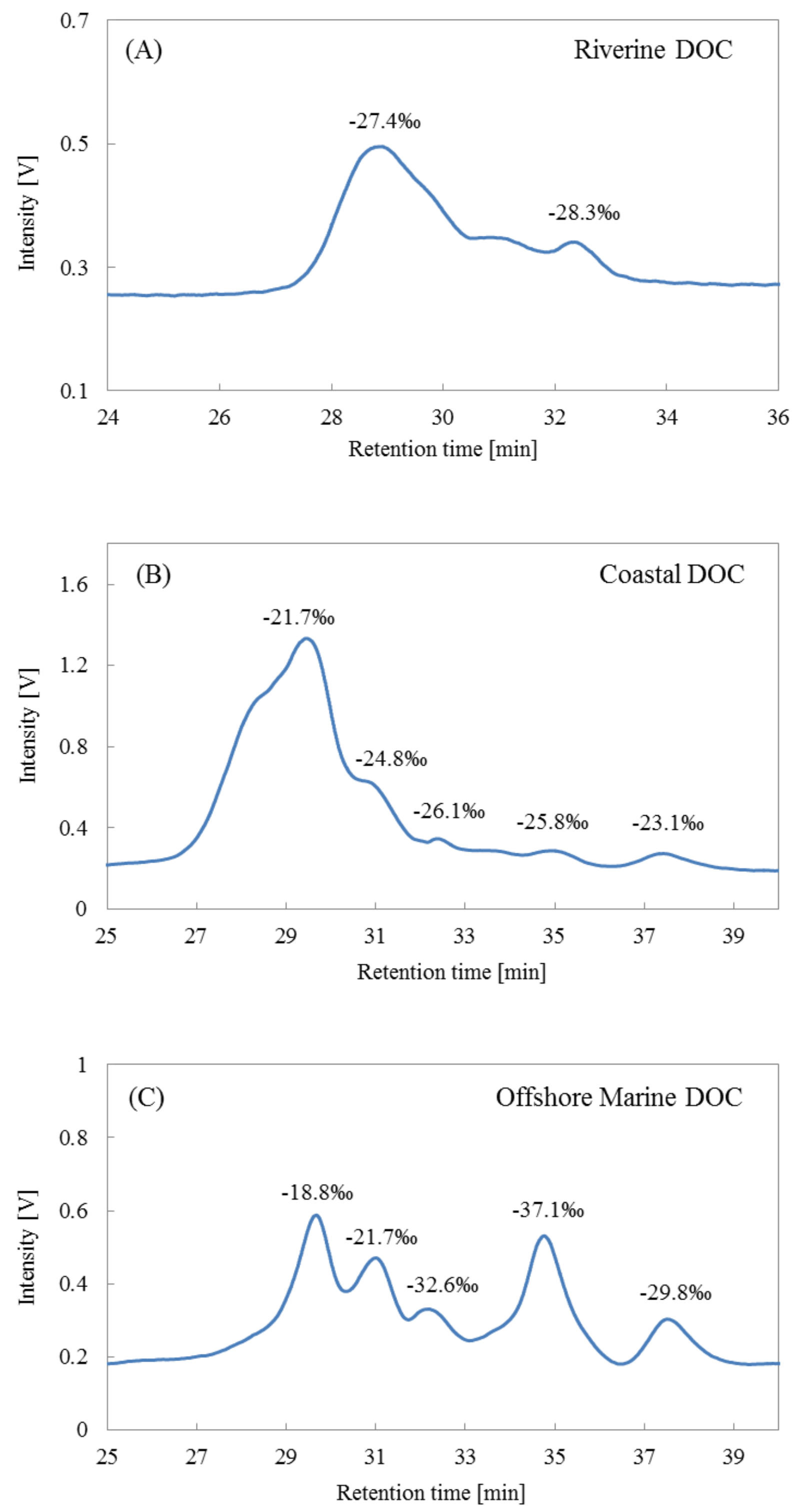

Figure 2.6: SEC-HPLC-IRMS chromatograms of DOC from (A) river Saale; (B) coastal and (C) offshore Arabian Sea water. δ^{13}C values of fractions are presented next to the peaks.

Here we tested the technique for marine and freshwater samples and the aim of this application was to demonstrate resolution of DOC into size fractions with distinct isotopic signatures. By coupling SEC with HPLC-IRMS in a continuous interface, the system offers potential for fast and reliable measurement of stable isotope ratios in terrestrial DOC size fractions which can be extended to freshwater and marine samples. Further work needs to be done to evaluate the technique to measure aquatic samples which often contain very small amounts of DOC. Improved chromatographic resolution using other modes of chromatography in the system can provide more insight into the composition of DOC and the source of various organic compounds.

Chapter 3

Soil microbial carbon turnover decreases with increasing molecular size

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Accepted: February 2013 in *Soil Biology and Biochemistry*

ABSTRACT:

It is well established that soil microorganisms play an important role in respiration of newly fixed plant carbon. Recent results show that they also contribute significantly to soil organic matter (SOM) formation. We hypothesized that different molecular size classes of compounds in soil microbial biomass (SMB) have variable turnover time and in consequence influence SOM formation differentially. Here we used natural differences in carbon stable isotope signatures (δ^{13}C values) after C3-C4 vegetation change to track newly fixed C4 plant carbon into SMB molecular size classes. SMB was obtained by chloroform fumigation extraction and δ^{13}C values of its size classes were measured using size exclusion chromatography coupled online to liquid chromatography – isotope ratio mass spectrometry (SEC-LC-IRMS). Resolved SMB was assigned to 5 size classes: 1800-9800 Da, 800-1800 Da, 380-800 Da, 180-380 Da and 50-180 Da. The contribution of recent C4 plant carbon to size classes of SMB decreased with increasing molecular weight (MW). It ranged from 77±19% in the lowest MW size class size class to 41±14% in the 1800-9800 Da size class in 'Sandy' soil and from 59±18% in the lowest MW size class to 8±15% in the highest MW size class in 'Clayey' soil. A decreasing carbon turnover of compounds in SMB extracts along a continuum of molecular size from small to large implies that low molecular weight microbial compounds are rapidly metabolized products that link to fast respiratory carbon fluxes, whereas high molecular weight ones could be products of microbial synthesis like structural compounds that have slower turnover rates and link to slower SOM formation.

INTRODUCTION:

Microorganisms play a central role in many soil processes, including decomposition and mineralization of organic matter. There is growing evidence that they are also responsible for carbon sequestration by production of compounds that persist in soil and form part of the stabilized soil organic matter (SOM) (Benner, 2011; Gleixner et al., 2002; Liang and Balser, 2011; Schimel and Schaeffer, 2012). It has been demonstrated that SOM is predominantly of microbial origin (sometimes up to 80%) as either living cells, their products or non-living biomass (Kindler et al., 2006; Miltner et al., 2012; Simpson et al., 2007). The macromolecular structure and biochemical composition of the microbially sourced organic matter varies with microbial species and cell type and largely controls the stability and persistence of SOM (Gleixner, 2013; Kiikkilä et al., 2012; Simpson, 2002). It is however unknown whether different molecular size classes of soil microbial biomass show differential reactivity, origin and turnover time.

Since most of SOM is of microbial origin and/or has been microbially processed, insights into the turnover of microbial biomass would help gain a better understanding of carbon cycling in soils. Stable isotope analysis provides a useful tool to track the flow of carbon in environments. Soils that have undergone vegetation change from C3 to C4 or vice versa are ideally suited to track carbon flow into different soil compartments at natural isotope abundance levels. In such a system with C3-C4 vegetation change, recent C4 plant carbon can be traced into the microbial biomass and be differentiated from SOM-derived carbon which has a C3 plant signature (Blagodatskaya et al., 2011; Kramer and Gleixner, 2008). An easy way to obtain microbial biomass residues from soil is biocidal fumigation which lyses microbial cells and releases their contents (Tate et al., 1988; Vance et al., 1987). Chloroform fumigation-extraction is widely used to estimate the amount of soil microbial biomass carbon (Franzluebbers, 1999; Philippot et al., 2012) and in combination with stable isotope analysis has been used to identify the source of

microbial biomass carbon as well as to measure its turnover rate (Dijkstra et al., 2006; Ryan and Aravena, 1994).

This study aims to relate the molecular weight of compounds extracted from soil microbial biomass (SMB) to their turnover. To achieve this we used a long term field experiment with C3-C4 vegetation change performed on two soil types and used stable isotope signatures to track photosynthetically fixed carbon into SMB. Here we demonstrate that compounds in SMB have decreasing carbon turnover along a continuum of molecular size from small to large.

EXPERIMENTAL:

We used the online size exclusion chromatography–liquid chromatography–isotope ratio mass spectrometry (SEC-LC-IRMS) to measure the $\delta^{13}C$ values of different size classes of microbial biomass (Malik et al., 2012; Scheibe et al., 2012). Soil extracts were obtained from two soils with C3-C4 vegetation change which allowed us to estimate the contribution of newly fixed C4 plant carbon (< 5 years) into different molecular size classes of microbial biomass. The experimental site, located at the Max Planck Institute for Biogeochemistry in Jena-Germany, consisted of two soil types that contrasted in soil texture and pH. The first "Sandy" (50 % sand, 44 % silt and 6 % clay; pH 6.9) was originally derived from forest A-horizon and the second "Clayey" (9 % sand, 75 % silt and 16 % clay; pH 7.8) from B-horizon of a calcareous soil. Both soils originally had C3 vegetation; since 2006 they were cultivated with C4 crops. A part of each soil plot was continued in C3 vegetation for comparison of $\delta^{13}C$ values. More details about the experimental site are provided elsewhere (Malik et al., 2012). Soil samples were collected in September 2011 (late vegetation period) from 0-10 cm depth using a 5 cm diameter stainless steel corer (n=3).

Soils were sieved <2 mm and chloroform fumigation extraction performed immediately based on the method by Vance et al., 1987 with slight modifications which were aimed at: 1) increasing the C concentration in the extracts (Needelman et al., 2001), 2) avoiding the necessity of dilution of salty extracts (Appel, 1998; Haney et al., 2001), 3) reducing the extraction time and preventing rapid microbial degradation of labile C compounds (Rousk and Jones, 2010). 7g wet soil was fumigated with chloroform gas for 24 h followed by repeated (3 times) evacuation with vacuum. A non-fumigated control was maintained with the same amount of soil. Organic carbon was extracted from fumigated and non-fumigated soils with 0.05M K_2SO_4 solution in a ratio of 1:4 (w/v). This mixture was homogenized on an orbital shaker (250 rev per min, 30 min), centrifuged for 5 min at 12000 g and then filtered using prewashed Whatman filter paper. The whole extraction procedure was completed in 40 min.

Soil extracts were acidified and purged with nitrogen gas in order to remove the dissolved inorganic carbon (DIC) and analyzed using SEC-HPLC-IRMS (Malik et al., 2012). Measurements were carried out using an HPLC system coupled to a Delta$^+$ XP IRMS through an LC IsoLink interface (Thermo Fisher Scientific, Germany). SEC was performed on a mixed bed analytical column (TSK-GEL GMPW$_{XL}$- 7.8 mm × 30 cm; Tosoh Bioscience, Germany) maintained at a temperature of 25 °C using a column oven. 100 μL aliquot of soil extracts was injected using an autosampler (Surveyor autosampler, Thermo Fisher Scientific) into the mobile phase that consisted of phosphate buffer 20 mM (pH 6.2) maintained at a constant flow rate of 500 μL/min using a Surveyor MS pump. All reagents and the eluent were degassed under vacuum (20 mbar) in an ultrasonic bath for 30 min, and to prevent regassing a constant stream of helium was maintained in solutions during analysis. Chromatographic runs were performed for 45 min. Based on

the calibration curves of polyethylene oxide and polyethylene glycol as molecular weight (MW) standards, the apparent MW of microbial biomass size classes was determined using their retention time (Malik et al., 2012).

RESULT AND DISCUSSION:

LC-IRMS can be used for fast and reliable measurement of carbon stable isotope ratios in aqueous mixtures like dissolved or water extractable organic carbon (Scheibe et al., 2012). We used the system to measure δ^{13}C values of SMB obtained by the chloroform fumigation extraction method, and to get additional insights we performed its online size fractionation. SEC of OC in the fumigated extracts were assigned to 5 size classes (Figure 3.1) eluting between 27-29 min (fraction 1/F1), 29-30 min (F2), 30-31 min (F3), 31-32 min (F4) and 32-33.5 min (F5). Apparent MW of size classes were estimated as 1800-9800 Da (F1), 800-1800 Da (F2), 380-800 Da (F3), 180-380 Da (F4) and 50-180 Da (F5) in the order of elution. In contrast, OC from non-fumigated soil extracts (often referred to as extractable organic carbon) appeared in only the two largest assigned size classes: F1 and F2 (Figure 3.1).

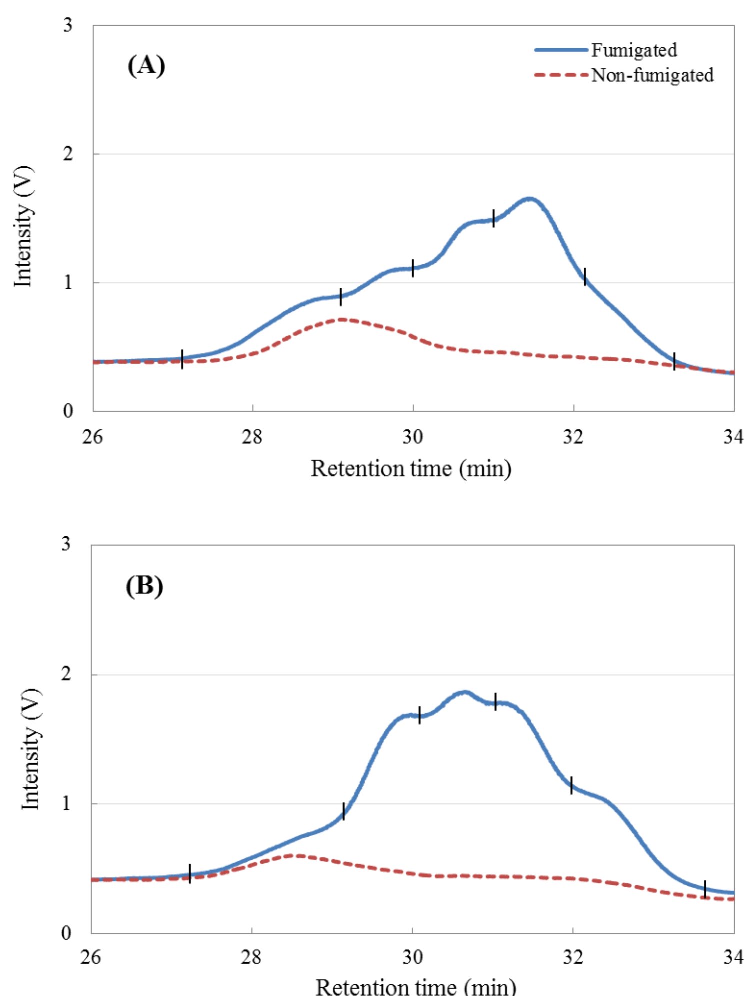

Figure 3.1: Size exclusion chromatographic separation of extractable organic carbon from fumigated and non-fumigated soils: (A) 'Sandy'; (B) 'Clayey'.

Traces of chloroform that might remain in the fumigated soils (Alessi et al., 2011) or other organic contaminants inadvertently added to the extracts did not elute with these size fractions (Appendix 2: Figure A-2.1). SEC showed only one late eluting peak (36-38 min) in Blank 1 (K_2SO_4 solution only) & Blank 2 (K_2SO_4 solution passed through pre-washed filters). This corresponds to the carbon in K_2SO_4 solution. In Blank 3 (K_2SO_4 solution passed through unwashed filters) an additional early eluting peak (30-31 min) is seen that originates from the organics in filters. Chloroform also elutes late (34-36min). The late eluting contaminant peak (35-37 min) that is seen in both fumigated and non-fumigated soil extracts (Appendix 2: Figure A-2.2) corresponds to the carbon in K_2SO_4 solution. In fumigated extracts it also includes leftover chloroform that shifts the resultant two-component peak to the left (eluting ~30 s early), but only in 'Sandy' soil extracts. This could suggest that chloroform contamination in the 'Clayey' soil used in the experiment was negligible. We excluded the late eluting contaminant peak from the SMB calculations and this improved the precision of measurement. Thus, the SEC-LC-IRMS measurement of SMB from chloroform fumigation extraction can boost the precision of its isotopic measurement by excluding the contaminant fraction from soil extracts.

SEC-LC-IRMS results suggest that most of the SMB residues consisted of compounds in the apparent size range of 180-800 Da. The most abundant size classes F3 and F4 made up 27 and 33 % of the total MB carbon (14.85 ± 2.1 mgC/L). Observations from SEC-FTIR (Fourier Transform Infrared Spectroscopy) and SEC-NMR (Nuclear Magnetic Resonance) measurements demonstrate that larger compounds in terrigenous OC (> 5 kDa) are mostly carbohydrates, alkenes and/or aliphatics, whereas mid-sized and smaller fractions contained more hydroxyl and carboxyl-rich molecules (Kiikkilä et al., 2012; Landry and Tremblay, 2012; Simpson, 2002; Woods et al., 2009). This suggests that the low MW compounds in SMB are more likely metabolites and the high MW compounds represent biosynthetic products like structural compounds.

The difference between fumigated and non-fumigated OC extracts gives a measure of the extractable microbial biomass C. $\delta^{13}C$ values of SMB size classes were thus calculated using a mass balance equation (Equation 3.1), where C_{fum} and $C_{non\text{-}fum}$ is the organic C concentrations of an individual size class in fumigated and non-fumigated samples, respectively; and $\delta^{13}C_{fum}$ and $\delta^{13}C_{non\text{-}fum}$ are their corresponding $\delta^{13}C$ values.

$$\delta^{13}C_{SMB} = (\delta^{13}C_{fum} * C_{fum} - \delta^{13}C_{non\text{-}fum} * C_{non\text{-}fum}) / (C_{fum} - C_{non\text{-}fum}) \qquad \text{Equation (3.1)}$$

$\delta^{13}C$ values of different size classes varied from −27.37 ‰ to −37.38 ‰ in the C3 control plots and from −19.29 ‰ to − 28.16 ‰ in the experimental plots with vegetation change (Figure 3.2). This demonstrates the isotopic heterogeneity of different microbial size classes which represents different microbial metabolic products, residues and structural components (Blair et al., 1985).

To estimate the contribution of fresh C4 plant biomass to size classes of SMB, the differences in $\delta^{13}C$ of each SMB size class in C3 and C3-C4 cultivated soils (Fig. 3.3A) was compared with the difference in $\delta^{13}C$ of plant biomass and SOM from C3 and C3-C4 cultivated soils. The difference in $\delta^{13}C$ for plant biomass and SOM was 15 ‰ and 1 ‰ respectively. The contribution of new C4 plant-derived carbon in different size classes of MB was calculated by using equation 3.2. The equation also takes into account the change in SOM $\delta^{13}C$ values that was minor (~1 ‰) as a result of the slow incorporation of C4 plant carbon into the SOM.

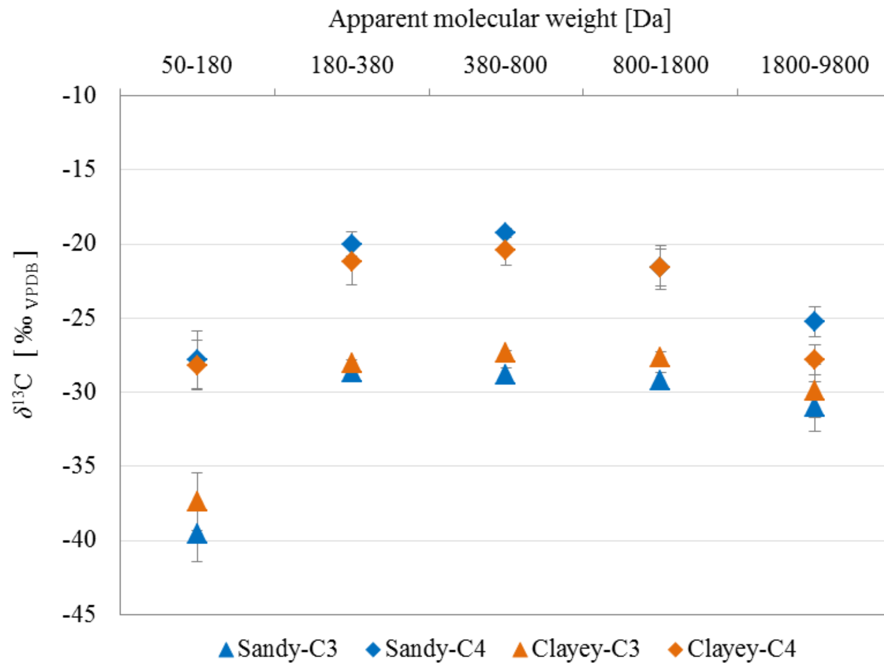

Figure 3.2: $\delta^{13}C$ values of different size classes in C3 control plots and experimental plots with vegetation change of both soil types. Note that the difference in $\delta^{13}C$ between C3 and C3-C4 cultivated soils of SMB size class decreases with increasing molecular weight.

