Metabolic Pathways of Amino Acids, Monosaccharides and Organic Acids in Soils assessed by Position-Specific Labeling

Dissertation
zur Erlangung des Grades
Doktor der Naturwissenschaften
(Dr. rer. nat.)
an der Fakultät Biologie / Chemie / Geowissenschaften
der Universität Bayreuth

vorgelegt von
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Bayreuth, den 11. September 2013

Die vorliegende Arbeit wurde in der Zeit von 01.03.2010 bis 11.10.2013 in Bayreuth am Lehrstuhl für Agrarökosystemforschung unter der Betreuung von Herrn Prof. Dr. Yakov Kuzyakov angefertigt.

Vollständiger Abdruck der von der Fakultät für Biolgie, Chemie und Geowissenschaften der Universität Bayreuth genehmigten Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.).

Dissertation eingereicht am: 11.09.2013
Zugelassen durch die Prüfungskommission: 18.09.2013
Wissenschaftliches Kolloquium: 31.01.2014

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IV. Abbreviations

ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
C	Carbon
CEC	Cation Exchange Capacity
DI	Divergence Index
DOC	Dissolved Organic Carbon
DON	Dissolve Organic Nitrogen
EA	Elemental Analyzer
FAME	Fatty Acid Methyl Ester
GC	Gas Chromatography
GC-C-IRMS	Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometer
HPLC	High Pressure Liquid Chromatography
IC	Ion Chromatography
IC-O-IRMS	Ion Chromatograph-Oxidation-Isotope Ratio Mass Spectrometer
IRMS	Isotope Ratio Mass Spectrometer
IS	Internal Standard
LMWOS	Low Molecular Weight Organic Substances
MS	Mass Spectrometer
N	Nitrogen
PEEK	Polyetheretherketon
PLFA	Phospholipid Fatty Acid
SIM	Selected Ion Mode
SOC	Soil Organic Carbon
SOM	Soil Organic Matter

V. Summary

Transformation of low molecular weight organic substances (LMWOS) is one of the most important steps in biogeochemical cycles since all high molecular substances pass this stage during their decomposition. Microbial utilization is the most relevant sink for LMWOS in soils and thus knowledge about microbial transformations of LMWOS is crucial for understanding the soil organic carbon (SOC) cycle and predicting its reaction to changes in controlling environmental parameters. Previous studies focused on determining fluxes through the LMWOS pool, but they rarely identified transformation steps. This thesis aims to establish position-specific isotope labeling as a tool in soil science to trace the pathways of LMWOS transformations.

In a medium-term field experiment six position-specific ^{13}C-labeled LMWOS from the three main LMWOS classes were applied: two amino acids (alanine and glutamate), two monosaccharides (glucose and ribose) and two organic acids (acetate and palmitate). ^{13}C remaining in soil and that incorporated into microbial biomass and specific microbial cellular compounds (phospholipid fatty acids (PLFA) and amino sugars) was determined by bulk and compound-specific ^{13}C analyses. Therefore, a new instrument coupling, an ion chromatograph with an isotope ratio mass spectrometer (IC-O-IRMS), and the respective methods for amino sugar analysis were established. The effect of altered environmental conditions and the relevance of further LMWOS sinks (sorption or plant uptake) were evaluated in several additional laboratory experiments based on position-specific ^{14}C-labeling. The divergence index (DI) was established to compare the position-specific fate of individual substances in various studies independent of the isotopic approach or experimental design used or the pool investigated.

Microbial utilization was the fastest process in the removal of LMWOS from soil solution and neither plant uptake nor sorption could out-compete microorganisms. The incorporation of individual molecule positions in soils, microbial biomass and distinct compound classes was clearly defined by the microbial metabolism: Glycolysis, oxidation by pyruvate dehydrogenase and the citric acid cycle were identified as the main metabolic processes. However, in addition to these oxidizing catabolic pathways, the anabolic pathways, i.e. building-up new cellular compounds, occurred in soils simultaneously. This involved an intensive C recycling and turnover within the microorganisms that was observed not only for cytosolic compounds but also for cell wall polymers. Intensive modifications and transformation within metabolic side branches, like the fatty acid formation and transformation pathways, were identified. These results for fatty acid transformations are crucial for their application as plant biomarkers in studies on palaeoenvironmental reconstruction.

The combination of position-specific ^{13}C-labeling with compound-specific isotope analysis of microbial biomarkers allowed the further identification of specific pathways of in-

dividual functional microbial groups in soils. Fungal metabolism was shown to be slower than bacterial intracellular C recycling and turnover, which provides the metabolic reason for the slow-cycling fungal-based and fast-cycling bacteria-based branch of the soil food web. Shifts in C allocation through various metabolic pathways were dependent on environmental factors: a gradient of C metabolism from starvation pathways via maintenance metabolism to metabolic pathways characteristic for microbial growth was observed with increasing substrate concentration. Sorption, also limiting the bioavailability of a substrate, caused similar shifts in metabolic pathways: the lower the bioavailability (e.g. due to sorption), the more C was allocated towards anabolic biosynthesis, i.e. into microbial products. Thus, these studies revealed that position-specific labeling is not only a valuable tool in biochemistry for metabolic flux analysis, but also enables the reconstruction of metabolic pathways of LMWOS within diverse microbial communities in complex media such as soil. Processes occurring simultaneously in soil i.e. 1) within individual, reversible metabolic pathways, 2) in various microbial groups or 3) in specific microhabitats (like on mineral surfaces, at the soil-plant interface or at hot-spots versus bulk soil) could be traced by position-specific labeling in soils *in situ*.

The main metabolic pathways of microbial LMWOS transformation by cata- and anabolism were traced by position-specific labeling. These pathways and their regulating factors are crucial for assessing C flows towards mineralization versus the formation of microbial biomass, the prerequisite for the formation of microbially-derived SOC. This molecular knowledge of transformation steps and their regulating factors is crucial to predict (i.e. by new process-based modelling approaches) and manipulate C allocation and stabilization in soils.

VI. Zusammenfassung

Die Transformation niedermolekularer organischer Substanzen (LMWOS) ist der zentrale Schritt in biogeochemischen Kreisläufen, da alle hochmolekularen Substanzen während ihres Abbaus den LMWOS Pool passieren. Mikroorganismen stellen die bedeutendste Senke für LMWOS dar, weshalb mikrobielle Transformationen von LMWOS essentiell für den Kohlenstoffkreislauf im Boden sind. Bisherige Studien quantifizierten meist Flüsse durch den LMWOS Pool, arbeiteten aber kaum an der Aufklärung der Transformationsprozesse. Im Rahmen dieser Dissertation soll die positionsspezifische Isotopenmarkierung als neue bodenkundliche Methode zur Aufklärung von LMWOS-Transformationswegen etabliert werden.

In einem Feldexperiment wurden sechs positionsspezifisch ^{13}C markierte LMWOS der drei wichtigsten Substanzklassen appliziert: zwei Aminosäuren (Alanin und Glutamat), zwei Monosaccharide (Glucose und Ribose) und zwei organische Säuren (Acetat und Palmitat). Die Analyse von verbleibendem ^{13}C im Boden, ^{13}C in der mikrobiellen Biomasse und in spezifischen Zellbausteinen (Phospholipidfettsäuren (PLFA) und Aminozucker) erfolgte durch gesamt- und komponentenspezifische ^{13}C Methoden. Hierfür wurde eine neue Instrumentenkopplung – ein Ionenchromatograph mit einem Isotopenmassenspektrometer (IC-O-IRMS) – etabliert und die darauf abgestimmte Aminozucker-Aufreiningungsmethode eingearbeitet. Der Effekt sich ändernder Umweltfaktoren sowie die Relevanz weiterer LMWOS-Senken (Sorption und Pflanzenaufnahme) wurden anhand mehrerer zusätzlicher Laborexperimente mit positionsspezifischer ^{14}C Markierung evaluiert. Die Einführung des Divergenz Index (DI) ermöglichte es den positionsspezifischen Einbau in verschiedenen Studien unabhängig vom applizierten Isotop, dem experimentellen Design und dem untersuchten Pool zu vergleichen.

Mikroorganismen waren die dominante Senke für LMWOS und weder Pflanzenaufnahme noch Sorption konnten in Rate und Kinetik mit mikrobiellen Aufnahmesystemen konkurrieren. Der Einbau einzelner Molekülpositionen in Boden, mikrobielle Biomasse und bestimmte Substanzklassen war durch den mikrobiellen Metabolismus bestimmt, v.a. durch Glykolyse, Oxidation durch Pyruvat-Dehydrogenase und Citratzyklus. Allerdings liefen parallel zu diesen oxidierenden, katabolen Stoffwechselwegen auch anabole Reaktionen, d. h. der Aufbau neuer Zellkomponenten, ab. Dies führte zu einem starken C-Umsatz und Recycling, nicht nur im Cytosol sondern z.B. auch von Zellwandpolymeren. Intensive Umsätze innerhalb metaboler Seitenäste, wie der Fettsäurebiosynthese, wurden identifiziert. Diese Ergebnisse zur Fettsäuretransformation sind wesentlich für die Anwendung von Fettsäuren als pflanzliche Biomarker in Paläoumweltstudien.