Plant derived carbon (%) = $((\delta^{13}C_{C4\ MB} - \delta^{13}C_{C3\ MB}) - (\delta^{13}C_{C4\ SOM} - \delta^{13}C_{C3\ SOM})) / ((\delta^{13}C_{C4\ Plant} - \delta^{13}C_{C3\ Plant}) - (\delta^{13}C_{C4\ SOM} - \delta^{13}C_{C3\ SOM})) \times 100$

Equation (3.2)

where $\delta^{13}C_{C4\ MB}$ and $\delta^{13}C_{C3\ MB}$ are the $\delta^{13}C$ values of a SMB size class, $\delta^{13}C_{C4\ SOM}$ and $\delta^{13}C_{C3\ SOM}$ are the $\delta^{13}C$ values of bulk SOM and $\delta^{13}C_{C4\ Plant}$ and $\delta^{13}C_{C3\ Plant}$ are the $\delta^{13}C$ values of plant material from the experiment plot with C3-C4 vegetation change and control C3 plot, respectively.

Contribution of recent C4 plant carbon to size classes of SMB showed a distinct trend in both soil types. The proportion of recent C4 plant carbon decreased with increasing molecular weight (MW) ranging between 41±14% and 77±19% in 'Sandy' soil and between 8±15% and 59±18% in 'Clayey' soil for highest and lowest MW size class, respectively (Figure 3.3B). With such source quantification it is possible to estimate the turnover time of microbial biomass size classes. Relatively higher turnover time of low MW "metabolite" fraction of SMB observed in this study could be associated to the rapid mineralization of plant carbon by microorganisms. On the contrary, the higher MW size classes of SMB which have slower turnover rates could be products of microbial biosynthesis. These products could persist in soils, become part of the stabilized soil organic matter and/or get leached in soil water. Thus, we suggest that smaller microbial compounds like organic acids and sugars are mostly used for respiration and energy metabolism whereas the renewal rate of larger structural compounds like membrane lipids is much slower. Hence the isotopic signal of the low MW compounds is different. Moreover, the observed size dependent differences of the turnover times explain why chloroform fumigation extracts show in general faster turnover than membrane lipids.

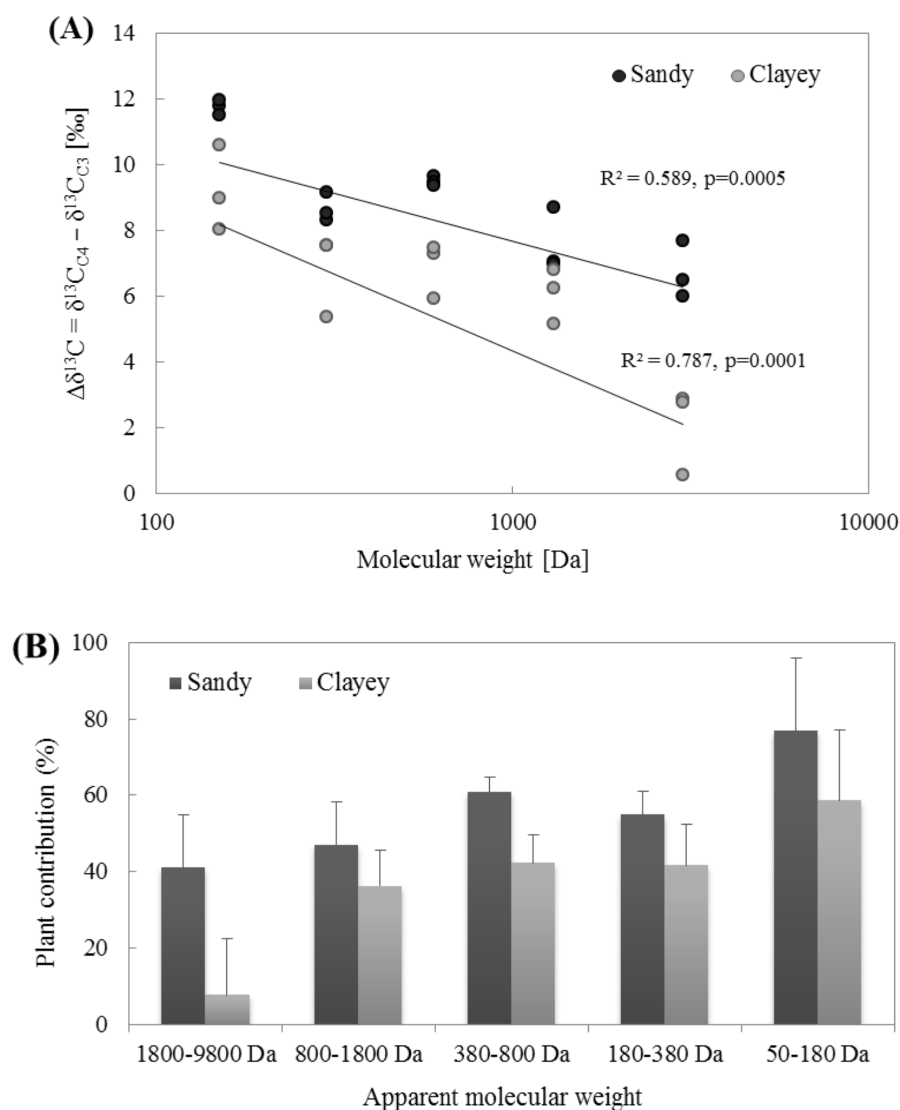

Figure 3.3: (A) Differences in $\delta^{13}C$ ($\Delta\ \delta^{13}C$) of microbial size classes in C3 and C4 cultivated soils as a property of its molecular weight. (B) Relative recent plant carbon contribution to different size classes of microbial biomass from the two soil types used in the experiment (n=3).

Plant contribution to bulk microbial biomass carbon was estimated at 54 ± 5% and 30 ± 2% in 'Sandy' and 'Clayey' soil, respectively. Thus, in the current study the recent plant contribution to microbial biomass was systematically higher in 'Sandy' soil compared to 'Clayey' soil. Most interestingly it was opposite to the SOM turnover which was higher in 'Clayey' soil. It remains open if differences in SOM quality, observed difference in the microbial community structure (unpublished results) or other ecosystem-specific effects (Throckmorton et al., 2012) are responsible for such difference in source contribution of microbial biomass in the two soils.

Chapter 4

Importance of Microbial Soil Organic Matter Processing in Dissolved Organic Carbon Production

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Accepted: July 2013 in *FEMS Microbiology Ecology*

ABSTRACT:

Soil dissolved organic carbon (DOC) sources and its seasonal dynamics are poorly known. We aimed to determine the contribution of plant and soil organic matter (SOM) to size classes of DOC in a field experiment with C3 to C4 vegetation change on two soil types through different seasons. Stable isotope ratios of DOC size classes were measured using size-exclusion chromatography (SEC) coupled on-line to liquid chromatography-isotope ratio mass spectrometry (LC-IRMS). SEC resolved DOC into three size classes: very high molecular weight/vHMW (>10 kDa), high molecular weight/HMW (0.4-10 kDa) and low molecular weight/LMW (< 0.4 kDa). HMW DOC was most abundant in all seasons, soil types and depths. In contrast, vHMW DOC was only seen post snowmelt in upper 20 cm and was mainly (87±9 %) plant-derived. Through all seasons, HMW and LMW DOC had less than 30% recent plant contribution. Similar size range and source of DOC size classes and soil chloroform fumigation extracts suggest microbial origin of DOC. Thus microbial SOM recycling is an important process in DOC production. We suggest that DOC molecules get partitioned manifold between soil solution and the mineral matrix (chromatography) thereby getting constantly decomposed, altered or produced anew by soil microorganisms (reactive transport).

INTRODUCTION:

Dissolved organic matter (DOM) is a small and reactive fraction of total organic matter in soil and is important in various biogeochemical processes (Battin et al., 2009; Kindler et al., 2011). Its movement through soil pores and interaction with solid organic matter makes it a highly dynamic carbon pool subject to physical, chemical and biological alteration. It primarily originates either from recent plant biomass (litter, root litter and/or root exudates) or from soil organic matter (SOM) (Kalbitz et al., 2000). Besides the substrate type/amount, microbial activity and community composition, abiotic factors like hydrologic variability and temperature also influence DOM production and flux (McDowell, 2003). The current understanding of DOM sources suggest that carbon derived from soil organic matter, and not from newly introduced plant organic matter, is the primary source of DOM in mineral soil (Fröberg et al., 2007a; Steinbeiss et al., 2008). Other studies demonstrate that plant residues or residues of microbial biomass feeding on the former are short-lived and beyond this pulse most of the DOM is derived from SOM (De Troyer et al., 2011; Gregorich et al., 2000). Thus, there is increasing evidence to suggest that DOC at depths originates primarily from within mineral soils being derived from highly altered SOM and is not merely a fraction of the fresh plant leachates transported in the soil solution (Karltun et al., 2005; Sanderman et al., 2008). There are other studies demonstrating the significance of abiotic processes like exchange, sorption or dissolution reactions in DOC production (Guggenberger and Kaiser, 2003; Toosi et al., 2012). In a conceptual model of the vertical movement of DOC, it is proposed that organic compounds in soil water get precipitated or sorbed followed by its microbial processing and re-release by desorption or dissolution (Kaiser and Kalbitz, 2012).

While most investigations on DOM relied on its bulk measurements to study its biogeochemical evolution, several other studies involving compositional and isotopic analysis of DOM suggest that different fractions have variable biodegradability or persistence (Kiikkilä et al., 2012; Landry and Tremblay, 2012; Qualls, 2005). By virtue of being persistent in the environment to a varying degree, DOM fractions could contribute differentially to soil organic matter cycling. We thus aimed at delineating the sources of DOM at molecular level to quantify the relative contribution of fresh litter, SOM and

microbial biomass to size classes of DOM. We hypothesized that different molecular size classes of DOM collected from a long term vegetation change field experiment originate from different sources. Stable carbon isotope ratios ($\delta^{13}C$) and its natural variability in plants with different photosynthetic pathways (C3 and C4) allow tracking of recent plant carbon into different soil carbon pools (Fröberg et al., 2007b; Kramer and Gleixner, 2008). In soils undergoing a vegetation change from continuous C3 to C4 plants, SOM is naturally labeled with C3 plant isotopic signature ($\delta^{13}C \approx -27$ ‰) and the recently fixed carbon introduced into the soil in the form of shoot litter or root litter/exudates is labeled with C4 plant signature ($\delta^{13}C \approx -12$ ‰). Comparing the isotopic composition in a plot continuously under C3 vegetation with that after vegetation change allows the quantification of plant carbon (both aboveground litter and roots) in soil DOM.

Online size-exclusion chromatography liquid chromatography-isotope ratio mass spectrometry (SEC-LC-IRMS) is a novel technique for $\delta^{13}C$ measurement of DOC molecular size classes (Malik et al., 2012). It couples the resolution of compounds in aqueous mixtures through a chromatographic column with the accurate isotopic measurement potential of an IRMS. DOC samples can be analyzed rapidly without sample processing or pre-concentration (Scheibe et al., 2012). Isotopic ratios of DOC size classes give additional insights into DOC cycling since components in this complex mixture show differential chemical reactivity and bioavailability (Kiikkilä et al., 2012; Qualls, 2005).

We analyzed soil water from a C3 to C4 vegetation change experiment in two soil types at three depths to quantify the relative contribution of recent plant- and old SOM- derived carbon in different size classes of DOC. In order to find systematic differences in the proportion of recently photosynthesized carbon in soil DOC the measurements were performed for different seasons representing snowmelt, early and later vegetation periods and winter. We also linked DOC sources to sources of microbial biomass carbon in order to discern the microbial involvement in DOM production and belowground carbon cycling.

MATERIALS AND METHODS:

Experimental site

The experimental design consisted of two soil plots with an area of 48 m^2 each that were established at the Max Planck Institute for Biogeochemistry, Jena, Germany in 2006. The plots were established using homogenized soil from different sites with varying properties and texture (Table 4.1); they were referred to as 'Sandy' and 'Clayey'. Both soils had previous continuous C3 vegetation. A half of each soil plot was subjected to vegetation change from C3 to C4 plants since 2007 for 5 years. The other half was maintained in C3 cultivation as control for comparison of $\delta^{13}C$ values. The annual cycle began post every winter when the soil was surficially tilled. This resulted in mixing and homogenization of the top 5 cm layer but a soil profile developed below with time. *Phacelia* (scorpion weed), *Helianthus annus* (sunflower) and *Triticum* spp. (wheat) were grown in the C3 control plots and *Zea mays* (maize), *Amaranthus* and *Sorghum* in the experimental C4 plots. Rotation or change in plant type over the years was done in order to prevent monoculture effect in the fields. Seeds were sown in spring and plants allowed to grow normally until harvest in autumn. Care was taken to remove unwanted plants and weeds growing in the plots as this could affect the isotopic signal. Post the harvest, the entire plant biomass was weighed, shredded and returned to the respective soil plots. Equal amount of plant biomass was returned to C3 and C4 plots. Plastic sheets then covered the plots until the next spring to prevent pollen and seed dissemination onto the plots.

Table 4.1: Characteristics of soil used for the vegetation change experiment

Soil type/ nomenclature	**'Sandy'**	**'Clayey'**
Soil parent material	Forest A-horizon soil	B-horizon of a calcareous soil
Soil texture	50% sand, 44% silt, 6% clay	9% sand, 75% silt, 16% clay
Soil pH	6.9	7.8
Organic C concentration	4.20 ± 0.19 %	1.96 ± 0.27 %
Inorganic C concentration	ND	0.78 ± 0.15 %
pH of soil water	7.3 (± 0.2)	8.0 (± 0.1)
SOM $\delta^{13}C$ in C3 plots	–27.8 ± 0.1 ‰	–29.0 ± 0.5 ‰
SOM $\delta^{13}C$ in C4 plots	–27.1 ± 0.2 ‰	–27.9 ± 0.5 ‰

Sample collection and preparation

Soil water samples were collected from the four plots using borosilicate glass suction plates (thickness- 9 mm, diameter- 120 mm, pore size- 1 µm; UMS, Germany) located at 10, 20 and 30 cm depth. One glass suction plate per depth per soil type was used in the experiments. Moreover, the plates have a large diameter which allows obtaining a representative sample. A vacuum of 200 mbar was applied to suck soil solution into 2 L borosilicate flasks. Soil water was collected every fortnight. Suction plates were chosen over zero-tension lysimeters as this allows soil water sampling almost throughout the year and not merely during storm events. Moreover, fortnight vacuum application that fades away successively implies that the soil solution collected corresponds to both slow flowpath that is more in contact with the soil matrix and fast flowpath that may represent fresh DOM.

Samples for SEC-LC-IRMS analysis were collected in January, June, September and December 2011 and March 2012. Soil abiotic parameters like moisture (Theta-Probe, ML2X, DeltaT, UK) and temperature (NTC 107, Campbell Scientific, Australia) were obtained from continuous measurements using sensors fitted at different depths (Table 4.2). March 2012 sampling was chosen to compare the effect of litter quantity on abundances of DOC size classes and their sources. This period also represents snowmelt or thawing however the litter input from the preceding harvest was lower (32.6 g plant litter C/kg SOC) compared to the harvest preceding January 2011 sampling (74.7 g plant litter C/kg SOC). Soil water samples were immediately frozen (−20°C) until analysis.

All samples were acidified and purged to remove the dissolved inorganic carbon (DIC). One mL of sample was placed in 1.5 mL brown glass vials (silanized), 20 µL of 8.5 % phosphoric acid (Merk, Germany) was added and vortexed for a minute. Samples were purged with a gentle stream of nitrogen (99.99% N_2) for 10 min using stainless steel syringe needles fitted to an automated 12-port-chamber (VisiprepTM, VisidryTM, Supelco, Sigma-Aldrich, USA). Acidified soil water samples were then analyzed by LC-IRMS in the column mode (HPLC mode). The soil solutions were further filtered through a 0.45 µm filter in the LC-IRMS interface in order to meet the operational description of DOM.

Table 4.2: Sampling time points through different seasons and the soil abiotic parameters measured. Note: harvest of aboveground plant biomass was done in November 2010 and October 2011.

Sampling date	January 2011	June 2011	September 2011	December 2011	March 2012
Description	Snowmelt, post-high litter input	Dry, early vegetation period	Moist, late vegetation period	Mild winter, post-low litter input	Snowmelt, post-low litter input
Soil moisture (Vol. %)	38.1 ± 0.8	21.8 ± 3.7	32.6 ± 5.3	33.3 ± 2.6	33.7 ± 1.5
Soil temperature (°C)	5.1 ± 0.1	18.8 ± 0.8	18.5 ± 0.2	3.4 ± 0.3	7.4 ± 0.8

DOC fractionation and $\delta^{13}C$ measurement

Stable isotope analysis of DOC size fractions was carried out using an HPLC system coupled to a $Delta^{+}$ XP IRMS through an LC-IsoLink interface (Thermo Fisher Scientific, Germany). Details of the LC-IRMS system and modifications included are given elsewhere (Malik et al., 2012; Scheibe et al., 2012). Size exclusion chromatography (SEC) was performed on a mixed bed analytical column (TSK-GEL $GMPW_{XL}$- 7.8 mm × 30 cm; Tosoh Bioscience, Germany) with a guard column (TSKgel PW_{XL}); both maintained at a temperature of 25°C. Using an autosampler (Surveyor autosampler, Thermo Fisher Scientific), 100 µL of analyte was injected into the mobile phase consisting of phosphate buffer 20 mM (pH 6.2) at a constant flow rate of 500 µL min^{-1}. Mobile phase solution and other reagents were degassed under vacuum (20 mbar) in an ultrasonic bath for 30 min and to prevent regassing a constant helium stream was maintained in solutions during analysis. Chromatographic runs were made for 45 min each and always duplicated. Isodat 2.0 SP 2.67 software (Thermo Fischer Scientific, Germany) was used to run the HPLC-IRMS system. After assessing the chromatograms, different size fractions as recurring peaks were assigned to 2 or 3 retention time intervals. Chromatographic resolution of the column was evaluated using polyethylene glycol (PEG) and polyethylene oxide (PEO) size standards. Molecular weight of DOC size classes thus achieved is only an apparent value, since the matrices of test substances used match only moderately to that of the DOC compounds. Linearity of the coupled system was ascertained using varying concentrations of different organic compounds. Additional information about the method and standardization are provided elsewhere (Malik et al., 2012; Scheibe et al., 2012).

$\delta^{13}C$ measurement of SOM, plant and microbial biomass

Soil samples for SOC measurement were collected in October 2010 and September 2011 using a 5 cm diameter stainless steel corer for different depth intervals: 5-10, 10-20, 20-30 cm. Samples were sieved < 2 mm, dried, ground and measured for C using an elemental analyzer (Elementaranalysator vario Max CN, Elementar Analysensysteme GmbH, Germany) and for $\delta^{13}C$ using an EA-IRMS (CE 1100 coupled via Con Flo III with a Delta+; Thermo-Fischer, Germany). $\delta^{13}C$ measurements of aboveground plant biomass collected following harvest every year were also performed after similar treatment on the EA-IRMS. Soil samples for microbial biomass extraction were collected in September 2011 using the same corer from the top 10 cm of all soil plots (n=3). Soils were sieved < 2 mm and chloroform fumigation extraction performed immediately. The fumigation procedure was based on the well-known method by Vance and colleagues (Vance et al., 1987) with slight modifications (Malik et al., 2013). 7 g wet soil was fumigated with chloroform gas for 24 h followed by repeated evacuation with vacuum to remove chloroform vapors from the soils. A non-fumigated control was maintained with the same amount of soil. Following fumigation DOC was extracted from all soils with 0.05 M K_2SO_4 solution in a ratio of 1:3. This mixture was homogenized on an orbital shaker (250 r min^{-1}, 1 h), centrifuged for 10 min at 12000 g and then filtered using prewashed Whatman filter paper. Soil extracts were treated in the same way as soil water samples before measurement on the HPLC-IRMS system (Malik et al., 2013).

Calculation of plant- and SOM- derived carbon

We used a two source model to calculate the contribution of differentially labelled substrates to DOC size classes and microbial biomass (Phillips and Gregg, 2001). The differences in $\delta^{13}C$ ($\Delta\delta^{13}C$) of each size class in C3 and C4 cultivated soils was compared with the difference in the $\delta^{13}C$ of plant biomass and SOM from the C3 and C4 cultivated soils (Kramer and Gleixner, 2008). The average $\delta^{13}C$ of C3 and C4 vegetation was −28.3 ± 1.1 ‰ and −13.4 ± 1.4 ‰, respectively. The average $\delta^{13}C$ of soil organic carbon from the plots without and with vegetation change was –28.1 ± 0.5 ‰ and – 27.2 ± 0.8 ‰, respectively. Thus, the $\Delta\delta^{13}C$ for plant biomass and SOM was calculated as 15 ‰ and 1 ‰, respectively. The contribution of plant-derived carbon in different fractions of DOC was calculated using equation 4.1. This equation not only considers the differences in the plant $\delta^{13}C$ values but also takes into account the differences in $\delta^{13}C$ values of soil organic matter that has a small value (~1 ‰) as a result of the slow incorporation of new plant carbon into the SOM.

$$\text{Plant derived carbon (\%)} = ((\delta^{13}C_{C4\ DOC} - \delta^{13}C_{C3\ DOC}) - (\delta^{13}C_{C4\ SOM} - \delta^{13}C_{C3\ SOM})) \,/\, ((\delta^{13}C_{C4\ Plant} - \delta^{13}C_{C3\ Plant}) - (\delta^{13}C_{C4\ SOM} - \delta^{13}C_{C3\ SOM})) \times 100$$

Equation (4.1)

RESULTS:

DOC Size Fractionation

SEC of soil DOC resulted in chromatograms with 2 or 3 discrete peaks each representing a molecular size class (Figure 4.1A-B). The earliest eluting peak between 20 and 27 min was assigned as the "very high molecular weight"- vHMW size class (10-150 kDa). The following peak from 27 to 31 min was called the "high molecular weight"- HMW size class (0.4-10 kDa, peak maxima at 1.5-3 kDa). The late eluting peak

with retention time between 31 and 35 min was assigned as the "low molecular weight"- LMW fraction (0.05-0.4 kDa). The vHMW size class of DOC was rare and only seen in water samples from one season (snowmelt, higher litter input) and the lower depths viz. 10 and 20 cm. Its average concentration across soil plots and depths was 2.3 ± 1.5 mg C L^{-1}. The HMW DOC size class was the most abundant and seen in all seasons and depths; its average concentration was 10.1 ± 7.6 mg C L^{-1} (n=30). The LMW size class of DOC was also present in soil water from all seasons. However its concentration was low at 2.5 ± 1.3 mg C L^{-1} (n=30). Total DOC concentration in the two soil types 'Sandy' and 'Clayey' was 16.7 ± 11.5 mg C L^{-1} and 7.9 ± 3.4 mg C L^{-1}, respectively (Appendix 3: Figure A-3.1).

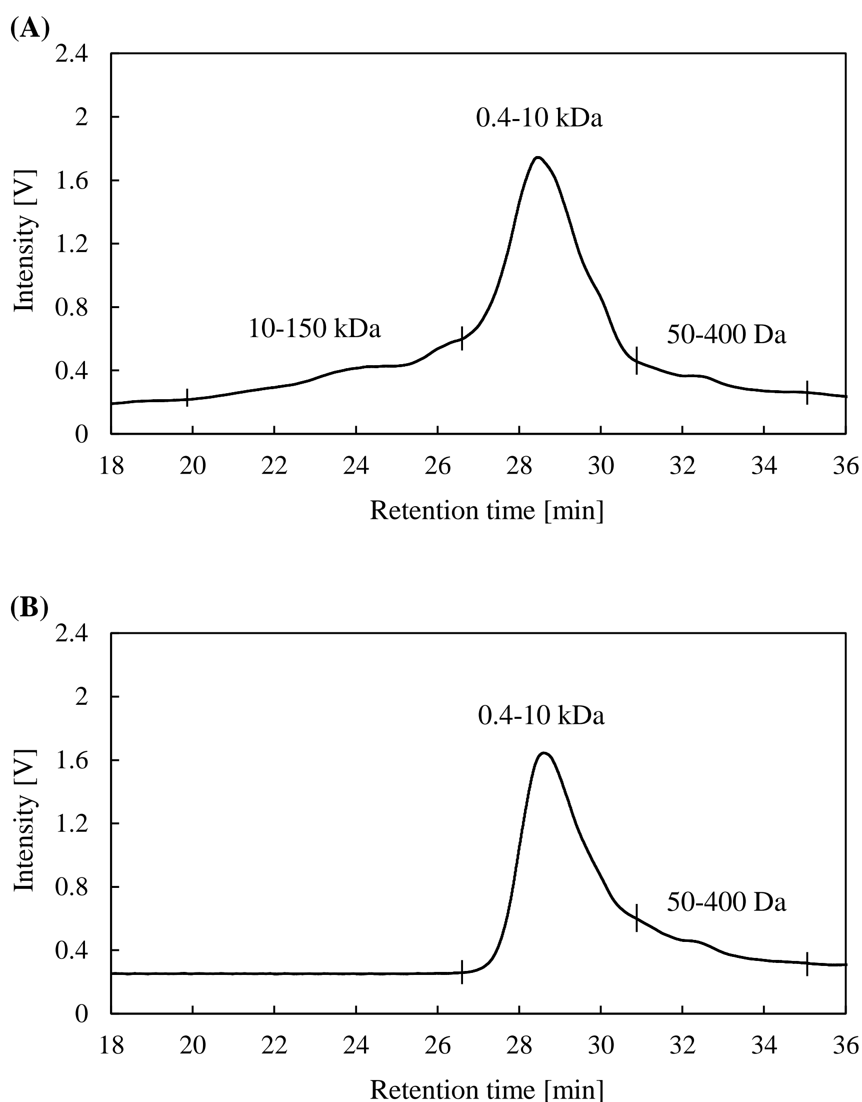

Figure 4.1: Representative SEC-LC-IRMS chromatograms showing resolution of DOC into three size classes in soil water from 'Clayey' soil plot collected in January 2011 (A). Soil DOC from all other sampling points resolved into only 2 size classes. Shown here is a chromatogram of DOC from the same plot sampled in September 2011 (B). Apparent molecular weights of size classes are presented next to the peaks.

The apparent DOC export for each size class was calculated by multiplying the concentration of each size class with the total volume of soil water collected and dividing it by the area of the suction plate and the time period from suction to sample collection (Kindler et al., 2011). DOC export for individual size classes showed a seasonal pattern, it was lowest in June 2011 (early vegetation period) in both soil types (Figure 4.2A-B). The yearly apparent flux across all depths sampled in the two soil plots 'Sandy' and 'Clayey' was 1.2-7 g C m^{-2} y^{-1} and 0.1-2.2 g C m^{-2} y^{-1}, respectively.

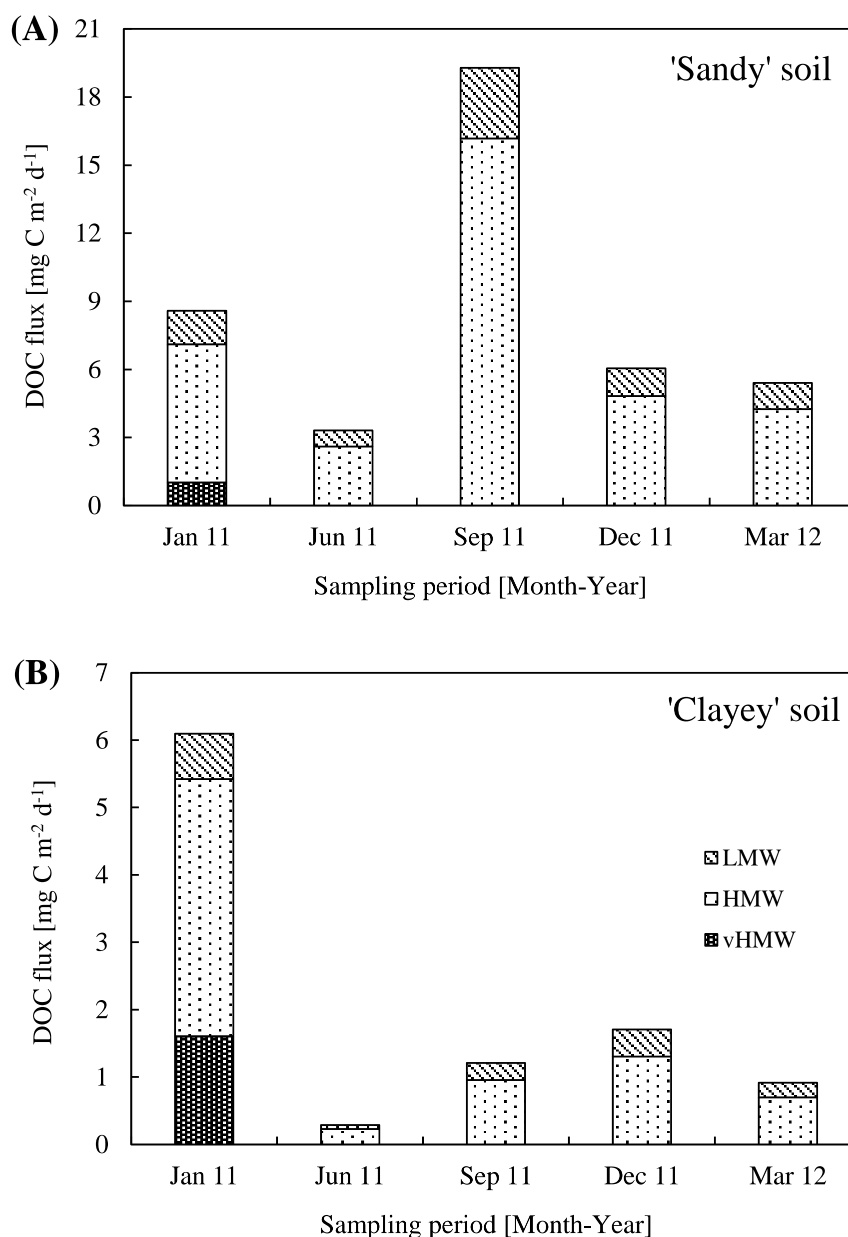

Figure 4.2: Seasonality in apparent DOC flux for individual size classes in 'Sandy' (A) and 'Clayey' soil (B). Labels- LMW: low molecular weight, HMW: high molecular weight, vHMW: very high molecular weight.

The absolute amount of plant carbon in total DOC export was highest in Jan 2011; it was 2.8 ± 2.4 mg C m^{-2} d^{-1} and 4 ± 2.6 mg C m^{-2} d^{-1} in 'Sandy' and 'Clayey' soil, respectively. It was lowest in June 2011 (early vegetation period, dry) at 0.1 mg C m^{-2} d^{-1} each in both soil types. In later sampling points the apparent DOC fluxes remained largely constant (0.3-0.9 mg C m^{-2} d^{-1}) except in September 2011 (late vegetation period) in 'Sandy' soil; here the DOC concentration and export (3 ± 0.8 mg C m^{-2} d^{-1}) was significantly higher than in 'Clayey' soil and other seasons.

δ^{13}C of DOC size classes

The δ^{13}C of DOC size classes showed distinct patterns based not only on vegetation type but also on sampling depth and molecular size of fractions. In the control plots without vegetation change DOC sources (plant and SOM) have similar isotopic signature (~ −28 to −29 ‰). Hence, these plots can be used to monitor the degree of isotopic fractionation in different DOC size classes. No isotopic fractionation was observed during HPLC separation in the SEC column as seen in the isotope ratio trace values of standard materials as well as environmental samples (Malik et al., 2012). In the C3 control plots, the vHMW and HMW DOC size classes were isotopically very similar to the substrate (average for the two soil types and three depths sampled: $\delta^{13}C_{vHMW}$ = −28.1 ± 0.9 ‰, $\delta^{13}C_{HMW}$ = −28.2 ± 0.5 ‰; n=30), whereas the LMW size fraction was isotopically depleted compared to the substrates available ($\delta^{13}C_{LMW}$ = −29.3 ± 0.7 ‰). On the contrary, DOC from plots with C3/C4 vegetation change was isotopically enriched. Here, the isotope ratio of the vHMW size class was close to the C4 plant signature (average $\delta^{13}C_{vHMW}$ = −14.8 ± 1.6 ‰). The HMW and the LMW size classes had isotopic values in-between that of C3 and C4 signatures ($\delta^{13}C_{HMW}$ = −23.9 ± 1.7 ‰, $\delta^{13}C_{LMW}$ = −24.6 ± 1.6 ‰; n=30) and thus show contribution of both new C4 plant carbon and old C3 labeled SOM-derived carbon.

Sources of DOC

To determine the carbon source of different DOC size classes, the differences in δ^{13}C ($\Delta\delta^{13}$C) of each fraction in C3 and C4 cultivated soils were calculated. The contribution of plant-derived carbon in different fractions of DOC was then calculated by using equation 4.1. The vHMW DOC size class was almost entirely derived from recent plant material (Figure 4.3A). The average plant contribution to this fraction of DOC was 87 ± 9 %. The other two size classes of DOC viz. HMW and LMW fractions were largely derived from SOM. The average plant biomass contribution to the HMW and LMW size classes across all seasons and soil types was 22 ± 16 % and 29 ± 17 %, respectively. The relative contribution of plant biomass to these size classes of DOC in 'Sandy' soil was lower (6-23 %) compared to that in the 'Clayey' soil (24-53 %). There was also a distinct depth-wise trend, the percentage of plant-derived carbon decreased with increasing depth in both soils (Figure 4.3B). It was 22-51 % at 10 cm, 10-39 % at 20 cm and 6-18 % at 30 cm. The seasonal pattern was not very clear to elucidate (Figure 4.3C, 4.4A-B, Appendix 3: Figure A-3.2). The vHMW size class was only seen in soil water from January 2011 (snowmelt, higher litter input) and was almost entirely plant-derived. The plant contribution in the HMW and LMW DOC size classes which were present in soil water from all seasons was relatively stable and did not vary much across seasons, although it was slightly but not significantly higher during the vegetation period in June 2011 and September 2011.

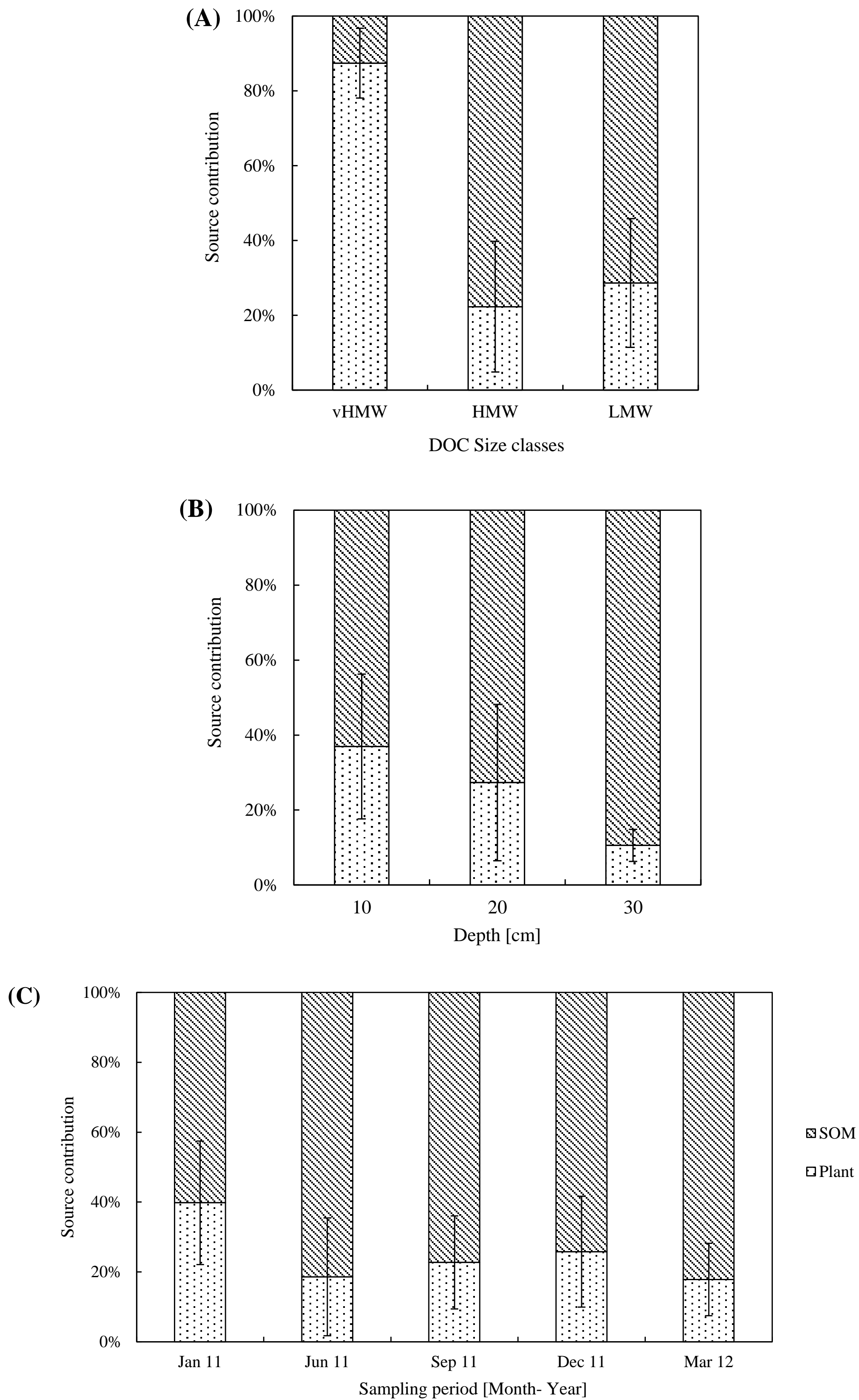

Figure 4.3: Average contribution of recent plant and old soil organic matter (SOM) to (A) the size classes of DOC; (B) total DOC for the different depths sampled; (C) total DOC for the different seasons (n=30).

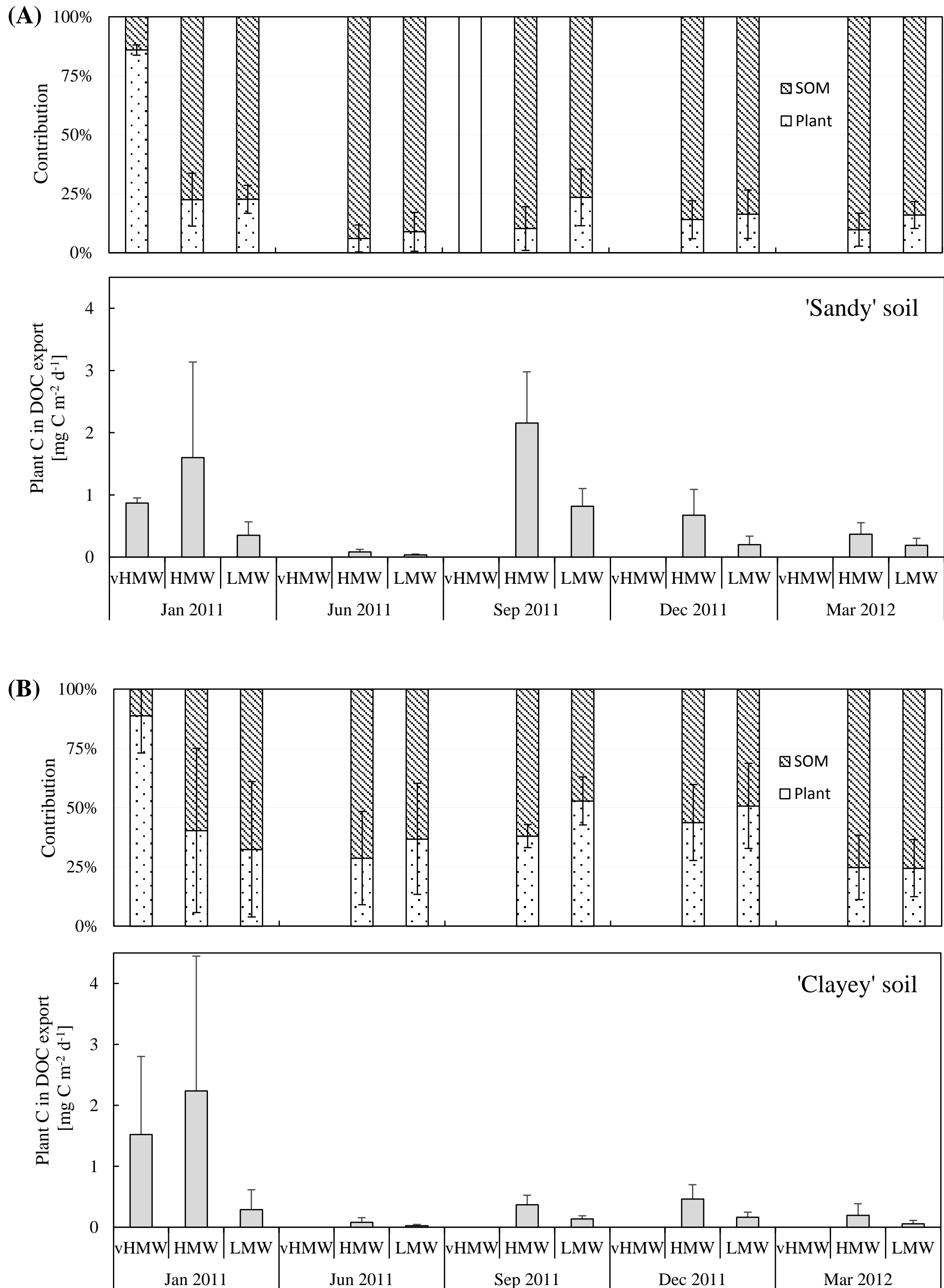

Figure 4.4: Seasonal variation in contribution of recent plant and old soil organic matter (SOM) to DOC size classes in 'Sandy' (A) and 'Clayey' soil (B). Also shown is the seasonality in the amount of plant carbon in DOC export. Error bars represent depth wise variation.