Die Kombination positionsspezifischer ^{13}C Markierung mit komponentenspezifischer Isotopenanalytik mikrobieller Biomarker erlaubte des Weiteren die Identifikation spezifischer Stoffwechselwege einzelner mikrobieller Gruppen. Pilze zeigten einen langsameren intrazel-

lulären C-Umsatz als Bakterien, was die metabole Grundlage für den langsam-zyklierenden, pilzbasierten und den schnell-zyklierenden, bakterienbasierten Zweig des Bodennahrungsnetzes liefert. Die Verschiebungen der Kohlenstoffflüsse durch verschiedene Stoffwechselwege wurden in Abhängigkeit von Umweltfaktoren identifiziert: Mit Zunahme der Substratkonzentration konnte ein Gradient von C-Mangel-Stoffwechselwegen über den Erhaltungsmetabolismus hin zu charakteristischen Wachstums-Stoffwechselwegen beobachtet werden. Eine Verringerung der Substratverfügbarkeit durch Sorption verursachte eine ähnliche Verschiebung der metabolen C-Flüsse: Je niedriger die Verfügbarkeit, desto mehr C wird in Biosynthesewege also mikrobielle Produkte, verlagert.

Diese Studien konnten zeigen, dass positionsspezifische Markierung nicht nur eine wertvolle Methode in der Biochemie darstellt, sondern auch die Aufklärung der Verstoffwechslung von LMWOS durch diverse mikrobielle Gemeinschaften in komplexen Medien wie dem Boden ermöglicht. Parallel ablaufende Prozesse in Böden wie z. B. 1) der Rückfluss durch reversible Stoffwechselwege, 2) Umsätze in verschiedenen mikrobiellen Gruppen oder 3) Umsätze in spezifischen Mikrohabitaten (an Mineraloberflächen, am Boden-Pflanze-Interface oder an Hot-spots versus dem Gesamtboden) können mittels positionspezifischer Markierung im Boden in situ verfolgt werden.

Der Umsatz von LMWOS in Kata- und Anabolismus wurde im Rahmen dieser Dissertation rekonstruiert. Das Verständnis für diese Stoffwechselwege und ihre Regulationsfaktoren ist entscheidend für die Beurteilung von C-Flüssen zwischen Mineralisation und dem Aufbau mikrobieller Biomasse – der Voraussetzung zur Bildung mikrobieller, organischer Bodensubstanz. Das Wissen über Transformationsschritte und ihre regulierenden Faktoren ist essentiell für die Vorhersage (z. B. mittels prozessbasierter Modellierung), aber auch für die Manipulation der C-Sequestrierung und Stabilisierung in Böden.

1 Extended Summary

1.1 Introduction

1.1.1 Low molecular weight organic substances in soils

1.1.1.1 Role and relevance of low molecular weight organic substances in soil

Soil organic carbon (SOC) is the largest terrestrial carbon (C) pool, with C stocks of around 1500 Pg (Batjes, 1996). On average 30-120 kg C m^{-2} is stored up to 1 m soil depth and 0.5 to 1% of this stock is annually respired (van Hees et al., 2005a) but the largest portion is stable or inactive. Traditionally, SOC has been divided operationally by chemical fractionation into structurally diverse fulvic and humic acids (van Hees et al., 2005a). More recent results revealed that a limited range of defined substance classes and their polymers build up the soil organic matter (SOM) (Schmidt et al., 2011; von Luetzow et al., 2006).

A minor portion of SOC constitutes dissolved organic matter (DOC), in most cases less than 2 mol C m^{-2} (van Hees et al., 2005a). Only up to 10% of DOC consists of identifiable compounds of low molecular weight. These low molecular weight organic substances (LMWOS) are defined as soluble substances with a molecular weight lower than 250 Da (Boddy et al., 2007) and mainly consist of aliphatic and aromatic carboxylic acids, amino acids and peptides, mono-, di- and small oligosaccharides, amino sugars, phenolic substances and siderophores (McKeague et al., 1986).

Although the portion of LMWOS in SOC is extremely low, they play a major role in ecosystem functions. Regarding the C cycle, the importance of LMWOS is not determined by their pool size (Fischer et al., 2007), but by their huge fluxes (>20 mol C m^{-2} y^{-1}) that pass through this pool. During decomposition of plant-derived organic matter the high molecular weight organic substances are degraded by exoenzymes into low molecular monomers and pass the pool of LMWOS. They can then be oxidized to CO_2 by microbial respiration. Van Hees et al (2005a) summarized for forest soils that although LMWOS comprise less than 0.05% of the C pool, they contribute to more than 10-20% to the soil respiratory fluxes, thus demonstrating the high relevance of this active, fast cycling C pool for the SOC turnover.

In addition to their function as energy and C source for microorganisms, they fulfill several important functions in soils: 1) Contribution to weathering and solubilization of nutrients for plants; 2) formation of soil structures like aggregates; 3) acceleration of re-

duction/oxidation processes; 4) contribution to translocation of Si, Fe, Al, Mn and some other elements within and from the soil profile; 5) contribution to metal ion detoxification; and many more (Kaiser and Kalbitz, 2012, Kuzyakov, 1996, van Hees et al., 2005). Hence, there is no doubt about the relevance of LMWOS for soil processes and within the last decades an increasing interest in their sources, sinks and their function in coupling plant-derived and microbial-derived SOC pools has arisen.

1.1.1.2 Sources and sinks of LMWOS

The pool of LMWOS has several sources and sinks which are subject to strong spatial and temporal variations and are controlled by several environmental factors (Figure S1). Plant residues and rhizodeposits are the main primary sources of organic matter and consequently LMWOS in soils (Rasse et al., 2005). While root exudates consist mainly of LMWOS (Farrar et al., 2003; Fischer et al., 2010a; Kuzyakov and Domanski, 2000), root and plant litter is built up by high molecular weight, insoluble polymers like cellulose and hemicellulose, suberin and cutin, proteins and lignin (Koegel-Knabner, 2002). During their decomposition, these macromolecules are split to their monomers by exoenzymes (Cadisch and Giller, 1996) and thus enter the pool of LMWOS. Microbial biomass grown on plant residues can be considered a secondary source for LMWOS and identical decomposition processes take place within the microbial necromass: it contains a small proportion of cytosolic LMWOS and many high molecular weight macromolecules (Kindler et al., 2006; Miltner et al., 2007) which are degraded exoenzymatically to LMWOS (Miltner et al., 2009).

Sorption to clay minerals and sesquioxides can act as a sink for LMWOS but the relevance of this process strongly depends on the LMWOS class: Uncharged molecules, like monosaccharides, show nearly no sorption (Jones and Edwards, 1998), whereas charged molecules like organic acids or amino acids reflect strong interactions with mineral phases (Jones and Brassington, 1998; Jones and Edwards, 1998). This, however, strongly depends on the structure and net charge of the individual carboxylic or amino acid (Jones and Brassington, 1998; Jones and Hodge, 1999). However, sorption is not an irreversible process and desorption can transfer sorbed LMWOS back to the pool of LMWOS. Recent concepts of interactions of DOC with the soil matrix reveal that sorption/desorption, microbial utilization and leaching cannot be regarded as separate processes but that aging of DOC and LMWOS pools with soil depth is visible (Kaiser and Kalbitz, 2012). Aging results from the retention of LMWOS either adsorbed or incorporated into microbial biomass. In both cases, desorption or degradation of the microbial necromass transfers this C back to the DOC and LMWOS pool.

The concept of direct interactions between LMWOS and SOM has changed within the last years. Traditional humification theories of spontaneous heteropolycondensation (Martin and Haider, 1971; Schnitzer and Kahn, 1972; Stevenson, 1994) were found to be less relevant than originally assumed (Sollins et al., 1996; von Luetzow et al., 2006). Direct interaction between LMWOS and the stable SOM pool was assumed to occur mainly by hydrophobic interactions (Lichtfouse et al., 1998a; Lichtfouse et al., 1998b), being summarized by the so-called pseudo-macromolecularity theory (Piccolo, 2002). Despite these being non-covalent interactions, entrapping of LMWOS can act as a LMWOS sink as they cannot be gained by simple extraction methods. Besides SOM being an LMWOS sink, it can also act as a source for LMWOS, e.g. if complex SOM compounds are degraded by exoenzymes to LMWOS.

In general, within the last decades, the view on the sources of SOM has changed: untransformed incorporation of plant material is assumed to be quantitatively less relevant than the incorporation and stabilization of microbial biomass compounds (Duemig et al., 2012; Miltner et al., 2011; Simpson et al., 2007). This new view enhances the relevance of LMWOS for soil C cycling as they are the preferred C and energy source for the soil microbial community as well as a product of microbial transformations.

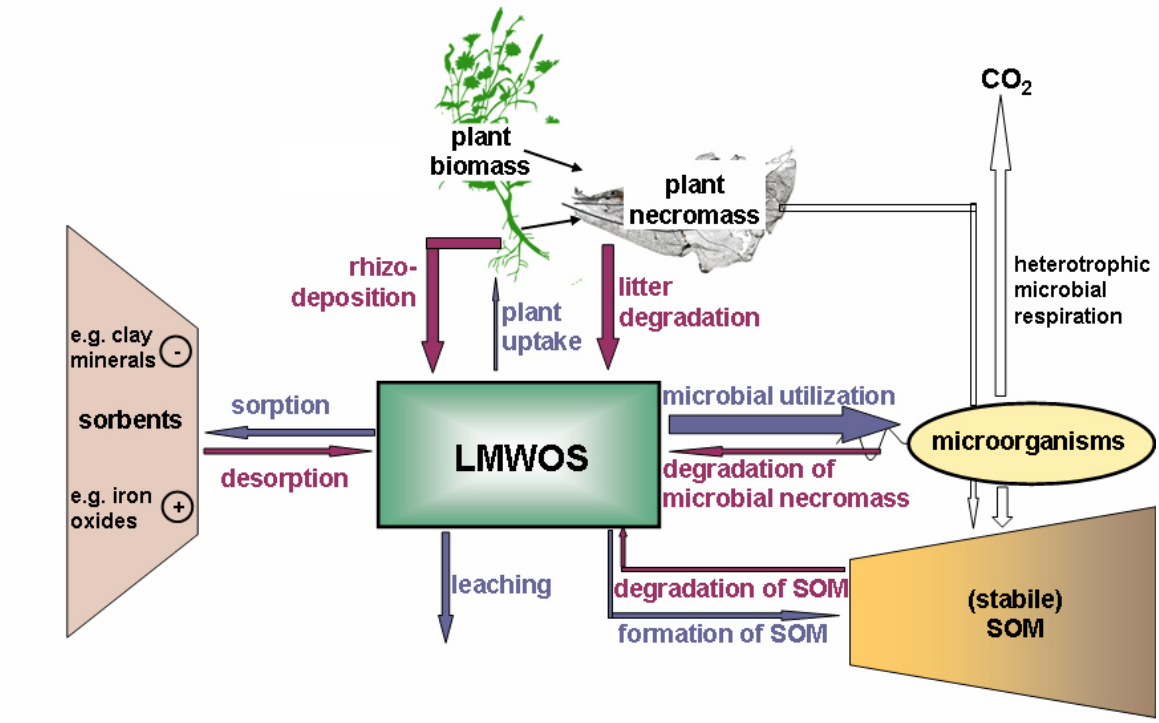

Fig. S1 Sources and sinks of low molecular weight organic substances (LMWOS) in soil. Blue arrows mark sinks and purple arrows sources of LMWOS

Figure S1 demonstrates an overview of the sources and sinks of LMWOS. Rather limited knowledge is available concerning the fluxes and underlying processes between LMWOS and microorganisms. However, this is the quantitatively most important sink, being responsible for the low concentration of LMWOS in soils (Hobbie and Hobbie, 2012). As the flux towards microorganisms is the most important, it affects all further processes like interactions with sorbents or stable SOM, leaching or the availability of LMWOS as a plant nutrient source. Therefore, a more detailed understanding of microbial uptake rates and C fluxes through the microbial metabolism as well as their controlling factors is required to understand and predict the fate of LMWOS – a key process in the SOC cycle.

1.1.2 Microbial utilization of low molecular weight organic substances

1.1.2.1 Microbial uptake of LMWOS: the most competitive process determining the fate of LMWOS-C in soil

Uptake kinetics of LMWOS by microorganisms (Anraku, 1980) and the competition of this process with further sinks of LMWOS are quite well understood: Utilization of many LMWOS is ubiquitous throughout the soil microbial community (Macura and Kubatova, 1973) and possible in a wide concentration range. Kinetic constants of uptake systems reveal that soil microorganisms are adapted to an extremely broad concentration range covering concentrations at hot spots (e.g. next to bursting cells) as well as concentrations in bare soil. Despite the ubiquitous ability for LMWOS utilization, specific microbial groups have higher competitiveness for LMWOS, show specific preferences for individual LMWOS or differ in their turnover of LMWOS-C (Moore et al., 2005). By combining isotope labeling approaches of LMWOS with the compound-specific isotope analysis of microbial biomarkers, this thesis aims to gain some further insights into LMWOS-C partitioning within the soil microbial community.

Sorption is another fast process that strongly reduces LMWOS concentration in soil solution and consequently reduces leaching of LMWOS from upper soil horizons to subsoil. However, if LMWOS are sorbed, this has an effect on the further microbial utilization (Jones and Edwards, 1998). In addition, there is clear evidence that microbial utilization outcompetes sorption in soils (Fischer et al., 2010b). However, little is known about the competition between sorption and microbial utilization, their interactions and their effects on each other. Plant uptake as an additional sink is known to be quantitatively less relevant for the LMWOS pool in soil due to the strong competition of microorganisms for LMWOS (Jones et al., 2005). However, a more process-orientated understanding of

competition between microbial uptake and utilization of LMWOS by various microbial groups on the one hand, and sorption and plant uptake on the other hand is needed to understand and predict the fate of LMWOS-C in soil. This thesis contributes to increase the mechanistic understanding of competing LMWOS sinks.

Microbial uptake is the main driver of LMWOS degradation leading to the short half-life time of most LMWOS in soils. Jones et al. (2004) used ^{14}C-labeled amino acids and determined the half-life time of amino acids in soil solution to be less than 4.0-7.5 minutes. Fischer et al. (2010b), working with soil suspensions, reported even lower values of around 2 minutes with sugars < amino acids < carboxylic acids. Consequently the LMWOS pool has an extremely high turnover which can reach up to 4000 cycles per year for individual compounds (Boddy et al., 2007). To summarize, although several sinks for LMWOS exist in soil, microbial utilization is the fastest and quantitatively most important process removing LMWOS. Therefore, the aim of this thesis was to gain a profound understanding not only on turnover rates and kinetics but also on the underlying processes.

1.1.2.2 Mineralization versus incorporation of LMWOS-C into microorganisms

Microbial metabolism is a crucial step for the fate of LMWOS-C in soils as it determines whether C is mineralized to CO_2 (catabolism) or incorporated into cellular compounds (anabolism). Total uptake as well as the ratio of mineralized LMWOS to total uptake (= sum of mineralized + incorporated) can vary for individual LMWOS. Hence, microbial incorporation as well as mineralization to CO_2 (i.e. cata- and anabolism) have to be regarded together to evaluate the fate of LMWOS-C (Jones, 1999; Kemmitt et al., 2008). For amino acids, the portion of mineralized LMWOS-C ranges from 20-40% of the uptake whereas 60-80% is incorporated into microbial biomass (Jones, 1999; Kemmitt et al., 2008). In contrast, carboxylic acids are predominantly decomposed to CO_2 and less C is spent on microbial biomass C (Jones et al., 1996). However, the pathways which cause these specifics of individual LMWOS are not understood in soils. Therefore, the aim of this thesis was not only to determine the fate of LMWOS but also the underlying mechanisms and pathways.

Half-life of LMWOS range from days to months receiving strongly different values in individual studies (reviewed by van Hees et al. 2005). In addition to abiotic factors such as temperature (Dijkstra et al., 2011c; Vinolas et al., 2001) or soil properties (Gonod et al., 2006; Kemmitt et al., 2008), the concentration of a substrate is a key driver of intracellular metabolism (Dijkstra et al., 2011a; Fischer and Kuzyakov, 2010b; Schneckenberger et al., 2008) and consenquently also affects the long-term fate of LMWOS-C in soils. However, as long as pathways and regulating factors of microbial metabolism are not

fully understood, a prediction of the effect of an environmental factor is hardly possible. Therefore, during this thesis, not only should pathways be reconstructed, but also regulating factors of C metabolism should be identified and their effects on C allocation into various microbial pathways and products evaluated.

Previous studies have mainly focused on the catabolic product of LMWOS metabolism: CO_2. However, tracing of anabolism is analytically much more challenging: it requires not only measurement of microbial biomass ^{13}C or ^{14}C incorporation, but also incorporation into specific cellular compounds e.g. by compound-specific isotope analysis. Within first studies, incorporation of LMWOS-C in membrane lipids (Garcia-Pausas and Paterson, 2011; Waldrop and Firestone, 2004), cell wall polymers (Bode et al., 2013; Indorf et al., 2012) and amino acids (Knowles et al., 2010) was determined. Knowles et al. (2010) even used dual-isotope-labeled amino acids, thus providing the first approach to trace not only the product of LMWOS metabolism but also to draw conclusions about the metabolic pathways used. However, knowledge about the formation pathways of the majority of microbial substance classes is rare and, consequently, the formation of some main SOM classes is still not understood. Hence, in addition to basic C cata- and anabolism, specific pathways for the formation of microbial biomass compounds should also be investigated within the scope of this thesis. This will gain a deeper understanding of SOM formation and the long-term fate of LMWOS-C in soils.

1.1.3 Metabolic tracing by position-specific labeling

The majority of the present studies on LMWOS transformations quantified uptake and turnover rates of a broad spectra of substances but rarely focused on the underlying pathways, mechanisms and regulating factors which control these fluxes. This was mainly attributed to the fact, that current techniques, based on determination of concentrations of LMWOS, their natural abundance isotope signature or even application of uniformly labeled LMWOS do not allow the reconstruction of transformation pathways. Therefore, position-specific labeling is a unique tool as it allows identifying the cleavage of applied LMWOS.