Based on relative terms, the amount of aboveground plant litter C added compared to the amount of native SOC was low; 2.5 % and 4.9 % in 'Sandy' and 'Clayey' soil, respectively. This estimate is low because it does not include belowground plant C input (from root biomass and exudates) which is 1.3 times higher than aboveground plant C input (Gleixner, 2013; Nguyen, 2009; Steinbeiss et al., 2008). In comparison, the mean overall relative total plant C contribution to DOC was much higher (14.5 % and 35.4 %, respectively). This relation suggests that 1 g of aboveground plant litter C contributes approximately 6.5 (2.8 in case of total plant C input) times more to DOC compared to 1 g of SOC, which mirrors the faster decomposability of plant C relative to SOC.

Sources of soil microbial biomass

Microbial biomass extracts were obtained using chloroform fumigation extraction from the topsoil during the vegetation period in order to compare its molecular size distribution and isotopic variability with that of DOC. Microbial biomass was resolved by SEC into various size classes (Malik et al., 2013). It was a complex mixture with an apparent molecular size ranging from 0.1-10 kDa with peak maxima between 0.2-1 kDa. δ^{13}C of microbial biomass in the C3 control plots was –30.6 ± 0.6 ‰ and –28.6 ± 0.2 ‰ in 'Sandy' and 'Clayey' soil, respectively. In the plots with vegetation change the δ^{13}C of microbial biomass was –22.2 ± 1.1 ‰ and –22.7 ± 0.1 ‰ in the two soils. Plant contribution to microbial biomass carbon was estimated at 54 ± 5% and 30 ± 2% in 'Sandy' and 'Clayey' soil, respectively.

DISCUSSION:

The C3/C4 vegetation change experiment allowed us to quantify the relative contribution of plant-derived carbon and from this the SOM- derived carbon in DOM assuming two end members. Fractionation of DOM by size exclusion chromatography coupled to an LC-IRMS gave additional insights into the carbon sources and turnover of individual size classes of DOM. There are certain assumptions that are made in quantifying the contribution of recent C4 plant versus old C3 SOM in a vegetation change experiment. We assume that same amount of DOM is produced and exported in both control and experimental plots. We also assume that isotopic fractionation, if any, during the production and degradation of DOM is similar in both the fields. However, the differences in δ^{13}C values of DOM size classes from the C3 and C4 soils were used to calculate the contribution of different sources; hence, any isotopic fractionation involved gets cancelled. In addition, the C3 control plots were used to monitor the degree of isotopic fractionation in different size classes as both the recent plant organic matter and the old SOM have similar isotope signature.

The DOM concentration and flux varied depending on hydrological conditions and other abiotic factors including soil properties. These factors in addition to the amount of litter added to the fields also determined the amount of plant carbon in DOM export. The advantage of the field experiment was that we could also consider the effects of abiotic factors in a natural environment on sources of DOM (Kalbitz et al., 2000) and thus the quantification was more reliable than previous experiments with controlled environmental conditions. In January 2011 sampling, the DOM export was quite high because of snowmelt or thaw. After the harvest in November 2010 a higher litter amount was added to the fields and since this was followed by a long and severe winter, most plant carbon leached into the DOM only during the snowmelt. June 2011

sampling period (early vegetation phase) was dry and so there was very little leached soil water. In later sampling points the DOM fluxes remained largely constant. Soil solution collected using tension lysimeters primarily corresponds to slow flowpath that is more in contact with the soil matrix and is different from the DOM collected using zero-tension lysimeters (Sanderman et al., 2008). However, application of vacuum that fades away during the course of DOM sampling allows collection of both fast and slow flowpath soil solution. We used such a set-up with large suction plates to collect soil water from different seasons.

Size fractionation resolved soil DOM into three size classes based on apparent molecular weights. The DOC detector also allows accurate estimation of carbon concentration of the size classes (Malik et al., 2012; Scheibe et al., 2012). The vHMW size class (10-150 kDa) was rare and less abundant. HMW size class contained midsized to large molecules ranging from 0.4-10 kDa. Majority of DOM compounds have this molecular size range and this fraction was found in all seasons. Very small molecules (< 0.4 kDa) that elute last were grouped into the LMW size class and though this fraction was seen in all seasons, its proportion was very small. Such fractionation of DOM has been demonstrated in other studies that estimated the molecular weights of organic matter fractions in water and by combining SEC with UV detectors or total carbon analyzers two to four fractions of DOC have been reported (Mueller et al., 2000; Her et al., 2002; Landry and Tremblay, 2012). However it should be noted that it is difficult to obtain separation solely based on size as there can be other forms of non-specific interactions between certain compounds in the DOM and the column material. In addition, there is a lack of suitable size standards for DOC measurements. Molecular weights of DOC size classes thus determined by SEC are only apparent. These are the limitations to be considered in interpretation of results obtained by SEC-LC-IRMS.

DOC is a complex mixture of different components that vary in biodegradability and chemical reactivity and may have different sources (Kiikkilä et al., 2005). SEC of DOC coupled to isotope analysis suggests that different molecular size classes of DOC indeed have variable sources. The vHMW DOC size class appeared only after a high litter application event and during snowmelt. Moreover this fraction is almost entirely plant derived. Under high moisture conditions like snowmelt DOC flux may be largely with preferential pathways, leading to kinetic restriction of sorption processes thus enabling young DOC to get transported to larger depths. Thus we hypothesize that the vHMW size class of DOC consists of non-degraded plant biomass leaching downwards. This finding is supported by observations from SEC-FTIR (Fourier Transform Infrared Spectroscopy) and SEC-NMR (Nuclear Magnetic Resonance) measurements, which suggest that larger compounds in terrigenous DOC (> 5 kDa) are mostly carbohydrates, alkenes and/or aliphatics (Landry and Tremblay, 2012; Woods et al., 2009). Presence of such compounds suggests more recent and reactive DOC. Our isotopic results prove that the vHMW fraction consists mostly of recent plant-derived carbon. The other two size classes viz. HMW and LMW were mostly derived from old soil organic matter that suggests the presence of highly processed material in these size classes of DOC. This observation is further substantiated by compositional studies of DOC (Woods et al., 2010; Landry and Tremblay, 2012) which suggest that compounds in this molecular size range contained more hydroxyl and carboxyl-rich alicyclic molecules (CRAM) (Hertkorn et al., 2006).

We also aimed to link DOM to microbial biomass residues in soil, since there is growing evidence that microorganisms are responsible for formation, stabilization and processing of SOM (Gleixner, 2013; Liang and Balser, 2011; Miltner et al., 2012). SEC of

microbial biomass from these soils obtained by chloroform fumigation extraction suggests that microbial residues or metabolic products fall mostly in the 0.3-1 kDa range. From its isotope analysis it was clear that microorganisms use plant carbon in the form of root exudates or soluble compounds from litter for respiration or energy metabolism and very little is used for biosynthesis, therefore, these fresh compounds are not seen in the DOC. Moreover, high contribution of old soil organic carbon to microbial biomass particularly the high molecular weight size classes (Malik et al., 2013) indicates a slow renewal rate of microbial biosynthetic compound. The similarities in molecular size ranges and isotope values of HMW and LMW DOC size classes with that of microbial biomass suggest that DOC is a footprint of microbial activity. Evidently, only when microbial activity and decomposition rates are lower plant-derived compounds like those in the vHMW size class appear in the DOC. On the contrary, when conditions are favorable for microbial growth and activity, more SOM related compounds are seen in the DOC. These compounds are most likely a result of microbial processing and alteration of soil organic matter (Kindler et al., 2009; Miltner et al., 2012; Steinbeiss et al., 2008).

The depth wise trend in plant contribution to DOC suggests that plant carbon is lost in the upper 20 cm. The DOC in higher depths has less than 18 % plant contribution. It is also important to note that the vHMW size class which consists of non-degraded plant biomass was seen only in the upper 20 cm. There was also a clear distinction between the soil types; DOC from 'Sandy' soil had a higher contribution of SOM than that from 'Clayey' soil. This may be because of the higher soil organic carbon concentration in 'Sandy' soil (Table 4.1).

The plant contribution to all DOC size classes except the vHMW fraction was consistently low across all seasons. Even after a very high biomass input plant-derived carbon in DOC was less than 40 %. The vHMW size class was absent in March 2012 soil water which was also a snowmelt period. This could be because the litter amount added in the preceding harvest was much lower compared to the earlier harvest. Consequently the plant contribution to HMW and LMW size classes was also much lower. Similar results demonstrating low input of plant biomass into DOC through litter decomposition experiments have been reported (Cleveland et al., 2004; De Troyer et al., 2011; Fröberg et al., 2007b; Sanderman et al., 2008). We show in this seasonal monitoring experiment that plant contribution both through litterfall and root litter/exudation is a less significant source of DOC in mineral soil. Even in the active vegetation period the contribution of fresh plant biomass to DOC was low, which suggest that incorporation of plant residues remobilizes some soil organic carbon which is leached into the dissolved phase (Marx et al., 2007). These results thus confirm that across all seasons and soil types majority of the compounds in the DOC are derived from SOM. Our observations thus lead to the conclusion that decomposition and remobilization of SOM and its processing by microorganisms are the most important processes in DOC production. In consequence, we suggest that DOC is reactively transported downward in soil whereby DOC molecules are partitioned manifold between soil solution and the mineral matrix (chromatography) thereby getting constantly decomposed, altered or produced anew by soil microorganisms (reactive transport) (Appendix 3: Text).

Chapter 5

Carbon flow from plants into the rhizosphere soil microbial loop

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In preparation

To be submitted to *Ecology Letters*

ABSTRACT

Flow of photosynthetically fixed carbon (C) from plants through the rhizosphere into the soil microbial food web represents a crucial link in the terrestrial carbon cycle. Soil microorganisms play an important role in respiring newly fixed carbon but also contribute to the maintenance of soil organic matter (SOM) through turnover and recycling of their biomass. It is likely that biochemical fractions of soil microorganisms vary in their turnover and therefore could contribute differentially to soil C storage. Here we sought to trace and compare the flow of plant C into different microbial biochemical fractions through a greenhouse-based, pulse chase $^{13}CO_2$ plant labeling experiment. Plant released ^{13}C was traced into rhizosphere microbial communities by measuring isotope abundance in DNA, RNA, fatty acids and cytosolic microbial biomass (chloroform fumigation extraction-CFE derived) using a combination of molecular biology and isotope ratio mass spectrometry approaches.

The amount of ^{13}C in total fatty acids ($\Delta\delta^{13}C$ = 32-54 ‰) was lower immediately after the pulse and remained so throughout the experiment. Initial highest ^{13}C enrichment was seen in cytosolic microbial biomass (96 ‰) and in nucleic acids (DNA: 38‰, RNA: 66‰). However, this was followed by a rapid, sharp decline in ^{13}C enrichment. A minor increase in ^{13}C amounts in DNA and RNA was observed towards the end of the experiment suggesting "cross-feeding" or secondary ^{13}C assimilation. Thus, the turnover rates of microbial components of differing biochemical properties were found to significantly differ implying that microbial cellular biochemistry is critical for the stabilization of microbial carbon in soil.

The uptake of plant released C by soil microbial functional groups was then assessed through phospholipid/neutral lipid fatty acid (PLFA/NLFA) and RNA ^{13}C analyses. Fatty acid-based measurements confirmed that mutualistic arbuscular mycorrhizal fungi (AMF) rely on root C and are important utilizers of the plant C initially released into the rhizosphere. Other saprophytic fungi and bacteria showed a delayed ^{13}C incorporation pattern which could suggest secondary ^{13}C assimilation indicative of trophic interactions. However, in contradiction to PLFA based observations, ^{13}C enrichment of bacterial RNA indicated that bacteria are also important as initial conduits of plant-released C. Moreover, the very slow turnover of PLFAs compared to RNA suggests that PLFAs are not suited to monitor fast C fluxes. Previous assessments of trophic interactions based on PLFA analyses thus raise the question whether differences in C turnover are due to differences in internal fatty acid turnover or as a result of trophic interactions. We thus dispute the current understanding of plant-soil C fluxes which underestimates the importance of rhizosphere bacteria as primary utilizers of plant-released C, and recommend more detailed quantitative investigations with nucleic acids biomarkers to better discern microbial trophic interactions in soil.

INTRODUCTION

Soil microorganisms are a crucial link between the above and belowground components of ecosystems. Moreover, their role in cycling the vast reserves of soil organic matter carbon (SOM-C) highlights their importance for ecosystem functioning (Gleixner, 2013). However, despite the relatively large size and temporal sensitivity of the soil organic carbon pool and its importance in maintaining agricultural productivity and mitigating atmospheric CO_2 levels, our knowledge of the soil processes involved in the formation and destabilization of SOM is still very limited (Allison and Martiny, 2008; Lal, 2010). This has led to large uncertainty in how soil carbon stocks globally will respond to climate warming (Canadell et al., 2007). This is primarily due to a lack of

understanding on whether environmental change will lead to a reduction or increase of soil carbon through altered microbial functioning (Wieder et al., 2013). Therefore there is a need to have a better mechanistic understanding of microbial processes contributing to the persistence and transformation of carbon in soil.

Root-associated microbial food webs are intrinsically linked to plant growth and the flow of energy and nutrients between plants and soil organisms (De Boer et al., 2006; Wardle et al., 2004). Important carbon sources for root-associated microorganisms are rhizodeposits and fine root turnover. It is generally acknowledged that a niche differentiation exists between bacterial and fungal decomposers with respect to substrate complexity (De Boer et al., 2006). Decomposition of labile substrates like root-exudates is often attributed to bacteria particularly in the favorable, resourceful rhizosphere environment (de Boer et al., 2005). Indeed, a variety of bacteria particularly from the Pseudomonadaceae or Burkholderiaceae family have been implicated in the rapid assimilation of root-derived plant carbon (Philippot et al., 2013; Vandenkoornhuyse et al., 2007). However, other studies have demonstrated a significant role of both symbiotic and saprophytic fungi in utilization of root derived carbon (Balasooriya et al., 2014; Drigo et al., 2010). Most significantly, arbuscular mycorrhizal fungi have been implicated in the rapid translocation of plant carbon belowground. It has been estimated that up to 20% of photoassimilates can be utilized by the AM fungi, thus making mycorrhizal hyphal turnover a substantial process for carbon input into SOM (Bago et al., 2000; Godbold et al., 2006). These results point to a lack of consensual knowledge concerning microbial trophic interactions and the flow of carbon through the plant-rhizosphere-soil continuum warranting a reappraisal of the root associated microbial food web.

Soil microorganisms mineralize most newly fixed carbon by respiration but they also contribute to the maintenance of SOM through turnover and recycling of biomass (Miltner et al., 2012; Simpson et al., 2007; Throckmorton et al., 2012). The contribution of non-living microbial biomass to maintenance and formation of soil organic matter is estimated to be as high as 80% of organic carbon in soil (Liang and Balser, 2011; Liang et al., 2011). The majority of plant carbon passes through microbial biomass before it is either respired as CO_2 or becomes stabilized within the soil organic carbon pool (Kögel-Knabner, 2002; Mambelli et al., 2011). Thus microbial carbon use efficiency, the proportion of microbial assimilated substrate carbon versus that lost as respired CO_2 is critical in determining the fate of soil organic carbon (Wieder et al., 2013). Stabilization of certain molecules in soil is largely attributed to the molecular transformation or resynthesis of compounds by microorganisms to form new products that persist in soil (Gleixner, 2013; Kindler et al., 2006; Simpson et al., 2007). Although microbial derived carbon inputs to soil are now increasingly acknowledged as a major source of SOM, the contribution of different microbial compounds to soil carbon storage remains poorly understood. Variable turnover rates of different microbial cellular fractions; by determining their retention times; are thought to contribute differently to soil carbon storage.

The flow of carbon from plants into soil can be examined by $^{13}CO_2$ pulse labeling of plants and subsequent isotopic analysis of different carbon pools in the plant-soil system (Griffiths et al., 2004). The translocation of ^{13}C from root exudates and fine root turnover into rhizosphere microorganisms can be traced by measuring the amount of ^{13}C in microbial biomarkers, also enabling carbon estimation of turnover times (Clayton et al., 2010; Leake et al., 2006; Ostle et al., 2003). Due to technological development in analysis of stable isotopes, it is now possible to measure ^{13}C content in various

biomarkers. The liquid chromatography- isotope ratio mass spectrometry (LC-IRMS) interface allows rapid and accurate compound specific isotope measurements in aqueous extracts (Malik et al., 2012; Scheibe et al., 2012). Measurement of ^{13}C labeled plant released carbon in major microbial functional compounds such as genomic DNA, ribosomal RNA, cellular proteins, cell wall lipid fatty acids, and cytosolic metabolites will allow an elucidation of the physiological mechanisms of microbial carbon metabolism in addition to an approximation of C turnover rates of these biochemical fractions. Stable isotope analysis of different phospholipid and neutral lipid fatty acid using gas chromatography- isotope ratio mass spectrometry (GC-IRMS) allows measurement of the plant tracer carbon into different microbial functional groups (Balasooriya et al., 2014; Drigo et al., 2010). This technique can be used in conjunction with ^{13}C analysis of bacterial RNA to assess the flow of plant carbon into the root-associated microbial food web.

We hypothesized that microbial cellular biochemistry by affecting the residence time plays an important role in stabilization of carbon in soil. Therefore, we sought to compare the turnover rates of microbial compounds differing in biochemical properties and size. Stable isotope probing with a multi-biomarker approach was employed to reappraise soil trophic interactions in terms of carbon flow through the plant-rhizosphere-soil continuum. A greenhouse-based, pulse chase $^{13}CO_2$ plant labeling experiment was performed in mesocosms with a mycorrhizal plant species and an inter-disciplinary analytical approach combining molecular biology and stable isotope analytical techniques was used to monitor carbon cycling in this system. Plant released ^{13}C was traced into rhizosphere soil microbial DNA, RNA, fatty acids and cytosolic microbial biomass (chloroform fumigation extraction- derived). Additionally, C flow into soil microbial functional groups was assessed through phospholipid/neutral lipid fatty acid (PLFA/NLFA) and RNA ^{13}C analyses. Microbial carbon fluxes were also linked to plant and soil C pools in order to investigate belowground carbon dynamics and deduce a mechanistic understanding of carbon flow in plant-soil systems.

MATERIALS AND METHODS

Plant and soil system

Soil was collected from the Jena Biodiversity Experiment site located in Jena, Germany in September 2012. Soil was sieved (< 2 mm), all visible roots were removed and prior to establishing mesocosms homogenized soil was stored at 4°C. Polystyrene pots (1L) were filled with 800 g of soil and incubated in the greenhouse (around 40 pots for destructive sampling on 10 occasions). Mesocosms were uniformly watered with an automated irrigation system that delivered water 2-3 times per day for 3 minutes each, with additional light (Son-T Agro 430 W HPS bulbs, primary light range = 520-610 nm, Philips Lighting Company, New Jersey, USA) provided 12 hours per day. Experimental mesocosms were left to equilibrate for 2 weeks before sowing *Chenopodium ambrosioides*, a temperate herb that is known to form mycorrhizal association. After developing true leaves (after 3-4 weeks), plants were thinned leaving 3 per mesocosm.

$^{13}CO_2$ labelling

After 3 months of plant growth, plants were exposed to $^{13}CO_2$ in an air tight glass chamber of 2000 L. Thirty planted pots were introduced into the chamber (3 control replicates were not subjected to the $^{13}CO_2$ pulse). Prior to $^{13}CO_2$ pulse, the chamber was flushed with CO_2 free synthetic air until the concentration of CO_2 fell

below 50 ppm. Enriched $^{13}CO_2$ (99 atom %) was introduced into the labelling chamber at a flow rate of 100 ml min^{-1} and was cycled throughout the chamber using an internal ventilation system to achieve uniform labelling. Photosynthetic uptake of CO_2 was monitored using a Picarro 2101i (Picarro Inc., Santa Clara, California, USA) and throughout the labeling period of 10 h, the CO_2 concentration in the chamber was maintained between 350-400 ppm. Photosynthetic rate was around 100 ppm h^{-1} at the beginning and dropped below 50 ppm h^{-1} towards the end of the labeling period. At the end of the labeling period (10 h), the chamber was opened, and the plants were returned to the greenhouse.