The first studies based on position-specific labeling started in the seventies with works of Haider and Martin (1975) and Martin and Haider (1976) and in the nineties with Kuzyakov and Galitsa (1993) and Fokin et al. (1993). They mainly aimed at tracing the fate of single functional groups and determined e.g. rates of decarboxylation of phenolic acids (Martin and Haider, 1976).

In parallel, position-specific labeling developed as a basic tool in biochemistry and enabled the quantitative modeling of C fluxes through metabolic networks (Zamboni et

al., 2005). Starting with biochemical pathways of microorganisms, this tool entered plant biochemistry (Ratcliffe and Shachar-Hill, 2006; Roscher et al., 2000) and also plant ecophysiology (Nasholm et al., 2001; Wegener et al., 2010). However, the first systematic approaches of position-specific labeling to trace C fluxes through the metabolic network in soils started only a few years ago (Dijkstra et al., 2011a; Dijkstra et al., 2011b; Dijkstra et al., 2011c; Fischer and Kuzyakov, 2010b) with ^{14}C and ^{13}C position-specific labeling of pyruvate and acetate, respectively.

In this thesis, ^{14}C as well as ^{13}C position-specific labeling was applied as the main methodological approach with various experimental designs under field and laboratory conditions. For the main classes of LMWOS (amino acids, monosaccharides and organic acids), all (purchasable) position-specific labeled isotopomers as well as the uniformly labeled substances were applied in separate treatments. In contrast to previous studies, transformation pathways of main LMWOS classes could be elucidated by this approach and are presented in this thesis. The combination with compound-specific isotope analysis allows the formation pathways of distinct microbial biomass compounds and of distinct microbial groups to be traced. In addition, changes in LMWOS-C fluxes due to changing environmental conditions could be explained by their regulation in microbial metabolism. In addition to uniform labeling, position-specific labeling not only allows the determination of fluxes and turnover rates of LMWOS-C, but also the identification of the underlying transformation pathways. It is a unique tool to gain detailed insights into submolecular transformation pathways and their regulation factors in soils. A first application of this tool was performed within the scope of this thesis and the results as well as perspectives are summarized in the following chapters.

1.1.4 Objectives

The main objective of this thesis was to establish position-specific ^{13}C- and ^{14}C-labeling and metabolic tracing as a tool in soil science, which enables a process-orientated view on the LMWOS cycle and can be applied for field and laboratory experiments. More specifically, the following objectives were aimed towards:

1) Determination of metabolic pathways of two representatives of the three main substance classes of LMWOS (amino acids, monosaccharides and organic acids) by position-specific labeling
2) Coupling of position-specific labeling with compound-specific isotope analysis of microbial biomarkers
    a. to follow the C incorporation into various cellular compound classes
    b. to identify specific metabolic pathways of individual members of the soil microbial community
3) Identification of specific metabolic pathways depending on certain environmental conditions:
    a. pathways of LMWOS sorbed on various soil components
    b. pathways under various concentrations of LMWOS
    c. extra- versus intracellular transformation pathways
4) Assessment of kinetics and ecological relevance of competing sinks for LMWOS:
    a. sorption versus microbial utilization
    b. plant uptake versus microbial utilization

1.2 Experiments and Methods

1.2.1 Field experiment

The field experiment was carried out on an agricultural field site close to Hohenpoelz (49°54' Northern latitude; 11°08' Eastern longitude, 500 m a.s.l.) in northern Bavaria. Mean annual temperature is +7 °C, mean precipitation is 870 mm and soil type is a haplic Luvisol (IUSS Working group WRB, 2007). 784 columns (Figure S2) were installed according to a randomized block design, where the four blocks represent the four replications of each treatment.

Fig. S2 Schematic (left) and photo (middle) of a labeling column of the field experiment. Right photo shows one of the four blocks, each with 196 columns.

In total, six individual LMWOS were applied: two amino acids (alanine and glutamate), two monosaccharides (glucose and ribose) and two organic acids (applied as acid anion: acetate and palmitate). Each of their purchasable isotopomers as well as their uniformly ^{13}C-labeled form was applied in separate columns (see Table S1). In addition, ^{15}N-labeled glutamate and alanine were added as well as labeled mineral nitrogen ($^{15}NO_3$- and $^{15}NH_4^+$). Consequently, this is the first experiment which allows metabolic tracing by two parallel approaches: 1) metabolic tracing based on individual C positions (Dijkstra et al., 2011a) and 2) metabolic tracing based on multiple isotope labeling (Knowles et al., 2010) (results of the second approach were not included in this thesis).

Each of the six substances had one background treatment where the same amount of non ^{13}C-enriched substance was applied. In each of the 22 labeled and 6 background treatments, the amount of C and N applied was as low as possible and identical for all columns to avoid potential disturbance of the microbial community. Each treatment was designed for seven sampling dates and in four replications (resulting in 784 soil columns).

To prevent rainfall and thus leaching, a roof was installed above the field site during labeling and for the first 10 days. Excluding additional rainfall water due to the roof and assuming no preferential flow in the freshly tilled soil, leaching can be regarded as negligible and mineralization is the only process removing ^{13}C from the soil. Only data from the first two sampling dates, reflecting the short-term dynamics of LMWOS, are presented in this thesis.

Sampling was performed by removing the entire column from the field. Length (i.e. volume) of soil column, fresh weight of soil and water content were determined. After homogenization, each sample was split and the subsamples were prepared and stored according to the different analyses.

Table S1 Treatments of position-specific ^{13}C and ^{15}N labeling. Applied amount of ^{13}C and ^{15}N, their isotopic enrichment, as well as the respective compound and labeled position are presented. Nat. abund. means application of non-enriched substances, x means"no application of ^{15}N in this treatment.

Position-specific ^{13}C labeling				^{15}N labeling	
substance class	substance	position(s)	applied amount of ^{13}C (µmol per column)	substance	applied amount of ^{15}N (µmol per column)
amino acids	alanine	C-1	94.2	alanine (nat. abund.)	x
		C-2	93.0		x
		C-3	94.7		x
		uniformly	96.3	^{15}N alanine (98 at%)	28.7
	glutamate	C-1	93.0	glutamate (nat. abund.)	x
		C-2	56.3		x
		uniformly	91.6	^{15}N glutamate (98 at%)	28.0
mono-sacharides	glucose	C-1	91.5	$(NH_4)_2SO_4$ (nat. abund.)	x
		C-2	93.4		x
		C-4	93.5		x
		C-6	91.8		x
		uniformly	93.4	^{15}N $(NH_4)_2SO_4$ (98 at%)	27.8
	ribose	C-1	93.3	$(NH_4)_2SO_4$ (nat. abund.)	x
		C-5	93.0		x
		uniformly	91.8		x
organic acids	acetate	C-1	94.6	KNO_3 (nat. abund.)	x
		C-2	94.1		x
		uniformly	95.8		x
	palmitate	C-1	47.5	KNO_3 (nat. abund.)	x
		C-2	47.1		x
		C-16	44.3		x
		uniformly	49.5	^{15}N KNO_3 (98 at%)	28.6

1.2.2 Laboratory experiments

Besides the field experiment, several laboratory experiments with modifications of environmental conditions were performed. In general, in laboratory experiments position-specific ^{14}C-labeled tracers were applied.

1.2.2.1 Experiment 1: Transformations of free alanine

The first experiment aimed at identifying the transformation pathways of amino acids depending on two factors: 1) the concentration of alanine (0.5, 5, 50, 500 and 5000 µM), and 2) the extra- and intracellular as well as abiotic processes (i.e. sorption) of alanine removal from soil solution, separated by selective inhibition. Alanine transformation products were operationally separated by sequential extraction into ion-exchangeable and ligand-exchangeable transformation products, whereas irreversible-bound transformation products remained in the soil.

1.2.2.2 Experiment 2: Transformations of sorbed alanine

In the sorption experiment, availability and microbial utilization pathways of absorbed alanine were investigated in two steps:

1) Labeled alanine was adsorbed to five sterilized sorbents commonly present in soils: two iron oxides with different crystalline structure: goethite and hematite; two clay minerals with 2:1 layers – smectite, and 1:1 layers – kaolinite; and active coal.

2) Thereafter, the sorbed alanine was mixed with the soil and incubated for 3 days. The effect of sorption on microbial utilization, especially their metabolic pathways, was elucidated.

1.2.2.3 Experiment 3: Plant uptake of intact alanine

Plant uptake was carried out with dual-isotope, position-specific labeled alanine in rhizotubes (Biernath et al., 2008; Rasmussen et al., 2010). Uptake of C and N from individual positions by *Zea mays, Lupinus albus* and *Cichorium intybus* was traced. As a control, ^{14}C acetate and mineral $^{15}NH_4^+$ and $^{15}NO_3^-$ were applied. Thus, passive uptake of LMWOS as well as the relevance of N-LMWOS uptake compared to mineral nitrogen was assessed. Position-specific labeling enabled the differentiation of intact alanine uptake versus the uptake of its transformation fragments.