Sampling

Destructive sampling was performed immediately, then at 3, 12 and 24 h, then 2, 4, 7, 14, 21 and 28 days after the pulse labeling. At each time point 3 mesocosms were sampled for rhizosphere soil and plant parts. After discarding the top 5 cm the rest of the soil was considered as rhizosphere soil, due to heavy colonization of roots. Soil was then sieved to <2mm and fine roots were extensively removed by hand. An aliquot of soil was stored at -80 °C prior to nucleic acids extraction. Additionally, soil for lipid and microbial biomass extractions was stored at -20 °C. A smaller aliquot for ^{13}C measurement of bulk soil organic matter was dried at 40°C. Plant roots, stems and leaves for ^{13}C measurement were washed with water in order to remove adherent soil residues and dried at 40°C.

Plant and soil organic matter ^{13}C analysis

Before ^{13}C analysis of bulk soil organic carbon, dried soil was ground using a ball-mill and carbonates were removed from the soil using 0.1 M HCl. After drying, plant parts were shredded and ground in a ball-mill. Bulk ^{13}C analysis of SOM and plant material was performed on an elemental analyzer coupled to an isotope ratio mass spectrometer (EA model CE 1100 coupled on-line via a Con Flo III[27] interface with a Delta+ isotope ratio mass spectrometer; all supplied by Thermo Fisher Scientific, Germany).

Plant respired $^{13}CO_2$ analysis

To measure the autotrophically or plant respired $^{13}CO_2$, three plant pots were placed into a 2000 L airtight glass chamber in dark (post sunset) at 1, 2, 4, 7, 14 and 21 days after pulse labelling of plants. The amount of stable isotope ($\delta^{13}C$ value) and concentration of the CO_2 in the chamber was continuously monitored for 90 min at each time point using a Picarro 2101i (Picarro Inc., Santa Clara, California, USA). To estimate the $\delta^{13}C$ value of plant respired CO_2, the reciprocal of CO_2 concentration was plotted against $\delta^{13}C$ value of chamber CO_2 over the analysis period, with the y-intercept representing the $\delta^{13}C$ value of plant respired CO_2.

Microbial biomass ^{13}C analysis

Microbial biomass from soil was obtained using the chloroform fumigation extraction (CFE) method described by Vance et al., 1987 including slight modifications (Malik et al., 2013). Soil (7 g wet weight) was fumigated with chloroform gas for 24 h followed by 8 vacuum evacuations. An identical mass of soil was also used as a non-fumigated control. Organic carbon was extracted from fumigated and non-fumigated

soils with 0.05M K_2SO_4 solution. Soil extracts were acidified and purged with nitrogen gas in order to remove the dissolved inorganic carbon (DIC) and analyzed in both the bulk (μEA) mode and the size exclusion chromatography (SEC) mode (Malik et al., 2013) on an HPLC-IRMS (HPLC system coupled to a Delta$^+$ XP IRMS through an LC IsoLink interface; Thermo Fisher Scientific, Germany). The SEC mode was used to measure ^{13}C incorporation into different molecular size classes of compounds in the CFE extracts.

Aliquots of soil extracts (100 μL) were injected using an autosampler (Surveyor autosampler, Thermo Fisher Scientific). The mobile phase during bulk measurements was degassed Millipore water at a constant flow rate of 500 μL min^{-1} maintained using a Surveyor MS pump. SEC was performed on a mixed bed analytical column (TSK-GEL $GMPW_{XL}$- 7.8 mm × 30 cm; Tosoh Bioscience, Germany) with a mobile phase of 20 mM phosphate buffer (pH 6.2) at a flow rate of 500 μL min^{-1}, each SEC run was performed for 45 min. The apparent molecular weight (MW) of differing microbial biomass size classes was determined by calibration with polyethylene oxide and polyethylene glycol MW standards.

^{13}C analysis of microbial nucleic acids

Microbial nucleic acids (DNA and RNA) were extracted from 0.5 g soil using a previously described method (Griffiths et al., 2000) including a double bead beating to improve the yield of nucleic acids. DNA and RNA were then purified and separated using an All Prep DNA/RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Following elution in molecular grade water, the purity and concentration of DNA and RNA were assessed by gel electrophoresis and Nanodrop quantification. The purity of total RNA was also assessed using Agilent TapeStation using R6K ScreenTape (Agilent Technologies, UK) which provides a better size resolution compared to agarose gel electrophoresis.

^{13}C analysis of DNA and RNA was performed on an HPLC-IRMS system in SEC mode as described above. This method is an improvement over previously described methods (Manefield et al., 2002; Miyatake et al., 2009; Thomson et al., 2013) as nucleic acids can be measured directly without any pre-processing. Moreover, online SEC-based separation of protocol contaminants as well as soil co-extracts from nucleic acids greatly improves the accuracy and precision of measurement. Samples (50 μL containing 100-600 ng DNA/RNA) were manually injected into the system. Subsequently, DNA or RNA chromatogram peaks were identified by correlating their retention times against standard yeast rRNA (Sigma-Aldrich, Germany), calf thymus DNA (Trevigen, USA) and RNA size ladder (Invitrogen-Life technologies, Germany).

Lipid biomarker ^{13}C analysis

Microbial lipids were extracted from approximately 50 g (dry weight) of soil according to a modified Bligh and Dyer extraction protocol (Bligh and Dyer, 1959; Kramer and Gleixner, 2008). Extractions were carried out using a mixture of chloroform ($CHCl_3$), methanol (MeOH) and 0.05 M phosphate buffer (pH 7.4) (1:2:0.8 v:v:v). Extracted lipids were separated into neutral lipids, phospholipids and glycolipids using silica columns. Fatty acid methyl esters (FAMEs) were then isolated by mild alkaline hydrolysis and methylation of fatty acids, followed by the removal of unsubstituted FAMEs. PLFAs were then separated into saturated fatty acids (SATFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). All extracts were dried under a nitrogen

stream, resuspended in a 200 µL stock solution containing n19:0 in isooctane as internal standard. Gas chromatography flame ionization detector (GC-FID, Hewlett Packard HP 6890 series GC-System coupled with a FID; Agilent Technologies, Palo Alto USA) was used to quantify the PLFA and NLFA content. ^{13}C analysis was performed using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS: HP5890 GC, Agilent Technologies, Palo Alto USA; connected to IRMS Deltaplus XL, Finnigan MAT, Bremen, Germany; via a combustion interface GC Combustion III Finnigan MAT, Bremen, Germany).

Isotope expression

^{13}C enrichment into the various carbon pools in the plant-soil system was estimated as $\Delta\delta^{13}C$ value which denotes the change in $\delta^{13}C$ value post labelling relative to the unlabeled control. Thus, the difference ($\Delta\delta^{13}C$) in the $\delta^{13}C$ values between labelled and unlabeled will be used throughout the results section.

RESULTS

^{13}C in plant respired CO_2, roots, shoots and soil

^{13}C incorporation following pulse labelling was chased into different plant pools including plant or autotrophically respired CO_2 (Figure 5.1A-C). The $\Delta\delta^{13}C$ of plant respired CO_2 was very high (3828.8 ‰) one day after the pulse, then dropped drastically over the following three days (891.4 ‰ on day 4) and finally reached a stationary phase towards the end of the experiment (159.6 ‰ after 3 weeks). As expected, ^{13}C uptake into plant shoots was higher than that of roots. However, the enrichment one day after the pulse was almost double in stems ($\Delta\delta^{13}C$ = 927.7 ± 206.5 ‰) than in leaves ($\Delta\delta^{13}C$ = 526.6 ± 83.6). Three weeks after the pulse labelling event the $\Delta\delta^{13}C$ value of leaves (536.2 ± 63.3 ‰) was similar to that observed immediately after the pulse. However, the $\Delta\delta^{13}C$ of stems three weeks post-labelling remained high at 762.2 ± 222.1 ‰, but this was lower than levels of enrichment immediately after the pulse. The $\Delta\delta^{13}C$ of main or primary roots was 400.9 ± 241.5 ‰ and 307.8 ± 123.4 ‰, compared to fine roots which had a $\Delta\delta^{13}C$ of 326.2 ± 72.9 ‰ and 158.7 ± 55.7 ‰ at 1 day and 3 weeks after the pulse event, respectively. Interestingly, the decrease in ^{13}C enrichment in primary roots was less than that of fine roots. The incorporation of ^{13}C into SOM was minor, with a mean $\Delta\delta^{13}C$ value of 2.7 ‰ throughout the experiment (Figure 5.1D). However, there was a significant increase between 1 and 4 days after pulse labelling.

^{13}C in bulk microbial biomass

The total SMB carbon content exhibited only minor variations across all time points suggesting a steady state; showing that the microbial biomass was constant throughout the experiment. When used in combination with stable carbon isotope analysis the CFE product can be used to track the source of microbial carbon. ^{13}C enrichment in total microbial biomass (TMB) was highest immediately after the pulse labelling of plants and remained so for at least 12 h after the pulse (Figure 5.2). The mean $\Delta\delta^{13}C$ of TMB 1, 3 and 12 h after pulse labelling was 95.5 ± 28.6, 102.4 ± 15.3 and 101.8 ± 7.3 ‰, respectively. This had decreased to 59.3 ± 14.9 ‰ at 24 h after pulse labelling and by the final sampling point 4 weeks after pulse labeling; the $\Delta\delta^{13}C$ had steadily decreased to 32.5 ± 1.6 ‰.

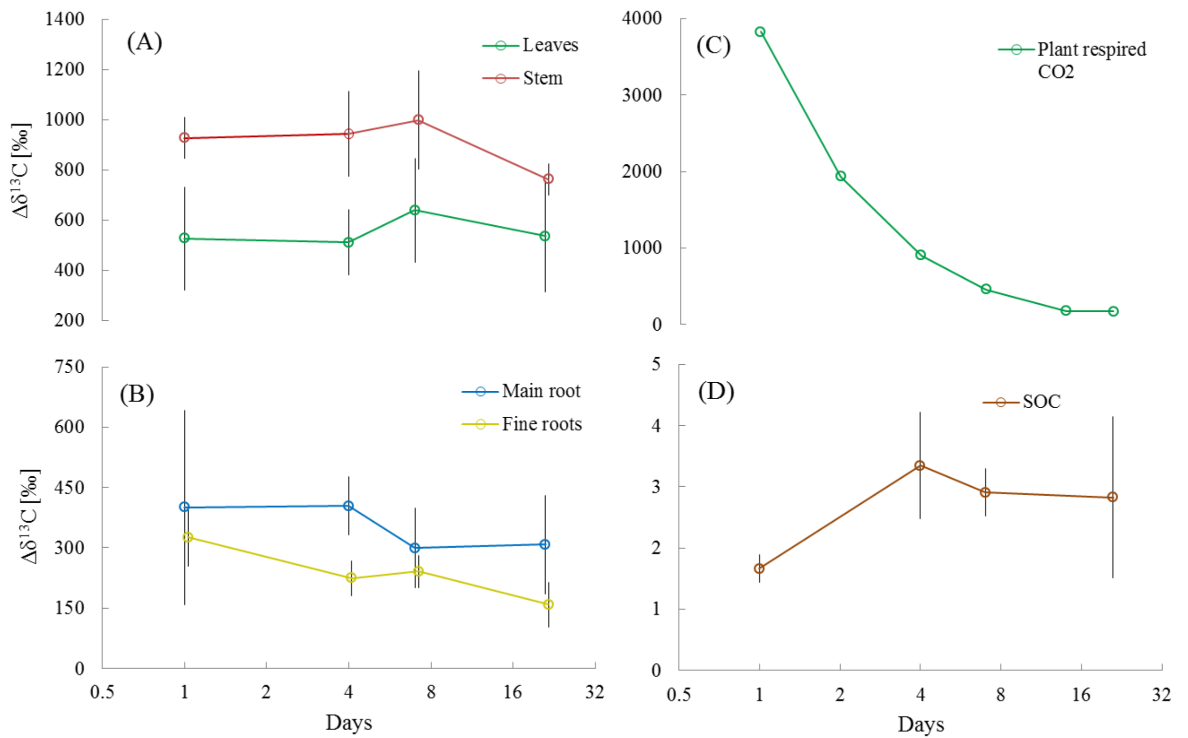

Figure 5.1: Isotope enrichment in different plant pools (A-C) and bulk soil organic matter (D). X-axis represents time after end of pulse labelling (n=3).

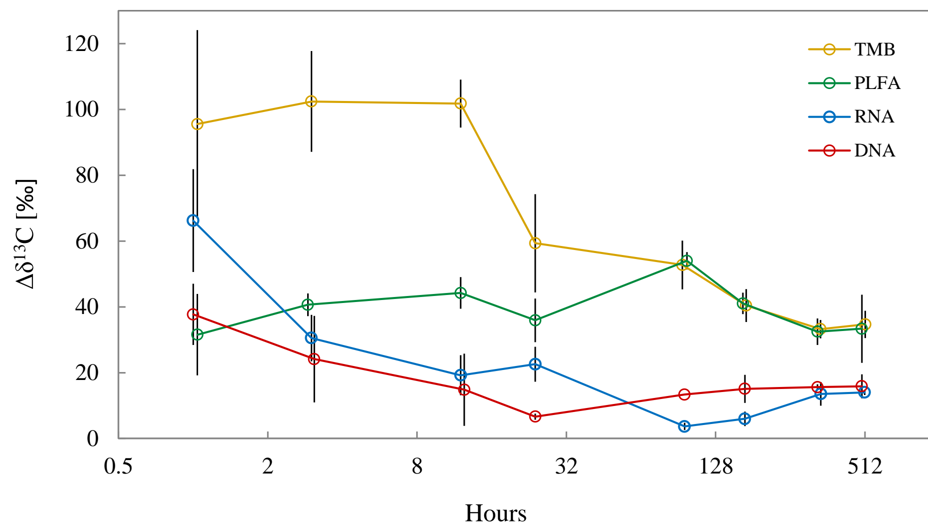

Figure 5.2: Isotope enrichment in different microbial cellular fractions (n=3). TMB: total microbial biomass obtained from chloroform fumigation extraction, PLFA: mean of all individual quantifiable PLFAs. X-axis represents time after end of pulse labelling.

^{13}C in microbial biomass size classes

Microbial CFE cell extracts separated by SEC were assigned to three size classes: low molecular weight (LMW), high molecular weight (HMW) and very HMW (vHMW). The relative abundance of the three size classes was 8, 80.4 and 11.6 %, respectively as a proportion of the total microbial biomass. The ^{13}C enrichment was highest in the LMW size class and decreased with increasing molecular weight (Figure 5.3). The mean $\Delta\delta^{13}C$ of LMW microbial biomass was 146.5 ± 43.4 ‰ at 3 h after pulse labelling, by 24 h after the pulse it had decreased to 78.9 ± 29 ‰ and by the end of the experiment it had more than halved to 31 ± 0.1 ‰. The successive decrease in $\Delta\delta^{13}C$ of HMW size class over time was less pronounced — 107.2 ± 16.5, 60.8 ± 14.6 and 33 ± 1.1 ‰ at 3 h, 24 h and 2 weeks after pulse labelling of plants, respectively. Finally, the mean $\Delta\delta^{13}C$ of vHMW fraction was the lowest amongst the microbial biomass size classes with measurements of 81 ± 29.9, 43.6 ± 18.7 and 20.3 ± 1.7 ‰ recorded 3 h, 24 h and 2 weeks after incubation with $^{13}CO_2$.

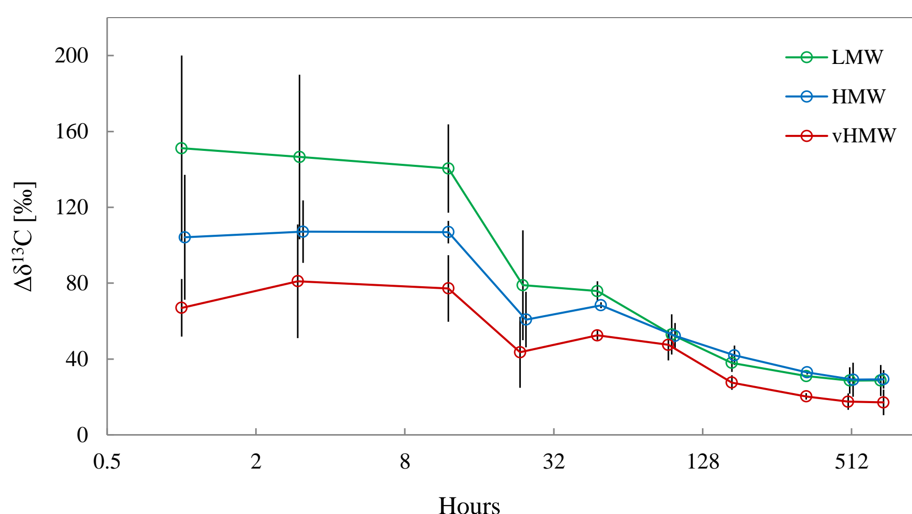

Figure 5.3: Isotope enrichment in different microbial size fractions (n=3). Labels- LMW: low molecular weight, HMW: high molecular weight, vHMW: very high molecular weight. X-axis represents time after end of pulse labelling.

^{13}C in microbial DNA and RNA

The incorporation of ^{13}C into DNA and RNA was also measured to assess the turnover of these compounds in soil microorganism. Soil microbial total DNA and RNA both showed highest ^{13}C enrichment immediately after pulse labelling of plants (Figure 5.2). ^{13}C enrichment in RNA decreased for up to 4 days, followed by a slight increase at 1 week which continued until the end of the experiment. The mean $\Delta\delta^{13}C$ of DNA 1 h after pulse labelling was 37.7 ± 9.3 ‰ and decreased to its lowest at 6.7 ± 0.9 ‰ at 24 h after the pulse event. This was followed by a minor increase to approximately 15 ‰ for the remaining time points. The ^{13}C enrichment in total RNA was also highest immediately after the pulse labelling event; 1 h after the pulse the mean $\Delta\delta^{13}C$ of RNA was 66.2 ± 15.6 ‰. It gradually decreased over the subsequent sampling points to the lowest level of enrichment (3.7 ± 1.1 ‰) 4 days after labelling. However, 2 weeks after

labeling there was a significant increase in the amount of ^{13}C in RNA ($\Delta\delta^{13}C$ = 13.6 ± 3.6 ‰) and this persisted until the final sampling point.

Size separation of the total RNA extracted from soil microorganism using the Agilent TapeStation resolved it into two distinct bands representing the bacterial small subunit 16S rRNA and large subunit 18S rRNA (Figure 5.4). Moreover, by comparing this to RNA extracted from yeast no fungal rRNA was observed in the soil total RNA extracts. On the contrary, there was no direct way to separate and identify the different constituents of DNA. Therefore, soil total DNA could represent bacteria, fungi as well as other soil fauna. However, with RNA we could identify the proportions of bacterial and fungal/eukaryotic RNA. Since the majority of soil RNA is bacterial in origin, it can be used as a biomarker to monitor bacterial carbon incorporation.

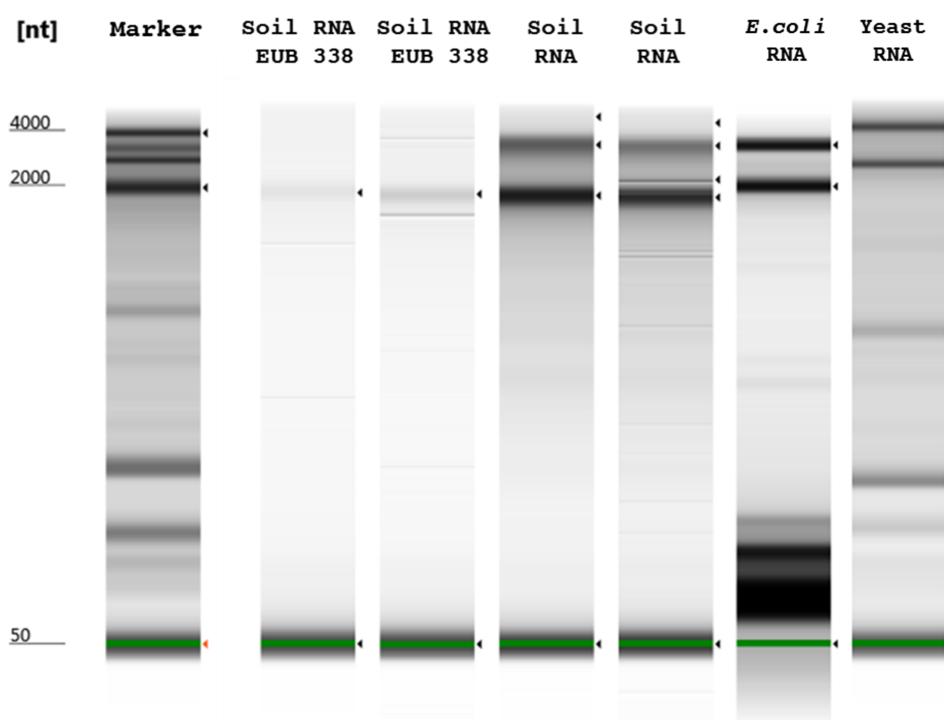

Figure 5.4: Size-based separation of RNA from soil microbial communities and pure cultures of *E. coli* and yeast on Agilent TapeStation-R6K ScreenTape. "Soil RNA EUB 338" is the captured 16S rRNA from the soil total RNA pool using magnetic bead capture hybridization. EUB 338 was the universal eubacterial probe used. E. coli and yeast were cultured in LB broth and RNA extracted using the standard phenol-chloroform extraction protocol (Griffiths et al., 2010).

^{13}C in PLFA, NLFA biomarkers

The abundances of total PLFAs and individual markers were consistent with only minor variation throughout the entire time series, corroborating the consistent microbial biomass amounts measured with CFE (data not shown). After pulse labelling of plants ^{13}C incorporation into total soil extractable PLFA remained largely similar (average $\Delta\delta^{13}C$ = 39.1 ± 7.5 ‰) throughout the experimental period (Figure 5.2) ranging between 31.5 ± 12.4 ‰ immediately after the labelling event and 54 ± 2.6 ‰ after 4 days. The absence

of a temporal trend in total PLFA ^{13}C enrichment could be due to the variable additive effects of the $\delta^{13}C$ values of individual PLFA markers. The ^{13}C incorporation patterns over time in different PLFA biomarkers were shown to be highly variable (Figure 5.5). Incorporation of ^{13}C was on average an order of magnitude higher in fungal PLFAs compared to bacteria (Figure 5.5). Highest enrichment was observed in the fungal PLFA 18:2ω6, with a mean $\Delta\delta^{13}C$ of 394.1 ± 36.7 ‰ and a minimum and maximum of 166.9 ± 61.4 ‰ and 621.3 ± 28.6 ‰, respectively. The $\Delta\delta^{13}C$ of the arbuscular mycorrhizal fungi (AMF) NLFA marker 16:1ω5 peaked 3 h after the pulse ($\Delta\delta^{13}C$ = 276.1 ± 352.4 ‰). The enrichment of the AMF marker decreased gradually over time to a minimum $\Delta\delta^{13}C$ of 26.2 ± 11.2 ‰. Contrastingly, ^{13}C enrichment of different bacterial PLFAs showed a divergent temporal pattern with roughly three discernable trends: 1) high enrichment immediately after the pulse event gradually increasing over time followed by a small drop after a week, eg. PLFA 18:1ω7; 2) consistent medium to low enrichment soon after the pulse with a gradual increase after 4 days, eg. PLFAs 15:0i, 16:0i, 17:0a, 17:0i; and 3) negligible enrichment immediately after the pulse followed by a small gradual increase towards the end of the experimental period, eg. PLFAs 17:0cy, 10Me17. The average $\Delta\delta^{13}C$ of representative Gram negative (18:1ω7) and Gram positive (16:0i) PLFA markers across all time points was 40.5 ± 4.3 ‰ and 16.6 ± 2.9 ‰, respectively. The $\Delta\delta^{13}C$ of the Actinomycetes biomarker PLFA 10Me17 was negligible post-labelling and gradually increased throughout the sampling regime to reach the highest value of 8.1 ± 2.6 ‰ after 3 weeks.

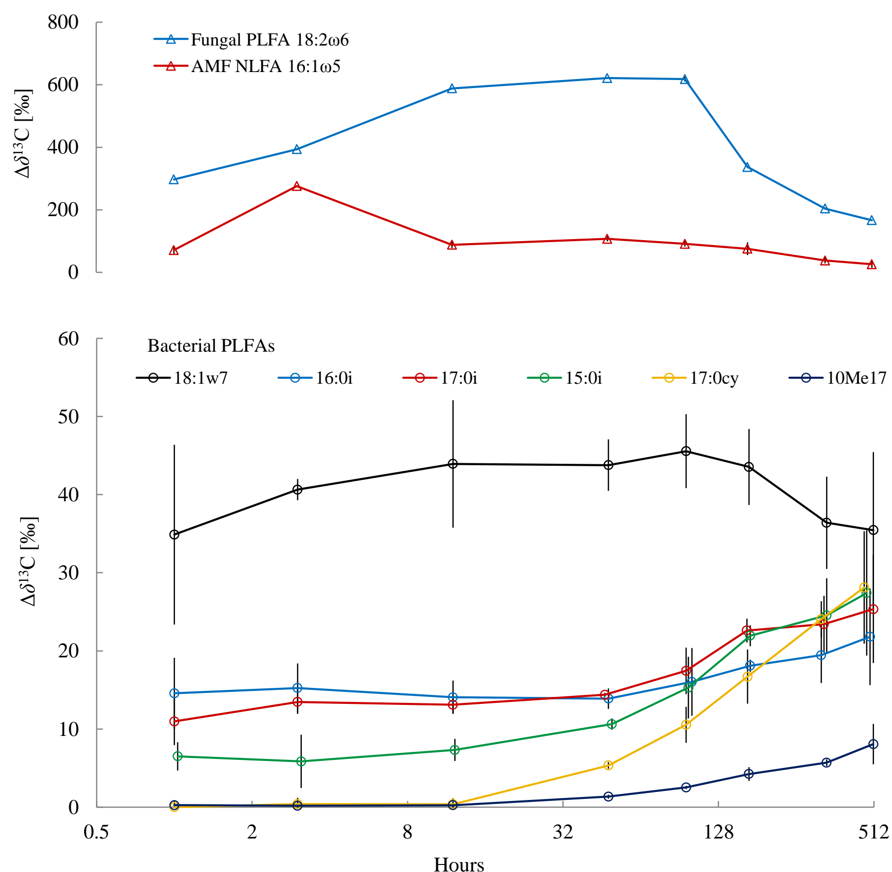

Figure 5.5: Isotope enrichment in different fungal and bacterial PLFA markers (n=3). X-axis represents time after end of pulse labelling.

DISCUSSION

This study used a novel approach combining stable isotope techniques and molecular biology to better understand C cycling in grassland systems. Here we have chased $^{13}CO_2$ into plants and subsequently into the soil microbial communities. Pulse $^{13}CO_2$ fixed by plants gets differentially allocated to different plant parts and is either used for respiration, biomass formation and/or storage. Up to 2 days after the pulse was applied, autotrophically respired CO_2 was highly enriched in ^{13}C indicative of fast respiratory fluxes. Based on this, it would be expected that the pulse ^{13}C be translocated into the rhizosphere as labile substances in the form of root exudates. These rhizodeposits serve as C source for rhizosphere microorganisms. As a consequence of this dependence, the ^{13}C incorporation patterns in the rhizosphere soil microbial compounds that are turned over faster reflect those of the labile plant pools. Due to the rapid growth and multiplication of microorganisms, ^{13}C from the pulse event was immediately incorporated into all microbial fractions; however, variable decomposition kinetics or ^{13}C dilution rates were observed.

Microbial biomass extracted by the widely used CFE procedure represents the cell lysis soluble extracts (Franzluebbers, 1999; Philippot et al., 2012). Faster carbon turnover of total microbial biomass extracts suggests that most microbial cytoplasmic compounds were rapidly renewed. The LMW size class of microbial biomass which was least abundant had the fastest turnover and can be linked to rapid respiratory carbon fluxes. Compounds in this size class are most likely sugars, amino acids, organic acids, etc. which are rapidly metabolized. The HMW (most abundant size class) and vHMW microbial compounds are thought to comprise of either large soluble polymers or mid-sized products of microbial degradation; and have relatively slower turnover. The size-turnover relationship of microbial cytoplasmic compounds suggests that smaller compounds are labile metabolic products; whereas larger compounds are microbial structural macromolecules which have a comparatively slow renewal rate and could be linked to slower SOM formation (Kindler et al., 2009; Malik et al., 2013; Miltner et al., 2012; Simpson et al., 2007).

Phospholipid fatty acids which form part of the microbial cell walls are steadily produced during cell growth and multiplication but have a relatively slow carbon turnover compared to other cellular macromolecules, based on ^{13}C incorporation values. On the contrary, higher ^{13}C enrichment was observed in DNA and RNA immediately after the pulse, and this decreased rapidly, suggestive of higher carbon turnover rates. As expected, DNA shows slower carbon turnover compared to RNA (Dumont et al., 2011; Manefield et al., 2002; Radajewski et al., 2003). This is because DNA is only renewed during cell multiplication and since DNA replication is semiconservative only half of the DNA is renewed (Meselson and Stahl, 1958). Moreover, DNA is often present in single copy numbers within cells and not all organisms in soil are actively replicating. Higher carbon turnover in RNA is reflective of the active anabolic function of the macromolecule; also its production is independent of cell replication. Interestingly, ^{13}C incorporation in both DNA and RNA remained high towards the end of the study, indicating cross-feeding or secondary assimilation of labelled substrates. This increase in ^{13}C enrichment was significant in RNA and suggests carbon recycling in the soil microbial loop. In nutrient-poor environments like soil, microorganisms would employ the salvage pathway of nucleotide synthesis rather than the *de novo* pathway (Nyhan, 2005). An indication that soil microorganisms use the former pathway to recycle nucleotides from previously degraded RNA is available from the late increase in ^{13}C enrichment in RNA towards the end of the time series period (Dumont et al., 2011). The initial ^{13}C dilution

rate or decrease in ^{13}C enrichment in all biomarkers except fatty acids coincided with a small yet significant increase in ^{13}C in bulk SOM. This confirms that microbial biomass contributes to SOM genesis through the iterative process of microbial metabolism, growth, multiplication and death (Miltner et al., 2012).

PLFA and NLFA markers when combined with carbon stable isotope analysis can be used to track the flow of labelled substrate into different functional groups of microorganisms (Boschker et al., 1998). A peak in ^{13}C incorporation in the arbuscular mycorrhizal fungi (AMF) represented by the NLFA 16:1ω5 marker was observed immediately following the pulse which indicates that the AMF are important in the initial carbon transfer from plants into soil microorganisms (Balasooriya et al., 2014; Drigo et al., 2010). The AMF form a symbiotic association with plant roots and depend on root exudates for their carbon requirement. The general fungal marker PLFA 18:2ω6 also had high ^{13}C incorporation soon after the pulse but peaked only later; this lag shows dependence of saprophytic fungi on complex organic matter from plants (fine root litter). The decline in ^{13}C content of this fungal PLFA marker correlates with an increase in most bacterial markers suggestive of a carbon transfer from fungi to bacteria (Balasooriya et al., 2014; Drigo et al., 2010). However different bacterial markers showed divergent patterns in ^{13}C incorporation highlighting the diversity of bacterial carbon use strategies (Kramer and Gleixner, 2008; Philippot et al., 2013; Vandenkoornhuyse et al., 2007). As a result, two ecological statuses of soil bacteria based on their C substrate utilization were discerned: the mutualistic rhizosphere bacteria mostly Gram negative types that rely on plant root exudates; and the saprophytes mostly Gram positive bacteria including Actinomycetes, which feed on complex organic matter from plants and/or other microorganisms. However, it should be noted that these differences in carbon turnover could also arise due to variable turnover of PLFAs in different bacteria (Ratledge and Wilkinson, 1989). It is known that Gram positive bacterial cell walls are more complex and this could be the reason for slower turnover rates of PLFA markers representative of this bacterial group. Therefore this factor should be taken into consideration when interpreting results from PLFA stable isotope probing.

Most RNA from the total RNA pool of soil microorganism is bacterial (Figure 5.4) (Urich et al., 2008). Therefore, we used total RNA from soil as a bacterial biomarker to monitor tracer carbon assimilation. The patterns of ^{13}C incorporation in bacterial RNA thus signify that bacteria are also important utilizers of the initial plant C released into the rhizosphere. This is in contrast to most existing literature based on PLFA stable isotope probing which underestimates the role of bacteria in initial plant carbon allocation largely due to the slow turnover of bacterial cell wall lipids (Balasooriya et al., 2014; Drigo et al., 2010). This could suggest that PLFA biomarkers are less appropriate for monitoring rapid carbon fluxes such as those occurring in the plant-rhizosphere-soil continuum.

CONCLUSION

Based on ^{13}C incorporation amounts, the turnover rates of microbial components of differing biochemical properties and size were found to significantly differ. The different degree of retention of microbial cellular fractions implies their differential contribution to soil C storage. Thus microbial cellular biochemistry plays an important role in stabilization of microbial carbon in soil. Based on the results of our multi-biomarker tracer study we present a mechanistic model of rhizosphere soil microbial carbon cycling in which there is continuous recycling of carbon in the microbial loop that could be an additive effect of exchanges within and between trophic levels. However, we question whether the current assumptions regarding plant-microbe C flux based on PLFA analyses underestimates the importance of rhizosphere bacteria as an initial conduit of plant-released C and recommend more detailed quantitative investigations with DNA and RNA biomarkers to better discern the trophic interactions in soil microbial food web.

Chapter 6

General Discussion

The thesis aims at understanding the mechanism of microbial control on carbon cycling in soil, specifically the turnover and recycling of carbon in the microbial food web. The flow of photosynthetically fixed plant C was traced into soil microorganisms using two isotope tracer experiments: 1) a greenhouse-based pulse chase $^{13}CO_2$ plant labeling experiment to monitor the plant-soil carbon flux over a short time scale; and 2) a C3-C4 vegetation change field experiment to investigate longer term C turnover dynamics. Studying physiology of microorganisms in terms of carbon metabolism in complex environments like soil is difficult mostly due to technological limitations. Therefore, the first aim was to develop tools that help gain new insights into microbial processes in natural environments. The goal was to standardize a new LC-IRMS based application that couples the online separation potential of size exclusion chromatography with the accurate isotope ratio measurement potential of mass spectrometers. This technique was then applied to follow the isotope tracers from plant and soil organic matter into the soil microbial and associated pools in the abovementioned experiments. This allowed approximation of carbon turnover rate of different microbial biomarkers like DNA, RNA and cytosolic compounds; since varying turnover could contribute differentially to microbial carbon storage in soil. In experiment 1, carbon flow was also monitored into different microbial functional groups using PLFA, NLFA and RNA carbon stable isotope analyses in order to reappraise carbon flow paths though the soil microbial food web and to highlight the key trophic interactions. Finally, with experiment 2, the sources of dissolved organic carbon (DOC) size classes were elucidated over a seasonal cycle and it was linked to soil microbial size classes to decipher microbial control over DOC production and degradation.

Online coupling of size exclusion chromatography with LC-IRMS allowed us to successfully measure the stable carbon isotope ratios of organic compound size classes (Malik et al., 2012). The interface enables isotopic measurement of aqueous soluble, nonvolatile compounds from complex mixtures. The method was first evaluated with different standard organic compounds and later applied to soil DOC (Chapter 2). After successfully standardizing the method with soil DOC from the C3-C4 vegetation change experiment, the technique was successfully applied to measure the stable carbon isotope ratios of marine and riverine DOC size classes without any sample pretreatment. Soil microbial biomass carbon obtained by chloroform fumigation exaction from both the experiments was effectively measured for bulk and size class $\delta^{13}C$ values using the μEA and SEC mode of LC-IRMS, respectively (Chapter 3). An advantage of the SEC separation is that it allows precise measurement of microbial biomass carbon and its isotope ratios by excluding contaminants like chloroform (Malik et al., 2013). In addition, the SEC mode was utilized to accurately analyze $\delta^{13}C$ values of soil microbial nucleic acids (Chapter 5). By separating soil co-extracts like humic acids and contaminating substances from protocol reagents like organic solvents, this technique results in improvement of precision and accuracy of analysis and allows measurement of smaller amounts of nucleic acid carbon. These measurements were effectively used to estimate the turnover time of microbial DNA and RNA in soil. Thus, the applications of the newly developed method provided insights into key processes in soil microbial carbon cycling.

Uptake rates of plant-derived carbon were found to differ depending on the intracellular biomarker assessed. It was established that the turnover rates of individual microbial components of differing biochemical properties were found to significantly vary (Chapter 5). The turnover of cell wall fatty acids was very slow. DNA and RNA showed faster turnover rates; and as expected RNA renewal was the fastest due to its rapid production by active microorganisms, independent of cell replication. It was also inferred that cytosolic soluble compounds are rapidly metabolized and linked to

respiratory C fluxes; and therefore have higher renewal rate. In soil, this variable turnover of individual microbial cellular fractions could lead to their different degree of retention implying differential contribution to soil organic carbon storage (Malik et al., in preparation). To conclude, cellular biochemistry plays an important role in stabilization of microbial carbon in soil.

The post-pulse labelling isotope dilution patterns of microbial cellular fractions indicated microbial "cross-feeding" or secondary plant carbon assimilation hinting at recycling of carbon in soil microorganisms (Chapter 5). Moreover, from the long term C3-C4 vegetation change experiment it was observed that old (>5 years) C3 soil organic carbon as a source accounts for as much as 70% of the microbial biomass carbon (Chapter 3). Based on these findings I conclude that there is continuous recycling of carbon in soil microorganism and propose a mechanistic concept, the soil "microbial carbon loop" (MCL) which emphasizes the microbial processing and reuse of plant and soil organic carbon. This can be linked to the recently recognized "microbial carbon pump" concept in marine systems, which highlights the microbial transformation of organic carbon from labile to recalcitrant forms (Jiao et al., 2010; Jiao and Zheng, 2011). Thus, the microbial carbon pump is thought to be responsible for the long residence time of a major fraction of the marine dissolved organic carbon. On similar lines, the soil MCL by continuous internal recycling and by processing organic carbon into compounds with longer persistence could lead to stabilization of soil carbon. The MCL could thus be a supplementary mechanism of microbial carbon storage in soil in addition to the well accepted concept of SOM genesis from microbial necromass (Liang and Balser, 2011; Miltner et al., 2012). This could be significant considering estimates which suggest that microbial carbon is globally equivalent to the standing plant biomass carbon (Whitman et al., 1998).

It was successfully illustrated that soil microbial carbon turnover decreases with increasing molecular size at varying time scales: short; daily to weekly (Chapter 5) and long; yearly (Chapter 3). Relatively higher turnover time of low molecular weight "metabolite" cytosolic fraction could be associated to the rapid mineralization of plant carbon by microorganisms. On the contrary, microbial compounds in the higher molecular weight range which have slower turnover rates could be microbial biosynthetic products. These structural compounds could persist in soils, become part of the stabilized soil organic matter and/or get leached in soil water. Proof to support this comes from the DOC seasonal monitoring experiment, where it was found that majority of DOC compounds are derived from SOM (Chapter 4). Across all seasons and soil types studied the plant contribution both through litterfall and root litter/exudation is a less significant source of DOC in mineral soil. Even in the active vegetation period the contribution of fresh plant biomass to DOC was low, which suggests that incorporation of plant residues leads to microbially mediated remobilization of some soil organic carbon which is leached into the dissolved phase. Thus, the conclusion was drawn that decomposition and remobilization of SOM and its processing by microorganisms are the most important processes in DOC production (Malik and Gleixner, 2013). In consequence, DOC is reactively transported downward in soil whereby DOC molecules are partitioned manifold between soil solution and the mineral matrix (chromatography) thereby getting constantly decomposed, altered or produced anew by soil microorganisms (reactive transport) (Kaiser and Kalbitz, 2012).

The final thesis objective was to reappraise the carbon flow path though the soil microbial food web using a multi-biomarker approach. Results from the isotopic incorporation into PLFA/NLFA reiterated the significance of fungi in general and AMF in particular in the initial translocation of plant carbon into the rhizosphere soil (Chapter 5). In this current view of plant-soil carbon transfer, bacteria are considered "latecomers" and quantitatively less significant. However, rapid pulse derived ^{13}C enrichment of bacterial RNA measured for the first time indicated that bacteria are important in the initial translocation of plant-released carbon. Thus, two groups of soil microorganisms based on their ecological status with respect to the carbon requirement were identified: the arbuscular mycorrhizal fungi (AMF) and mutualistic rhizosphere bacteria that rely on plant root exudates; and the saprophytic bacteria and fungi that feed on complex organic matter from plants and/or microorganisms. These trophic interactions were added to the proposed MCL concept which now suggests that microbial processing and continuous recycling of plant and soil organic carbon in the "microbial carbon loop" is an additive effect of exchanges within and between trophic levels (Figure 6.1). While SOM genesis from dead microbial biomass contributes significant to carbon stabilization in soil, the MCL could represent an important carbon storage mechanism through living microbial biomass.