1.2.3 Methods to trace ^{13}C and ^{14}C in transformation products of LMWOS

An overview over the methods, applied in this thesis, to trace ^{13}C, ^{15}N and ^{14}C in specific transformation products and more unspecific SOC fractions is presented in Table S2. Methods are briefly described in the following chapters and in detail in the Material and Methods section of the respective studies.

Table S2 Applied ^{13}C- and ^{14}C-labeling approaches as well as analytical methods for the individual studies; First line shows whether samples were derived from field or laboratory experiments; PS indicates position-specific labeling.

	Field experiment					Laboratory experiment		
Study	Study 1	Study 2	Study 3	Study 6 + 7	Study 8	Study 4	Study 5	Study 9
Topic	LMWOS comparison	method development	amino acid	monosaccharides	organic acids	free amino acids	sorbed amino acids	plant uptake of amino acids
Labeling	Uniformly ^{13}C		PS ^{13}C	PS ^{13}C	PS ^{13}C	PS ^{14}C	PS ^{14}C	PS ^{14}C & ^{15}N
^{13}C in bulk soil	X		X	X	X			
^{15}N in bulk soil								X
^{15}N in plant biomass								X
microbial biomass ^{13}C	X		X	X	X			
^{13}C in PLFA	X		X	X	X			
^{13}C in amino sugars		X		X				
^{14}C in soil solution						X	X	
^{14}C in bulk soil						X	X	X
^{14}C in soil extracts						X		
^{14}C in CO_2							X	

1.2.3.1 Bulk-isotope measurements by EA-IRMS

^{13}C and ^{15}N remaining in soil was quantified by determination of $\delta^{13}C$ and $\delta^{15}N$ values of bulk soil samples. Incorporation of ^{13}C in microbial biomass was calculated from the $\delta^{13}C$ value of fumigated and unfumigated soil extracts, gained by the chloroform-fumigation-extraction method (Brookes et al., 1985; Wu et al., 1990). For all $\delta^{13}C$ measurements, the samples were freeze-dried and measured by Elemental Analyzer-Isotope Ratio Mass Spectrometer (EA-IRMS). ^{13}C incorporation was calculated according to the mixing model referenced on the respective $\delta^{13}C$ values of the background treatment.

1.2.3.2 Compound-specific isotope analysis of microbial biomarkers

^{13}C incorporation into microbial membrane lipids, the phospholipids fatty acids (PLFA), was determined by compound-specific isotope analysis. For this, the Bligh-and-Dyer extract of intact polar lipids was performed. PLFA were purified by liquid-liquid extraction and column chromatography and derivatized to their fatty acid methyl esters (FAMEs). $\delta^{13}C$ value of FAMEs was determined by gas chromatography-combustion-

isotope ratio mass spectrometry (GC-C-IRMS) and ^{13}C incorporation was calculated by mixing models with the respective background treatments as a reference.

In addition, ^{13}C incorporation into microbial cell wall monomers, the amino sugars, was measured in this thesis. However, GC-C-IRMS methods are not sufficiently reliable and existing liquid chromatography-oxidation-isotope ratio mass spectrometry methods were not able to determine the $\delta^{13}C$ value of bacterial muramic acid. Therefore, a new instrument coupling, using an ion chromatograph (IC) instead of a classical liquid chromatograph for IC-O-IRMS measurements and amino sugar $\delta^{13}C$ determination was established in study 2 (see Figure S3). Thus, previous purification methods (Bode et al., 2009; Glaser and Gross, 2005; Indorf et al., in press) had to be optimized and an IC-O-IRMS measurement had to be elaborated (see study 2). Briefly, after acid hydrolysis, iron and salts were removed by precipitation (Zhang and Amelung, 1996) and neutral compounds were separated from amino sugars by a cation exchange resin (Indorf et al., 2013). Liquid chromatography was optimized (Bode et al., 2009) and ^{13}C incorporation into microbial cell walls was calculated using the analogy of incorporation into PLFA.

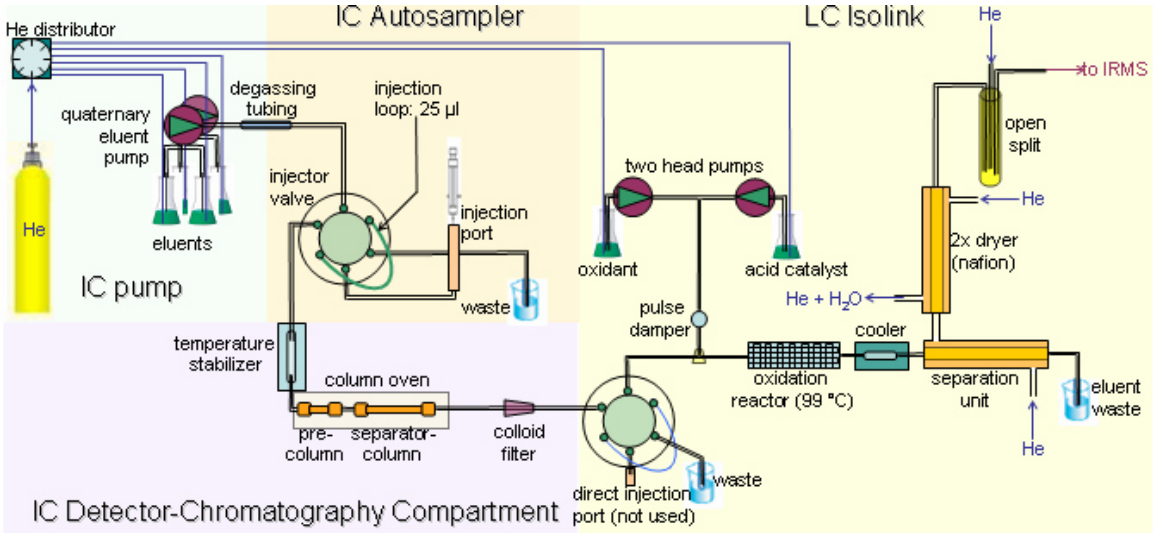

Fig. S3 Overview of the instrument coupling: Ion Chromatograph is shown on the left side with the pump, autosampler and detector-chromatography compartment. Connection to isolink occurs via a PEEK capillary with interposed colloid filter. Scheme of LC Isolink is adapted from Krummen et al. (2004).

1.2.3.3 Radiochemical analyses

Incorporation of ^{14}C into the transformation products was performed after various sampling and extraction methods based on scintillation counting. For this purpose, solid samples were combusted and $^{14}CO_2$ was trapped in NaOH. Bound ^{14}C was determined by scintillation counting. ^{14}C in soil extracts or suspensions could be directly measured

after mixing a subsample with a scintillation cocktail. Direct measurement of decomposed $^{14}CO_2$ was performed on experiments in well plates. Therefore, a CO_2 trap based on 24-segment filter paper was constructed above the 24-well plate. Construction of this trap was optimized and efficiency evaluated in study 5.

1.2.4 The Divergence Index

It remains challenging to compare the position-specific fate of individual LMWOS, especially if C is transformed into strongly differing C pools. To enable the comparison of the individual studies independent of the isotopic approach (^{13}C or ^{14}C) or experimental design used or the pool investigated, the Divergence Index DI_i was introduced in this thesis (equation 1).

$$DI_i = \frac{n \cdot [C_i]}{\sum_{i=1}^{i=n} [C_i]} \qquad (1)$$

This index shows the fate of individual C atoms from the position i within a transformation process relative to the mean transformation of the n total number of C atoms in the substance. Thus, a DI_i of 1 means that the transformation of this C atom in the investigated pool corresponds to that of uniformly labeled substance (average of all C atoms of the substance). The DI_i ranges from 0 to n, and values between 0 and 1 reflect reduced incorporation of the C into the investigated pool, whereas values between 1 and n show increased incorporation of the C atom into this pool as compared to the average. This index is not dependent on absolute amounts or proportions of the substance used in individual processes. Therefore, it enables comparison of the distribution of individual C atoms over the whole range of investigated concentrations, the size of C pools, the process rates, etc.

1.3 Results and Discussion

1.3.1 Overview: main results of the studies

An overview over the objectives and the main results and conclusions of the individual studies is presented in Table S3.

Table S3 Title of the individual studies as well as their objectives and main conclusions.

Study	Objectives	Main Conclusions
Study 1: Fate of low molecular weight organic substances in soil: from microbial uptake to utilization and stabilisation	• Overview of microbial metabolism and C allocation within the microbial metabolism • Identification of specifics in the LMWOS utilization of various microbial groups in soil	• The entry steps of an LMWOS to the basic C metabolism accounts for the fate of the 3 classes of LMOWS in soil
Study 2: Improved $\delta^{13}C$ analysis of amino sugars in soil by Ion Chromatography - Oxidation - Isotope Ratio Mass Spectrometry	• Establishment of a new instrument coupling of ion chromatograph with isotope ratio mass spectrometers for routine measurements of water-dissolvable metabolites by IRMS • Development of an IC-O-IRMS method for parallel amino sugar quantification and $\delta^{13}C$ determination	• IC-O-IRMS enables reliable and routine measurements of amino sugars • Parallel quantification and $\delta^{13}C$ determination of basic and acidic amino sugars is possible in soils • IC-O-IRMS has great advantages compared to classical LC-O-IRMS
Study 3: Biochemical pathways of amino acids in soil: Assessment by position-specific labeling and ^{13}C-PLFA analysis	• Identification of transformation pathways of two representative amino acids (alanine and glutamate) • Assessment of specifics in amino acid metabolism of individual microbial groups in soils	• Basic C metabolism accounts for the majority of the observed alanine transformations • Incorporation of glutamate C-2 reflects specific microbial pathways
Study 4: Biogeochemical transformations of amino acids in soil assessed by position-specific labeling	• Effect of concentration (i.e. C availability) on alanine transformations • Kinetics of sorption, extracellular transformation and microbial uptake and utilization of alanine	• Biotic processes outcompete sorption in soils • Extracellular LMWOS transformations are only relevant at low concentrations or in special microhabitats • Concentration strongly affects the metabolic pathway and fate of alanine C in soils
Study 5: Sorption affects amino acid pathways in soils: Implication from position-specific labeling of alanine	• Microbial availability of LMWOS sorbed to various sorbents • Effect of sorption on microbial utilization and transformation pathway of LMWOS	• Transformation of sorbed LMWOS follows classical biochemical pathways: no abiotic transformations occur • C allocation through specific pathways in microbial metabolism is strongly affect by the sorption mechanism and bioavailability of LMWOS

Study 6: Biochemistry of hexose and pentose transformation in soil analyzed by position-specific labeling and ^{13}C-PLFA	• Tracing metabolism pathways for hexoses and pentoses in soils • Identification of specifics in bacterial and fungal metabolism in the use of monosaccharide C for PLFA formation	• Glycolysis and pentose phosphate pathway as well as backflux could be traced in soils *in situ* • Glucose – as a ubiquitous substrate – is spread over entire metabolism, intensively recycled and a large portion is used for microbial biomass formation
Study 7: Metabolic pathways of fungal and bacterial amino sugar formation in soil assessed by position-specific ^{13}C-labeling	• Reconstruction of the main pathways for amino sugar biosynthesis in soils • Identification of specifics in bacterial and fungal metabolism during cell wall formation	• Formation of amino sugars follows several pathways: 1) direct intact glucose utiliation, 2) glycolysis and backflux and 3) pentose phosphate pathway and backflux • Fungi showed a lower metabolic activity than bacteria in maintenance metabolism
Study 8: Formation and transformation of fatty acids in soils assessed by position-specific labeling of precursors	• Reconstruction of the main pathways for fatty acid formation and transformation in soils • Identification of microbial group specific fatty acid metabolism • Assessment of microbial transformation of the free fatty acid pool in soils	• Acetate ^{13}C was rarely used for building of fatty acid backbones but mainly for elongations or introductions of functional groups • Fatty acids were intensively modified in soils according to the demand of the microbial community which has to be considered for paleoenvironmental applications
Study 9: Organic N uptake by plants - Reevaluation by position-specific labeling of amino acids	• Determination of the overestimation of uniformly labeling approaches • Evaluation of the relevance of intact amino acid uptake for the N nutrition of agricultural plants	• Quantification of intact amino acid uptake based on uniform labeling causes an 1.2-3fold overestimation • Microbial utilization strongly dominated the fate of organic substances in soils • The majority of amino acid uptake by plants was explained by passive uptake of microbial transformation products

1.3.2 Determination of metabolic pathways of amino acids, monosaccharides and organic acids

1.3.2.1 Similarities and differences of individual LMWOS

Study 1 aimed at investigating the similarities and differences of the fate of the applied LMWOS, to gain insights in their microbial utilization and decomposition. The percentage of ^{13}C-LMWOS remaining in the SOM and incorporated into the microbial biomass pool after 3 days was similar for the substances investigated (Figure S4). However, after 10 days, significant differences between the incorporation of individual substances

arose. The initial rapid uptake was quite similar for all investigated LMWOS as they are ubiquitous substrates for many members of the microbial community. However, intracellular metabolism may have accounted for the observed differences in the fate of LMWOS-C in soil and this effect became evident after 10 days of continued transformation of the LMWOS (Figure S4).

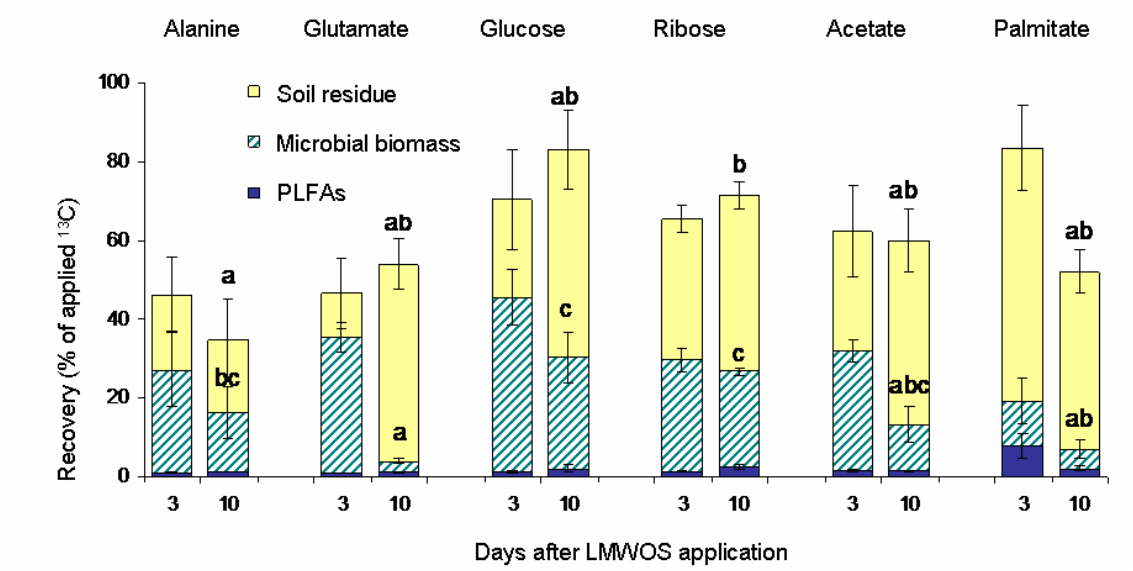

Fig. S4 *^{13}C recovery (in % of applied ^{13}C) from six LMWOS in soil, microbial biomass and PLFA, 3 and 10 days after addition. Letters indicate significant differences in ^{13}C incorporation of the individual substances if occuring.*

LMWOS specifics of incorporation into the microbial biomass depend on their entry point into the basic C metabolism of microorganisms (Figure S5): Microbial uptake of both amino acids was similar on day 3, but much less glutamate C than alanine C remained in microbial biomass on day 10 (Figure S4). This reflects the fact that substrates with direct incorporation into the oxidizing citric acid cycle (Figure S5), such as glutamate, are preferentially oxidized for energy production compared to LMWOS, like alanine, which enter glycolysis. The high and rapid glucose uptake is connected to the fact that glucose is the most abundant sugar in soils (Derrien et al., 2006; Derrien et al., 2004; Fischer et al., 2010a). In comparison to amino acids and carboxylic acids, glucose was preferentially incorporated into microbial biomass. This reflects the preference of glycolysis substrates for anabolic utilization compared to catabolism. For carboxylic acids, the ^{13}C incorporated in microbial biomass declined by a factor of two from day 3 to day 10, which also reflects the preferred catabolic oxidation of substances entering the citric acid cycle (Figure S5).

Incorporation of LMWOS ^{13}C into microbial membrane lipids, the PLFA, enables the identification of preferences of individual functional microbial groups for certain substrates

(Waldrop and Firestone, 2004). The incorporated ^{13}C portion ranged between 0.8 and 2% of the initial applied tracer and no differences were detected between amino acids, monosaccharides and acetate. However, up to 8% of the applied palmitate was incorporated in PLFA. This shows that, due to economic reasons in the construction of complex cellular compounds, microorganisms prefer the most-direct precursor for the formation of cellular compounds (Lengeler et al., 1999).