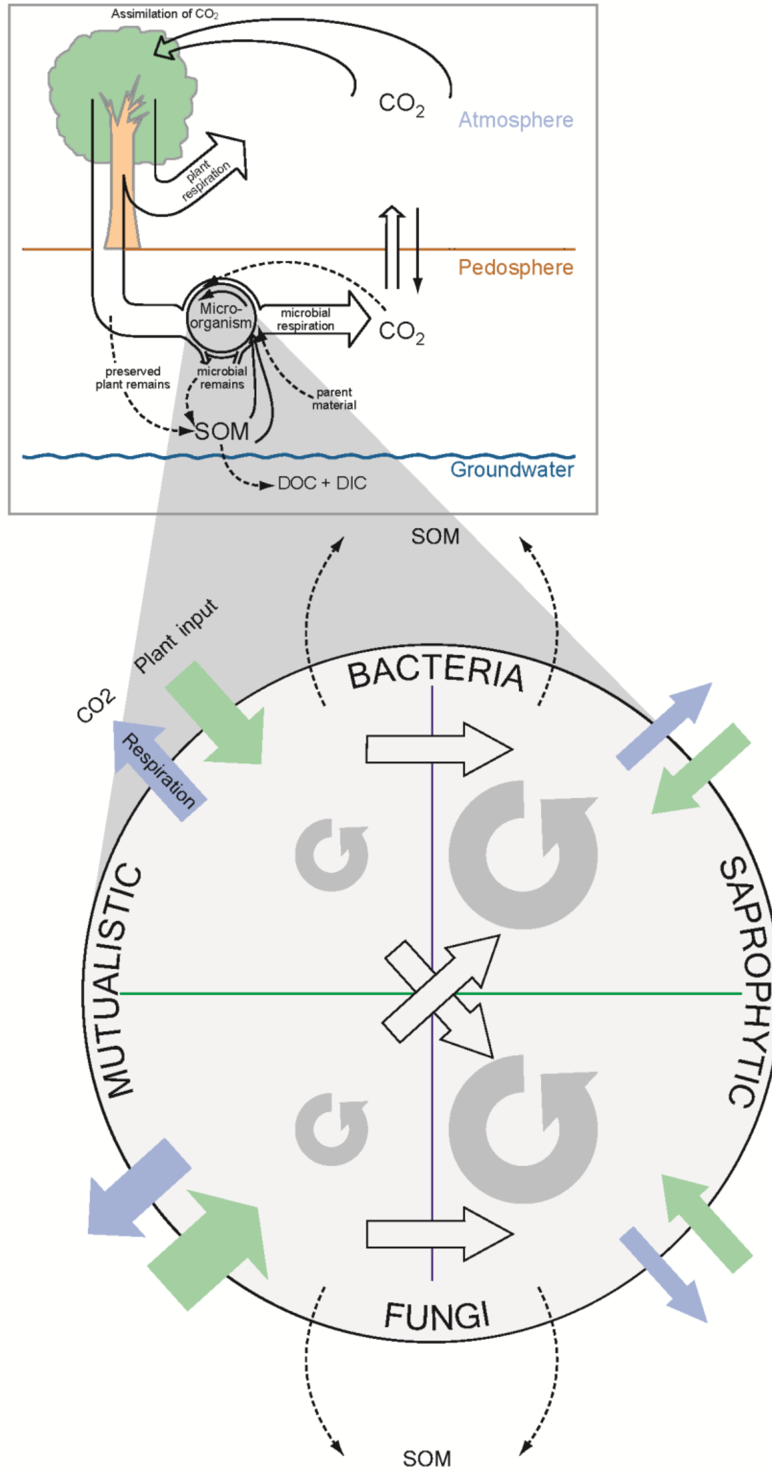

Figure 6.1: A schematic view of the terrestrial carbon cycle with a focus on the proposed mechanistic concept of "Microbial Carbon Loop-MCL". MCL emphasizes on the microbial processing and continuous recycling of organic carbon within and between trophic levels. Figure modified after Gleixner, 2013.

Outlook:

The thesis provides evidence that cellular biochemistry affects microbial soil carbon maintenance. Further molecular characterization of microbial compounds in soil coupled with isotope tracers will help gain better mechanistic understanding of microbially mediated soil carbon stabilization. More detailed molecular investigations with new analytical tools like SEC coupled to FTIR (Fourier Transform Infrared Spectroscopy) is necessary to further corroborate microbial control on key soil processes (Landry and Tremblay, 2012). Secondly, the trophic interactions in the rhizosphere soil food web were reevaluated and the reformed view was incorporated in the mechanistic MCL concept. I question whether the current assumptions based on PLFA analyses underestimates the significance of root-associated bacteria as an initial conduit of plant-released C and recommend more detailed quantitative investigations with biomarkers like DNA and RNA to better discern trophic interactions in the soil MCL. In this regard, quantifying tracer incorporation into phylogenetically or functionally separated RNA pools using magnetic bead capture hybridization stable isotope analyses might be fruitful. Protein stable isotope probing and coupled in situ hybridization nano-SIMS (Secondary Ions Mass Spectrometry) both offer immense potential in linking microbial identity to function in terms of plant or soil organic carbon assimilation (Murrell and S. Whiteley, 2011). Finally, we recommend the use with further verification of the proposed conceptual framework of "microbial carbon loop" as an important mechanism of long-term carbon storage in soil.

Many studies are now investigating how anthropogenic change (eg. warming, change in land-use, loss of plant diversity, drought and fire) affects soil biodiversity and soil carbon. Typically it is observed that intensification of management or alterations of aboveground diversity brings about associated change in both soil biodiversity and carbon storage. However, the explicit linkages are harder to discern – i.e. is it the environment/aboveground change which is altering soil biodiversity causing change in carbon storage, or is soil biodiversity simply responding to increased/decreased carbon inputs. It is widely believed that warming induced changes in microbial physiology affect microbial carbon-use efficiency and either limit or enhance soil microbial decomposition making it critical in controlling the fate of soil carbon (Wieder et al., 2013). Yet, the sensitivity of microbial carbon-use efficiency and the mechanism of their physiological adaptation to environmental change still remain unclear. This work opens new avenues to discern whether change brings about differences is carbon storage within different microbial cellular components. There is therefore a need to roll out the approaches described here in further studies examining the mechanisms by which change affects both soil biodiversity and carbon storage.

Summary

Soil microorganisms play an important role in respiring newly fixed C but also contribute to the maintenance of soil organic matter through turnover and recycling of their biomass. Microbial growth and activity largely regulates the soil-atmosphere carbon exchange which makes soil microorganisms central in terrestrial C cycling. The thesis sought to understand the physiological mechanism of the microbial control on processes involved in decomposition and stabilization of organic matter in soil. It was hypothesized that microbial fractions differing in biochemical properties and size vary in their turnover and therefore could contribute differentially to soil C storage. In addition, a reappraisal of the root-associated microbial food web was pursued with a multi-biomarker stable isotope probing approach. The final aim was to link microbial processes to the large soil organic carbon (SOC) and the small yet highly reactive dissolved organic carbon (DOC) pool.

A C3-C4 vegetation change experiment was employed to investigate long term microbial C turnover dynamics and a $^{13}CO_2$ pulse plant labeling experiment was used to trace and compare the flow of plant C into different microbial compounds over a short time scale. Tracer carbon was chased into rhizosphere soil microbial DNA, RNA, fatty acids (FA) and microbial biomass (chloroform fumigation extraction-CFE derived) size classes using a combination of molecular biology and mass spectrometry approaches. A novel LC-IRMS (liquid chromatography-isotope ratio mass spectrometry) based size exclusion chromatography application was developed to measure stable carbon isotope ratios of organic compound size classes. The interface enabled accurate isotopic measurement of aqueous soluble, non-volatile compounds from complex mixtures, such as CFE-derived microbial biomass, soil microbial nucleic acids and DOC.

It was established that microbial cellular compounds have differing carbon turnover time. The turnover of DNA, RNA and the cytosolic "metabolite" fraction was much faster compared to cell wall fatty acids. Microbial carbon turnover decreased with increasing molecular size both at short and long time scales. Variable turnover rates of microbial biochemical fractions suggest that cellular biochemistry plays an important role in stabilization of microbial C in soil. Pulse carbon "cross-feeding" or secondary assimilation indicated internal recycling of carbon by microorganisms. This was further corroborated by evidence from the field experiment, where up to 70% of the microbial biomass carbon was derived from old soil organic matter. This implies that microbial processing and recycling of plant and soil organic carbon could be an important mechanism leading to soil carbon stabilization.

Phospholipid/neutral lipid fatty acid (PLFA/NLFA) and RNA ^{13}C analyses was employed to assess plant pulse ^{13}C flow into soil microbial functional groups. FA-based observations confirmed that symbiotic arbuscular mycorrhizal fungi are important in the initial conduit of plant-released C. Other saprophytic fungi and bacteria demonstrated delayed ^{13}C assimilation which could suggest secondary ^{13}C assimilation indicative of trophic interactions. However, RNA ^{13}C incorporation pattern indicated that bacteria are also important utilizers of the plant C initially released into the rhizosphere; contradicting FA-based observations. The very slow turnover of PLFAs compared to RNA suggests that PLFAs are not appropriate to monitor rapid C fluxes. Moreover, different microbial groups show variable internal PLFA turnover rates; Gram-positive bacteria due to their complex cell walls could turnover more slowly than Gram-negative bacteria. This raises the question whether previous assessments using PLFA analyses accurately represent trophic interactions or if observed differences in C flow dynamics are simply due to the differences in internal turnover of fatty acids. Thus, the current FA-based assumptions which underestimate the importance of bacteria in rhizosphere carbon

cycling are highly disputable. There is therefore a need for detailed quantitative investigations on plant-soil C fluxes with nucleic acid biomarkers to better discern soil microbial trophic interactions.

Finally, based on the findings I propose a mechanistic concept termed the soil "microbial carbon loop" (MCL) which explains the microbial processing and continuous recycling of plant and soil organic carbon within and between trophic levels. While microbial necromass contributes significantly to SOM formation, the MCL could represent an important microbial physiological mechanism leading to carbon storage in soil. We recommend the use of the proposed conceptual framework of "microbial carbon loop" to further investigate and verify microbial mechanism of carbon cycling in soil. The interdisciplinary approaches described in the thesis can be applied in further studies to examine the microbial physiological adaptation to environmental change that is potentially altering both the soil biodiversity and carbon storage.

Zusammenfassung

Bodenmikroorganismen spielen eine wichtige Rolle neu fixierten Kohlenstoff als Stoffwechselprodukt in Form von CO_2 wieder in die Atmosphäre zu entlassen, tragen aber auch zur Erhaltung der organischen Substanz im Boden durch Umsatz und Recycling ihrer Biomasse bei. Das Wachstum und die Aktivität der Mikroorganismen regulieren weitgehend den Kohlenstoffaustausch zwischen Boden und Atmosphäre. Damit spielen die Bodenmikroorganismen eine zentrale Rolle im terrestrischen Kohlenstoffkreislauf. Ziel dieser Arbeit war es, die physiologischen Mechanismen der mikrobiellen Kontrolle über Prozesse, die an der Zersetzung und Stabilisierung organischer Substanz im Boden beteiligt sind, zu verstehen. Die Hypothese war, dass die mikrobiellen Fraktionen, die sich in ihren biochemischen Eigenschaften und ihrer Größe unterscheiden, einen unterschiedlichen Umsatz aufweisen und somit einen differenzierten Beitrag zur Kohlenstoffspeicherung im Boden liefern. Darüber hinaus wurde eine Neubewertung des Wurzel-assoziierten mikrobiellen Nahrungsnetzes durchgeführt, indem die stabilen Isotope in verschiedenen Biomarkern untersucht wurden (Stable Isotope Probing, SIP). Das Hauptziel dieser Arbeit bestand darin, einen Zusammenhang zwischen den mikrobiellen Prozessen und einerseits dem großen organischen Kohlenstoff (Soil Organic Carbon, SOC)-Speicher im Boden sowie andererseits dem kleinen, aber hochreaktiven Vorrat an gelösten organischen Kohlenstoff (Dissolved Organic Carbon, DOC) herzustellen.

Ein C3-C4-Vegetationswechselexperiment wurde durchgeführt, um die Dynamik des langfristigen mikrobiellen Kohlenstoffumsatzes zu untersuchen. Außerdem wurde ein $^{13}CO_2$-Pulsmarkierungsexperiment angewandt, um den Fluss von Pflanzenkohlenstoff in verschiedene mikrobielle Substanzklassen innerhalb kurzer Zeit zu verfolgen und zu vergleichen. Der markierte Kohlenstoff wurde in die DAN (Desoxyribonukleinsäure, deoxyribonucleic acid), RNA (Ribonukleinsäure, ribonucleic acid) und Fettsäuren (FA) der Mikroorganismen der Rhizosphäre sowie in die Größenklassen mikrobieller Biomasse (extrahierte Biomasse nach Begasung mit Chloroform, Chloroform Fumigation Extraction, CFE) verfolgt, indem molekularbiologische Methoden in Kombination mit Massenspektrometrie-Verfahren eingesetzt wurden. Eine neue Größenauschlusschromatographie-Methode (Size Exclusion Chromatography, SEC) die auf Flüssigkeitschromatographie-Isotopenverhältnismassenspektrometrie (Liquid Chromatography-Isotope Ratio Mass Spectrometry, LC-IRMS) basiert, wurde entwickelt. Diese Methode ermöglicht es die stabilen Kohlenstoffisotopenverhältnisse verschiedener Größenklassen organischer Verbindungen zu messen. Diese Kombination von SEC mit LC-IRMS ermöglicht die genaue Isotopenanalyse von wasserlöslichen, nicht-flüchtigen Verbindungen in komplexen Gemischen, wie in mittels CFE-extrahierter mikrobieller Biomasse, mikrobiellen Nukleinsäuren aus Böden und DOC.

Es wurde gezeigt, dass die zellulären mikrobiellen Fraktionen unterschiedliche Kohlenstoffumsatzzeiten haben. Der Umsatz von DNA, RNA und der zytosolischen "metabolischen" Fraktion war deutlich schneller im Vergleich zum Umsatz der Fettsäuren der Zellmembran. Der Umsatz des mikrobiellen Kohlenstoffs nahm mit zunehmender Molekülgröße sowohl über kurze als auch längere Untersuchungszeiträume hinweg ab. Unterschiedliche Umsatzraten mikrobieller biochemischer Fraktionen deuten darauf hin, dass die zelluläre Biochemie eine wichtige Rolle bei der Stabilisierung des mikrobiellen Kohlenstoffs im Boden spielt. Eine Kreuzverteilung des markierten Kohlenstoffs (pulse carbon "cross-feeding") oder sekundäre Assimilation weisen auf ein internes Recycling des Kohlenstoffs durch die Mikroorganismen hin. Dies wird zusätzlich gestützt durch Erkenntnisse aus dem Feldexperiment, die zeigten, dass bis zu 70 % des Kohlenstoffs in der mikrobiellen Biomasse aus altem organischem Bodenmaterial stammte. Dies könnte bedeuten, dass

die mikrobielle Verarbeitung und das mikrobielle Recycling des Kohlenstoffs aus Pflanzen und organischem Bodenmaterial ein wichtiger Mechanismus zur Stabilisierung von Kohlenstoff im Boden sein könnte.

Die ^{13}C-Analysen der Phospholipidfettsäuren (Phospholipid Fatty Acid, PLFA), der neutralen Fettsäuren (Neutral Lipid Fattic Acids, NLFA) sowie der RNA wurden zur Einschätzung des ^{13}C-Flusses nach der Pulsmarkierung der Pflanzen in die funktionellen Gruppen der Bodenmikroorganismen eingesetzt. Beobachtungen basierend auf den Fettsäureanalysen bestätigen, dass symbiotische arbuskuläre Mykorrhizapilze wichtig für die initiale Weiterleitung des durch die Pflanzen freigesetzten Kohlenstoffs sind. Andere saprophytische Pilze und Bakterien zeigten eine verzögerte ^{13}C-Assimilation, die auf eine sekundäre ^{13}C-Assimilation aufgrund trophischer Interaktionen hinweisen könnte. Jedoch deutete das ^{13}C-Aufnahmemuster in die RNA darauf hin, dass Bakterien ebenfalls wichtige Konsumenten des zunächst in die Rhizosphäre entlassen Pflanzenkohlenstoffs sind, womit den Beobachtungen aus den Fettsäureuntersuchungen widersprochen wird. Der sehr langsame Umsatz von PLFAs im Vergleich zu RNA deutet darauf hin, dass PLFAs nicht geeignet sind, um schnelle Kohlenstoffflüsse zu beobachten. Darüber hinaus zeigen verschiedene mikrobielle Gruppen unterschiedliche interne PLFA-Umsatzraten. Dabei könnten Gram-positive Bakterien aufgrund ihrer komplexen Zellwände PLFA langsamer umsetzen als Gram-negative Bakterien. Es stellt sich die Frage, ob vorangegangene Bewertungen mit PLFA-Analysen trophische Interaktionen exakt wiedergegeben haben oder ob die beobachteten Unterschiede in der Dynamik der Kohlenstoffflüsse nur auf unterschiedliche interne Umsätze der Fettsäuren zurückzuführen sind. Damit sind die momentanen fettsäurebasierten Annahmen, die die Bedeutung der Bakterien im Kohlenstoffkreislauf der Rhizosphäre unterschätzen, in Frage gestellt. Es besteht daher der Bedarf detaillierte quantitative Untersuchungen zu Kohlenstoffflüssen zwischen Pflanzen und Boden mittels Nukleinsäurebiomarkern durchzuführen, um mikrobielle trophische Interaktionen besser zu erkennen.

Auf Grundlage der Ergebnisse schlage ich schließlich ein mechanistisches Konzept mit der Bezeichnung „mikrobieller Kohlenstoffkreislauf im Boden“ (soil "Microbial Carbon Loop", MCL) vor. Es erklärt die mikrobielle Verarbeitung und das ständige mikrobielle Recycling von organischem Kohlenstoff aus Pflanzen und Bodenmaterial innerhalb und zwischen trophischer Ebenen. Während mikrobielle Nekromasse wesentlich zur SOM-Bildung beiträgt, könnte der MCL einen wichtigen physiologischen Mechanismus der Mikroorganismen darstellen, der zur Speicherung von Kohlenstoff im Boden führt. Wir empfehlen die Verwendung des vorgeschlagenen Rahmenkonzepts MCL, um den mikrobiellen Mechanismus des Kohlenstoffkreislaufs im Boden weiter zu untersuchen und zu bestätigen. Die interdisziplinären Ansätze, die in dieser Arbeit beschrieben sind, können in zukünftigen Studien angewandt werden. Diese Folgestudien sollten die physiologische Anpassung der Mikroorganismen an Umweltveränderungen, welche möglicherweise sowohl die Biodiversität im Boden als auch die Kohlenstoffspeicherung verändern, untersuchen.

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Appendix 1

Supporting Information for Chapter 2

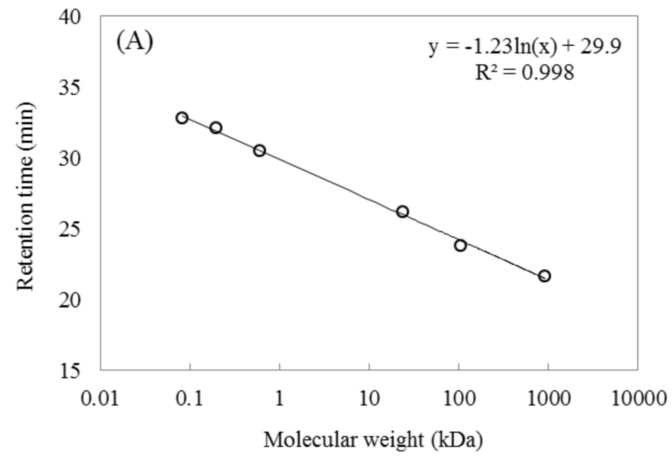
Experimental site

A long term vegetation change field experiment with two types of soils began in 2006 at the Max Planck Institute for Biogeochemistry, Jena, Germany. Two soils (Table S-1) that had always carried C3 type plants were subjected to a vegetation change from C3 to C4. A half of each soil plot was continued in C3 vegetation (as control) to compare δ^{13}C values. Thus, there were four plots altogether: Sandy C3, Sandy C4, Clayey C3 and Clayey C4. Since 2006, different plant species were grown in the C3 and C4 plots. *Phacelia* (scorpion weed), *Helianthus annus* (sunflower) and *Triticum* spp. (wheat) were the C3 plants grown. The C4 plants grown were *Eragrostis, Zea mays* (maize), *Amaranthus* and *Sorghum*. Rotation or change in plant species over the years was done in order to prevent monoculture in the fields.

The annual cycle began post winter when the plastic sheets that cover the plots were removed and the soil was tilled superficially. Seeds were sown in spring and allowed to grow normally till its harvest in autumn. Care was taken to maintain the same amount of biomass in both vegetation types so that roughly the same amount of carbon enters the soil. Care was also taken to remove unwanted plants and weeds growing in the plots as this could affect the δ^{13}C signal. Post the harvest, the entire plant biomass was weighed, shredded and then returned to the respective soil plots (equal amount of plant biomass returned to C3 and C4 plots). The plastic sheets then cover the plots until the next spring to prevent pollen and seed dissemination onto the plots.