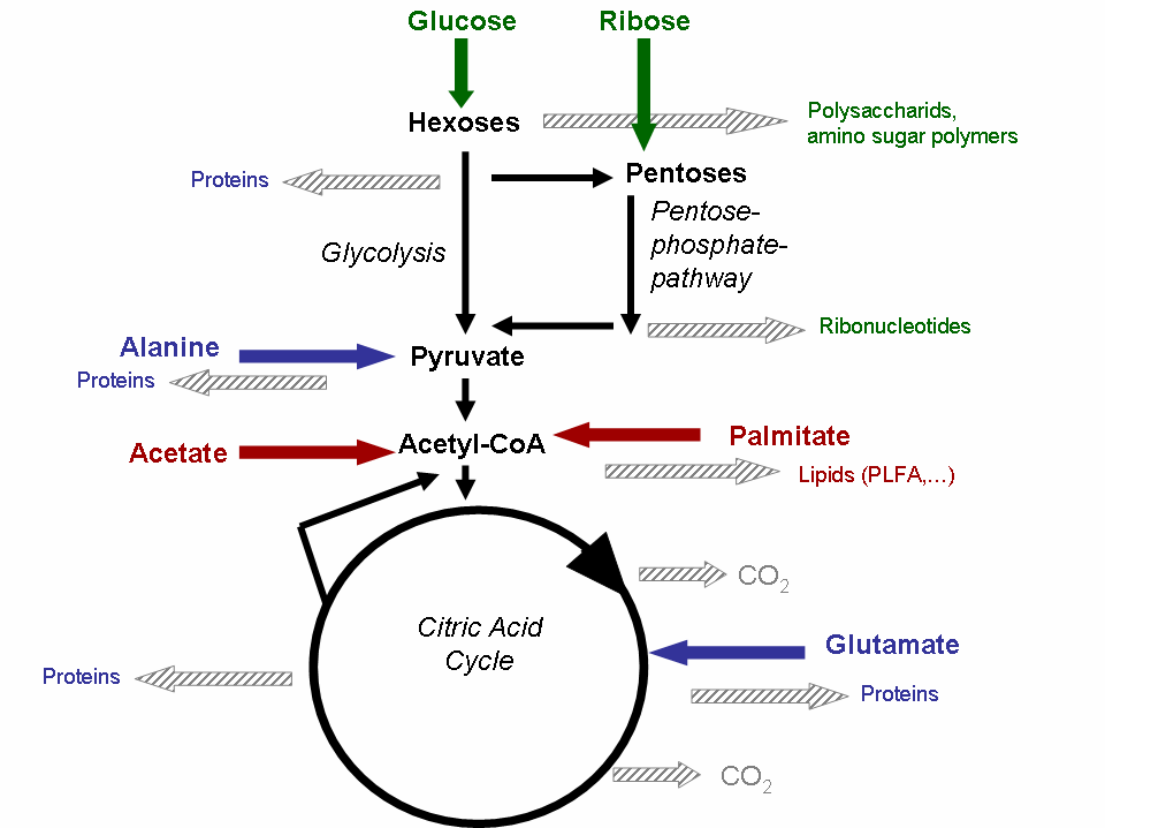

Fig. S5 Primary metabolic pathways of the six representatives of three LMWOS classes (amino acids (blue), sugars (green) and carboxylic acids (red)). Thick arrows reflect the entry points of LMWOS in the metabolic pathways; black fine arrows show the basic C metabolism and shaded arrows reflect anabolic pathways for the formation of cellular compounds.

Study 1 revealed that the fate of individual LMWOS in soil was clearly affected by the C partitioning between catabolism and anabolism within microbial cells. Study 1 allowed the conclusion that the entry steps of a LMWOS to the basic C metabolism and consequently the main transformation pathway of this LMWOS accounts for the fate of LMWOS C in soil. Substrates entering at glycolysis were preferentially incorporated into microbial biomass, whereas LMWOS entering the citric acid cycle were preferentially mineralized. However, based on the transformation of uniformly labeled substances the C allocation through the metabolic pathways shown in Figure S5 could not finally be proven but only supposed. Therefore, this basic investigation was followed by several studies

based on position-specific labeling, which enabled the identification of specific transformation steps and the reconstruction of underlying metabolic pathways.

1.3.2.2 The main pathways for LMWOS metabolization by soil microorganisms

General pathways for LMWOS metabolization could be identified by the incorporation of individual C positions of the six applied LMWOS into the microbial biomass – an approach commonly called metabolic tracing (Dijkstra et al., 2011a). Recent studies focused on glucose (Scandellari et al., 2009) and pyruvate (Dijkstra et al., 2011a) to trace the metabolism. In this thesis, a broader spectra of metabolic tracers were used, which entered at various steps of basic C metabolism (see Figure S5) to enable a more detailed look at specific steps of the microbial metabolic network.

Study 3 revealed that the carboxyl C of both amino acids is oxidized rapidly in soils (90% within 3 days), whereas methyl C from residual alanine and glutamate molecules showed lower mineralization (<70% within 3 days), but within the C remaining in soil a preferential incorporation into microbial biomass compounds (>40% within 3 days) was observed. This allows the general conclusion that highly oxidized, functional groups are more prone to mineralization in soils, whereas more reduced C groups like methyl groups are more likely to be stabilized in soils.

The underlying reason for these results can be found in microbial metabolism: In accordance with previous studies based on pyruvate (Dijkstra et al., 2011a; Wegener et al., 2010), alanine C-1 was decarboxylated by pyruvate dehydrogenase. Therefore, alanine (in analogy to pyruvate) is an appropriate tracer in soil for pyruvate dehydrogenase activity: This central step in C metabolism leads to a loss of alanine C-1 position and transfers C from the glycolysis to the citric acid cycle precursor acetyl-CoA – and consequently to an oxidizing, catabolic pathway (Figure S6, middle). Within the citric acid cycle, alanine C-2 and C-3 could be fully oxidized. However, a complete oxidation of the remaining C-2–C-3 - backbone does not necessarily occur. C-2 would be oxidized by an immediate step, whereas C-3 can cycle several times through the citric acid cycle. If C is allocated from the citric acid cycle to anabolic pathways, the C-3 could be transferred to various microbial biomass compounds (Keseler et al., 2009, Caspi et al., 2008). This further use of citric acid cycle metabolites (containing only C-3) in anabolism was observed in study 4: Preferential C-3 incorporation (compared to C-2) in various microbial metabolites, e.g. the ligand-exchangeable metabolite fraction (see study 4, Figure 5), was traced.

Results of alanine were confirmed by the position-specific fate of glutamate (Study 3) – an amino acid which (after deamination to oxoglutarate) directly enters the citric acid cycle. The immediate oxidation step in the first cycle causes glutamate C-1 oxidation,

whereas C-2 could be allocated to various microbial metabolites. Besides glutamate, acetate (see study 8) also enters the citric acid cycle and shows a similar pattern. The first oxidation step causes loss of C-1 and, as a result, the preferential incorporation of C-2 into microbial products. These results show that the citric acid cycle affected the transformation of several LMWOS. Consequently, various LMWOS can be used to follow the allocation of C from the citric acid cycle towards anabolism. These analogies in the fate of molecule positions from individual LMWOS due to pyruvate dehydrogenase oxidation and citric acid cycle are summarized in Table S3.