Table A-1.1: Characteristics of soil used to create the dump plots for vegetation change experiment

Soil type/ nomenclature	**Sandy (S)**	**Clayey (C)**
Soil parent material	Forest A-horizon soil	B-horizon of a calcareous soil
Soil texture	50 % sand, 44 % silt, 6 % clay	9 % sand, 75 % silt,16 % clay
Soil pH	6.9	7.8
Organic C concentration	4.20 ± 0.19 %	1.96 ± 0.27 %
Inorganic C concentration	ND	0.78 ± 0.15 %
Organic N concentration	0.27 ± 0.01 %	0.21 ± 0.02 %
pH of soil water	7.3 (± 0.2)	8.0 (± 0.1)
Conductivity of soil water	573 (± 349) µS cm^{-1}	1257 ± 376 µS cm^{-1}



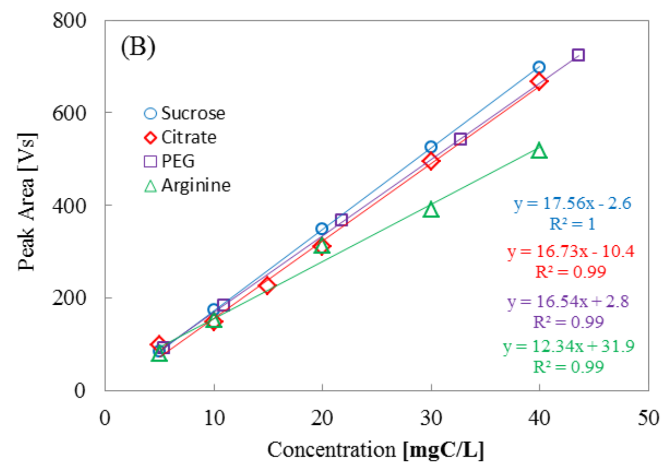
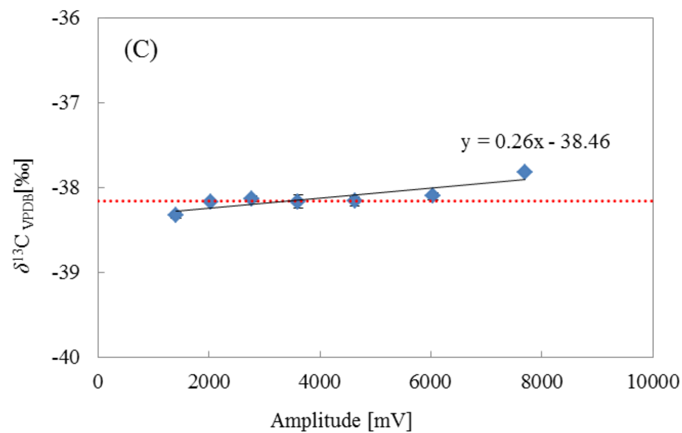


Figure A-1.1(A-B): Standard curves for calibration of HPLC column (A) with size standards and (B) different concentrations of standards.



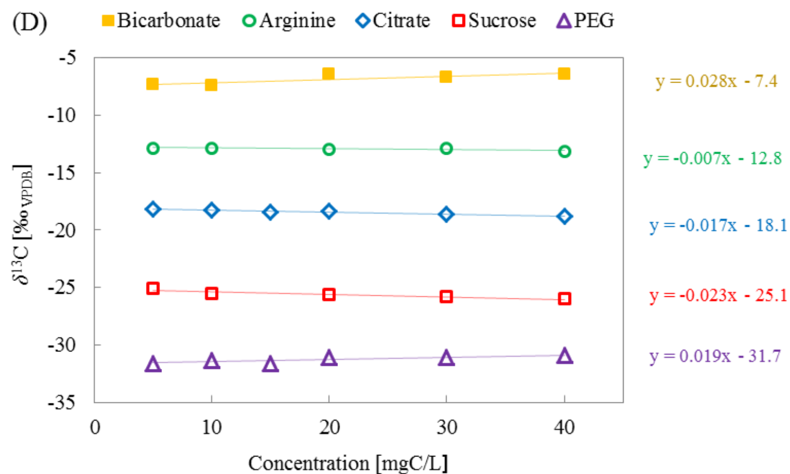


Figure A-1.1(C-D): Measurement of stability and linearity of the IRMS: (C) Stable carbon isotope ratios obtained with multiple injections of CO_2 at varying pressures. The dotted line signifies $\delta^{13}C$ value for CO_2 reference gas measured using a standard IRMS (Finnigan MAT 252). (D) Effect of different concentrations of standards on carbon stable isotope ratio measurement.

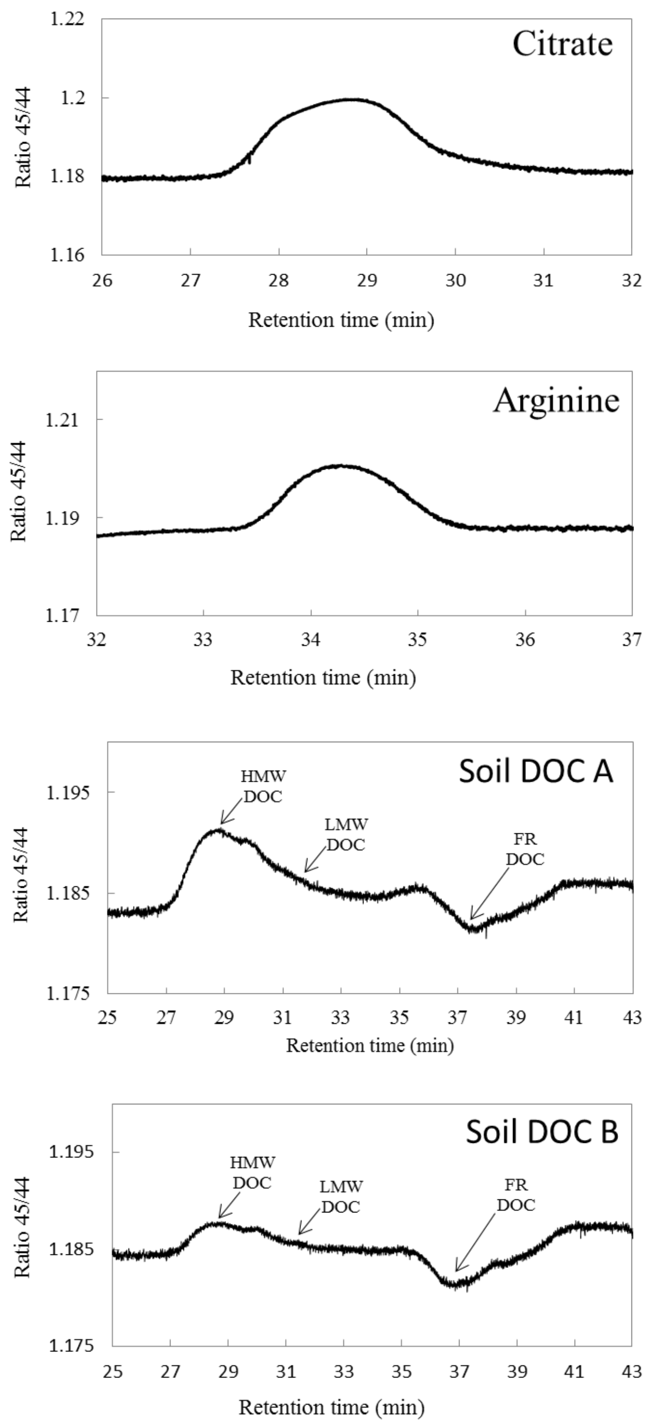


Figure A-1.2: Ratio trace of standards and soil water samples. Most standards tested as well as DOC samples did not show any isotopic fractionation normally seen as huge isotopic swings.

Appendix 2

Supporting Information for Chapter 3

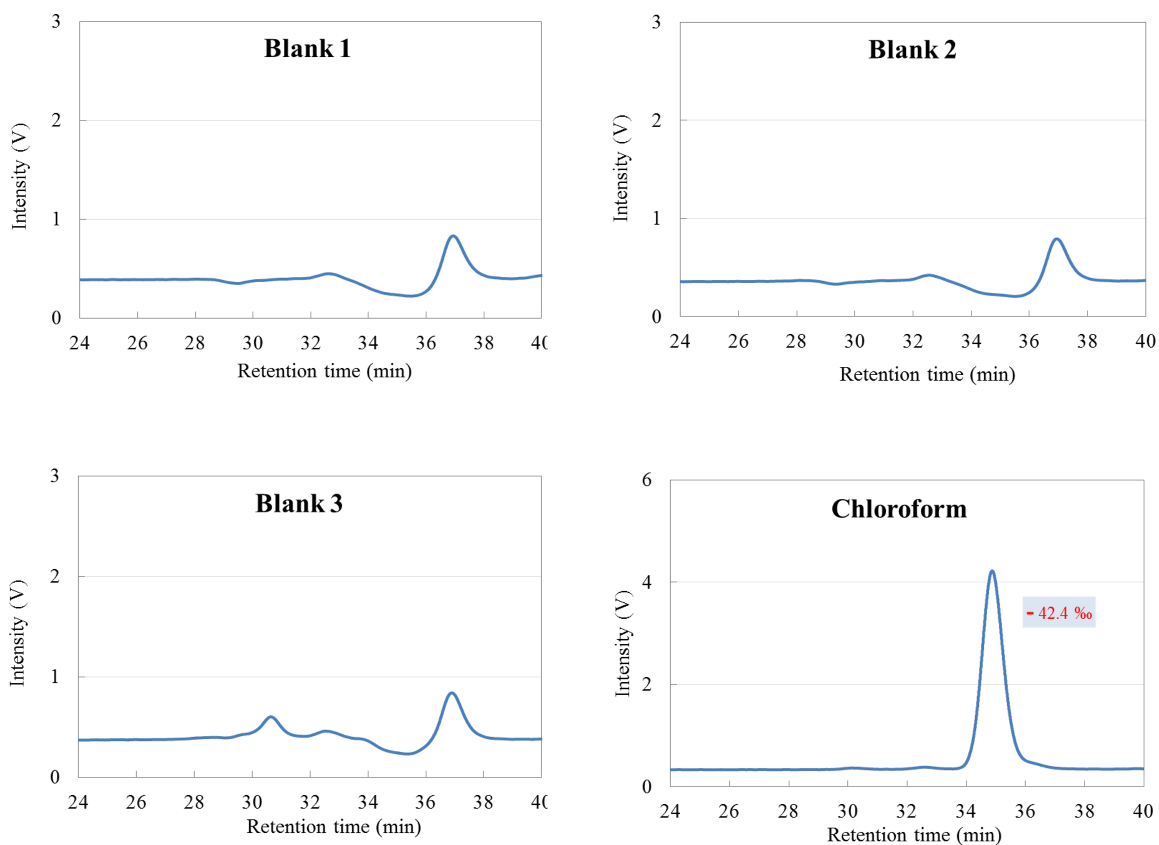


Figure A2.1: SEC-LC-IRMS chromatograms of blanks: Blank 1- K_2SO_4 solution only, Blank 2- K_2SO_4 solution passed through pre-washed filters, Blank 3- K_2SO_4 solution passed through unwashed filters.

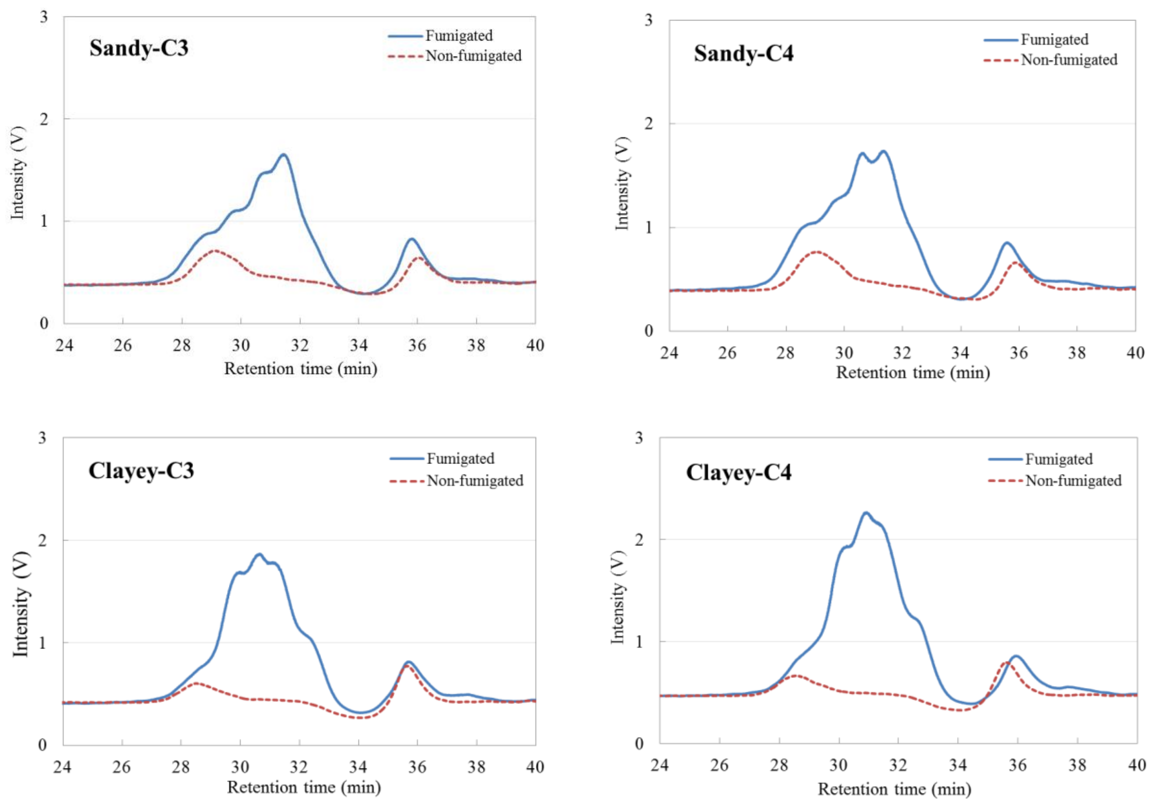


Figure A2.2: SEC-LC-IRMS chromatograms of fumigated and non-fumigated soils from the two soil plots 'Sandy' and 'Clayey' with (C4, experimental) and without (C3, control) vegetation change. Note the late eluting contaminant peak in both fumigated and non-fumigated soil extracts. This peak was not included in the final results.

Appendix 3

Supporting Information for Chapter 4

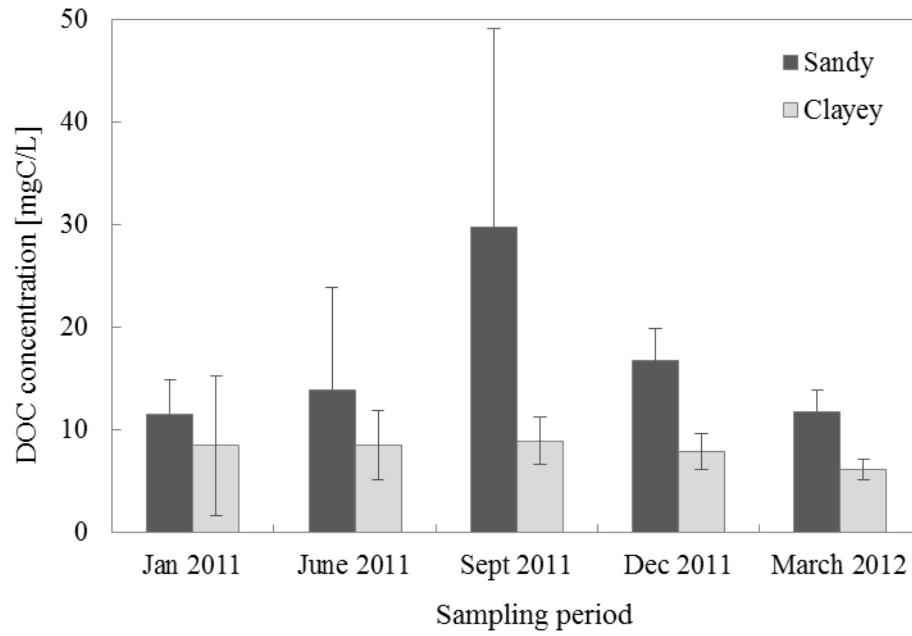


Figure A-3.1: Seasonal trend in total DOC concentration in the two soil types expressed as a mean of all depths and vegetation types.

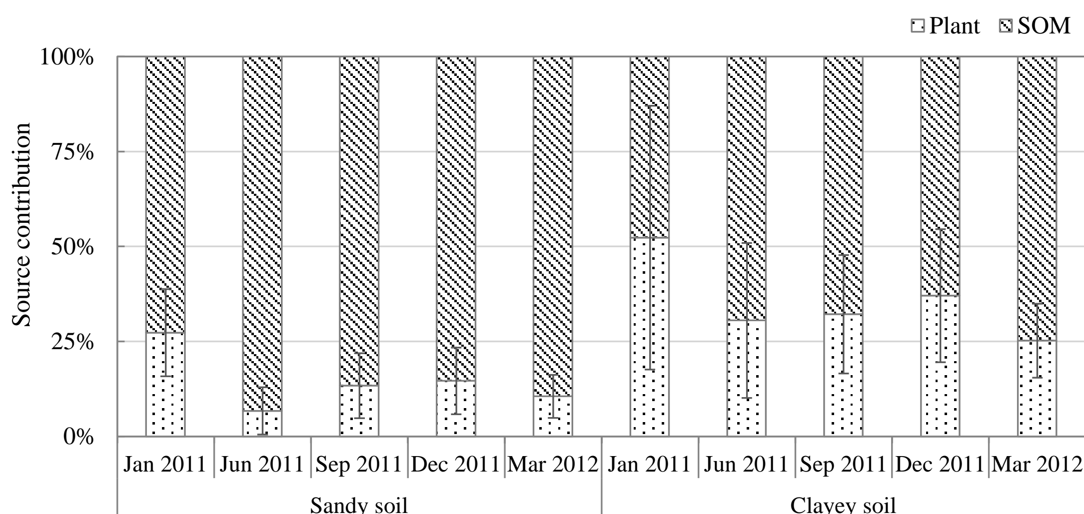


Figure A-3.2: Seasonal variation in contribution of recent plant and old soil organic matter (SOM) to total DOC in the two soil types.

Soil dissolved organic matter dynamics: conceptual model

Based on our results from DOM size fractionation and online carbon stable isotope analysis we propose a mechanism of soil DOM cycling. We suggest that DOM molecules get partitioned manifold between soil solution and the mineral matrix (chromatography, not "chromatographic stripping") thereby getting constantly decomposed, altered or produced anew by soil microorganisms (reactive transport). These conclusions are in-line with the Kaiser and Kalbitz (2012) conceptual model of DOM vertical movement in soil water which assumes that organic compounds in soil water get precipitated or sorbed followed by its microbial processing and re-release by desorption or dissolution. It is also mentioned that this view is a deviation from the view that soil acts as a chromatographic column toward DOM.

The old literature states soil as a chromatographic system, implying an ion exchanger. This means that some DOM molecules get bound to mineral exchangers and some are not retained and move through the soil. Kaiser and Kalbitz (2012) and Steinbeiss et al. (2008) stated that DOM gets repeatedly sorbed and desorbed at the mineral surfaces and also gets additionally decomposed and recycled in the soil. The first part; multiple sorption and desorption between two non-mixable phases is called chromatography. We thus suggest that soil acts as a chromatographic system towards DOM whereby its molecules get partitioned manifold between the soil mineral matrix (stationary phase) and the soil solution (mobile phase) by precipitation/dissolution and sorption/desorption; additionally getting processed, decomposed or produced anew by soil microorganisms.

Declaration of independent work:

I hereby declare that I have written this thesis independently using the mentioned resources, personal communication and cited literature.

Selbständigkeitserklärung:

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, 25.07.2014

Ashish Malik

Author contributions to the manuscripts

Manuscript 1:

Malik, A., Scheibe, A., LokaBharathi P. A., Gleixner, G. Online stable isotope analysis of dissolved organic carbon size classes using size exclusion chromatography coupled to an isotope ratio mass spectrometer. *Environment Science & Technology*, 2012, 46 (18), pp 10123–10129.

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- Ashish Malik: designed research, performed sampling and analyses, analyzed data and wrote the paper
- Andrea Scheibe: contributed soil water samples, introduced and helped with analyses and reviewed the paper
- P. A. LokaBharathi: contributed marine water samples
- Gerd Gleixner: designed research and reviewed the paper

Manuscript 2:

Malik, A., Blagodatskaya, E., Gleixner, G. Soil microbial carbon turnover decreases with increasing molecular size. *Soil Biology & Biochemistry*, 2013, 62, pp 115-118.

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- Evgenia Blagodatskaya: introduced and helped with analyses, reviewed the paper
- Gerd Gleixner: designed research and reviewed the paper

Manuscript 3:

Malik, A., Gleixner, G. Importance of microbial soil organic matter processing in dissolved organic carbon production. *FEMS Microbiology Ecology*, 2013, 86, pp 139–148.

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Manuscript 4:

Malik, A., Dannert, H., Griffiths, R., Thomson, B., Gleixner, G. Carbon flow from plants into the rhizosphere soil microbial loop. In preparation (to be submitted to *Ecology Letters*).

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- Helena Dannert: performed the experiment, sampling and analyses, analyzed data
- Robert Griffiths: contributed new reagents and analytical tools, reviewed the paper
- Bruce Thomson: contributed new reagents and analytical tools, introduced and helped with analyses, reviewed the paper
- Gerd Gleixner: designed research and reviewed the paper