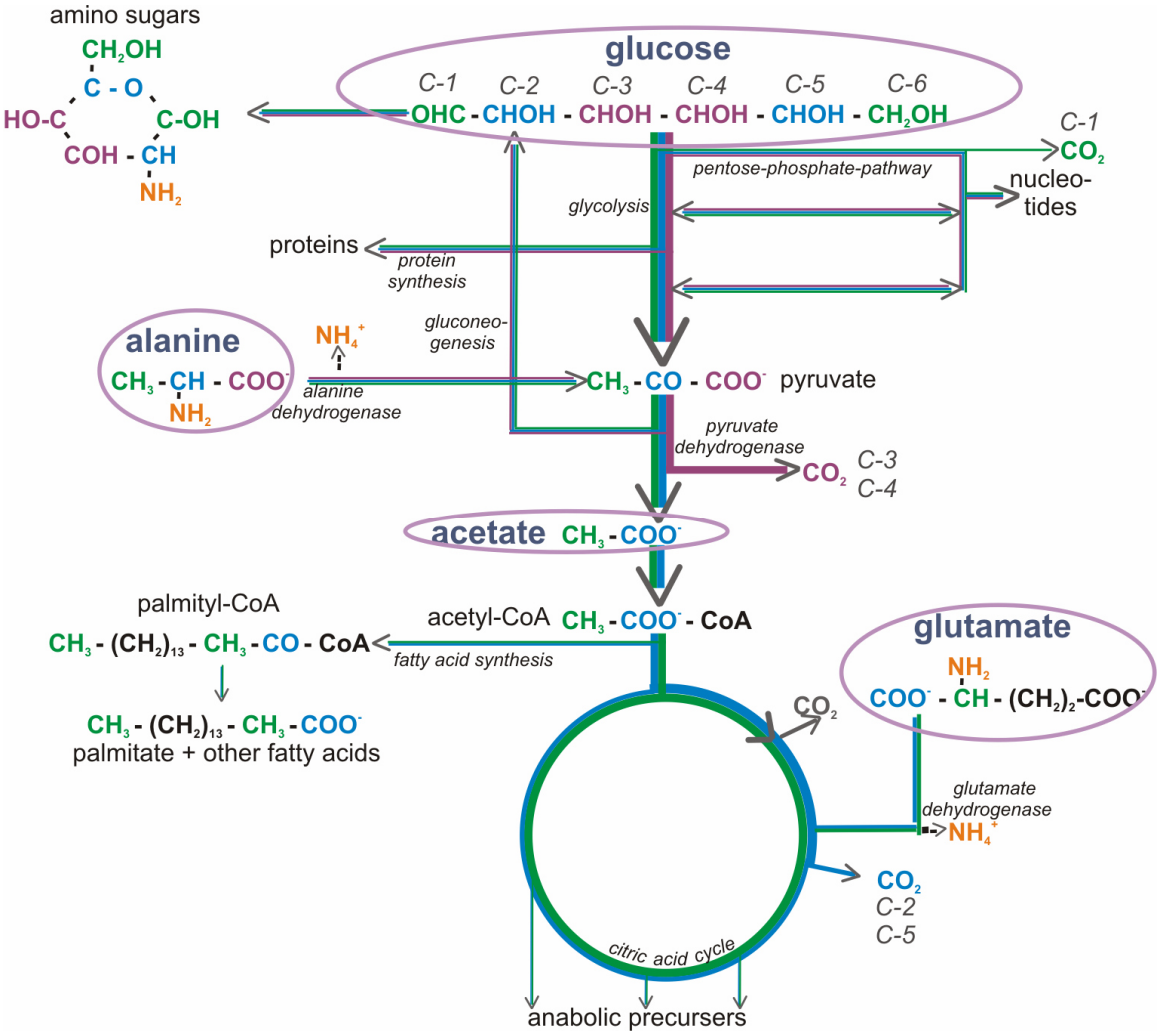

Fig. S6 Basic C metabolism of heterotrophic organisms: colored arrows show the fate of C from individual molecule positions of four LMWOS (glucose, alanine, acetate and glutamate). Main oxidizing steps causing the preferential oxidation of specific C positions and consequently a minor incorporation in microbial biomass are (from up to down): 1) oxidizing branch of the pentose phosphate pathway, 2) the pyruvate dehydrogenase reaction and 3) oxidation within the citric acid cycle.

Study 6 revealed that glucose transformations also allowed the reaction of pyruvate dehydrogenase to be observed, but hardly those of the citric acid cycle. Glycolysis splits

hexoses between C-3 and C-4 and results in two symmetric trioses: C-1 to C-3 and C-6 to C-4. If glycolysis and pyruvate dehydrogenase oxidation were the only pathway to use glucose (Table S4), this would result in a symmetry in the position-specific fate of individual glucose C positions with C-6 = C-1 ~ C-5 = C-2 > C-4 = C-3 = 0 (Scandellari et al., 2009) (Figure 5 in study 6). Preferential incorporation of C-6 (corresponding to C-3 of alanine, see Table S3) compared to preferential oxidation of C-4 (corresponding to C-1 of alanine, see Table S3) was observed in study 6 and confirms the transformation of glucose by glycolysis and pyruvate dehydrogenase.

Table S4 Analogies in the behavior of individual C positions of LMWOS entering the main branch of the basic C metabolism (glycolysis, pyruvate dehydrogenase and citric acid cycle). Analogies were concluded from the basic metabolic pathways shown in Figure S6. Positions within one column are equivalent within these pathways.

LMWOS	Individual C positions		
glucose	C-1 = C-6	C-2 = C-5	C-3 = C-4
alanine	C-3	C-2	C-1
acetate	C-2	C-1	
glutamate	C-2	C-1	

Two conclusions of study 6 – using monosaccharides as metabolic tracers – have to be considered for the further application of metabolic tracing in soils:

1) Glucose transformation did not reflect the classical fingerprint of citric acid cycle metabolism (i.e. a preferential incorporation of C-6 and C-1 compared to C-5 and C-2). As too many other pathways branch off from glycolysis to citric acid cycle, these side branches overprint the citric acid cycle transformations (Scandellari et al., 2009). Therefore, tracers which directly enter a pathway are advantageous, if specific pathways should be traced, especially in media as complex as soils.
2) Classical fingerprints (e.g. preferential oxidation of glucose C-4) of specific pathways were lost from day 3 to day 10, especially in the case of monosaccharides (study 6). This showed the parallel existence of anabolic and catabolic pathways in soils (Derrien et al., 2007). Sampling times after tracer application therefore have to be considered: Short intervals have to be chosen to trace one-directional pathways (≤ 3 days). In contrast, longer time intervals allow an integrated view on C allocation into cata- and anabolism.

In general, the basic biochemical pathways led to a specific fate of C from individual molecule positions. Further, specific pathways imply a position-specific fate which deviates from the pattern expected from the transformations of basic C metabolism (Figure S6 and Table S4). This was, for example, shown by a preferential C-1 oxidation com-

pared to C-6 of glucose (study 6 and study 7), which reflects glucose transformation by the pentose phosphate pathway (see Figure S6).

To summarize, the basic C metabolism as well as some side branches could be traced by the chosen spectra of LMWOS within this thesis and fundamentals for a systematic application of position-specific labeling in soil science were investigated. Glucose and ribose transformations were characterized by glycolysis and pentose phosphate pathway. Alanine transformation was dominated by pyruvate dehydrogenase oxidation. Acetate, palmitate and glutamate directly entered the citric acid cycle and hence could be used to trace catabolic reactions as well as anabolic pathways starting from citric acid cycle intermediates. Consequently, the basic C metabolism (and its oxidation steps) determined the fate of all investigated LMWOS in soils. Although further abiotic or biotic sinks and transformations of LMWOS are described in the literature (sorption, abiotic condensation reactions, etc.), the microbial metabolism dominated the results of these studies overall: Transformations of LMWOS occurred mainly by microbial metabolism and position-specific ^{13}C-labeling allowed the individual transformation steps to be traced.

1.3.2.3 Metabolic pathways for the formation of specific cellular compounds

Incorporation of individual LMWOS positions into microbial biomass enabled specific oxidation steps to be traced as individual positions get lost e.g. by pyruvate dehydrogenase oxidation or citric acid cycle oxidation (Figure S6). Moreover, if specific anabolic pathways, i.e. the formation of new cellular compounds from applied LMWOS, should be traced, the direct incorporation of individual molecule positions into these compounds has to be determined (Scandellari et al., 2009, Knowles et al., 2010). This requires the combination of position-specific labeling of the LMWOS with compound-specific isotope analysis (CSIA) of the newly formed cellular products. Within this thesis, this combination was performed for the first time in soil science.

Firstly, phospholipid fatty acid formation was traced by CSIA of microbial PLFA in studies 3, 6 and 8 for each of the applied LMWOS. Fatty acid formation branches off from acetyl-CoA (Figure S6). Therefore, all LMWOS that enter glycolysis pass the pyruvate dehydrogenase oxidation before entering the fatty acid formation pathway (Scandellari et al., 2009). This was proven by various studies within this thesis, for example:

1) Study 3: no allocation of alanine C-1 into the PLFA
2) Study 6: no allocation of glucose C-4 into the PLFA

In study 3, fatty acid formation from glutamate a more oxidized, citric acid cycle metabolite was determined. Allocation of C from the citric acid cycle to anabolic pathways like gluconeogenesis and fatty acid formation required special pathways, which are only used

under special conditions, e.g. C deficiency (Knowles et al., 2010). The glyoxolate bypass may serve as an example (Caspi et al., 2008). The allocation of glutamate C-2 into PLFA revealed such special, anabolic pathways which are indicative for certain ecophysiological conditions.

Further insights into an anabolic pathway can be gained if its direct precursors are labeled. Intact incorporation of that precursor (which leads to an identical incorporation of all positions) can be distinguished from the incorporation of various metabolites of C metabolism (Sauheitl et al. 2009). The latter leads to the preferential incorporation of non-oxidized positions into newly formed compounds. This was performed for two compound classes within this thesis: glucose was used as a direct precursor of amino sugars in study 7 and acetate and palmitate were applied as direct precursors of fatty acids in study 8.

Study 7 revealed that although glucose is a direct precursor for amino sugar synthesis, it is only partially used directly for amino sugar formation. Up to 55% of the ^{13}C that is incorporated into amino sugars is derived from glucose metabolites; e.g. glucose was transformed via glycolysis and thereafter glucose C was transferred back via gluconeogenesis to the amino sugar formation pathway (Figure S6). This showed that oxidizing catabolic pathways occur in parallel with anabolic pathways in soils, which was similarly observed by Derrien et al. (2007).

In study 8, acetate and palmitate were used as precursors for PLFA formation. Palmitate incorporation into PLFA was significantly higher than acetate incorporation (study 8, Figure 1). Consequently, an already existing fatty acid was a preferred precursor for direct PLFA formation to avoid additional costs for the formation of new monomers (Lengeler et al., 1999). However, the incorporated palmitate is successively modified according to the fatty acid demand of the microbial community (Figure S7). During 10 days, 65% of the incorporated palmitate was transformed to various fatty acids. Within these transformations, desaturation was performed faster than elongation or branching. In contrast, acetate was rarely used to form completely new fatty acids but was mainly a precursor for the transformation of existing fatty acids. For instance, acetate C was introduced by elongation or branching into the PLFA.

Palmitate as well as acetate labeling revealed a high internal turnover of fatty acids in soil (Lichtfouse et al., 1995). Therefore, C might be recycled several times within a metabolic side branch but by undergoing continued transformations. These transformations of bound and free fatty acids cause consequences for the utilization of fatty acids as plant and microbial biomarkers. If the uptake and transformation of long-chain, plant-derived fatty acids occur in a similar range in soils as for palmitate, then the interpretation

of plant-derived fatty acid fingerprints and their isotope signature for paleoenvironmental studies is hampered.

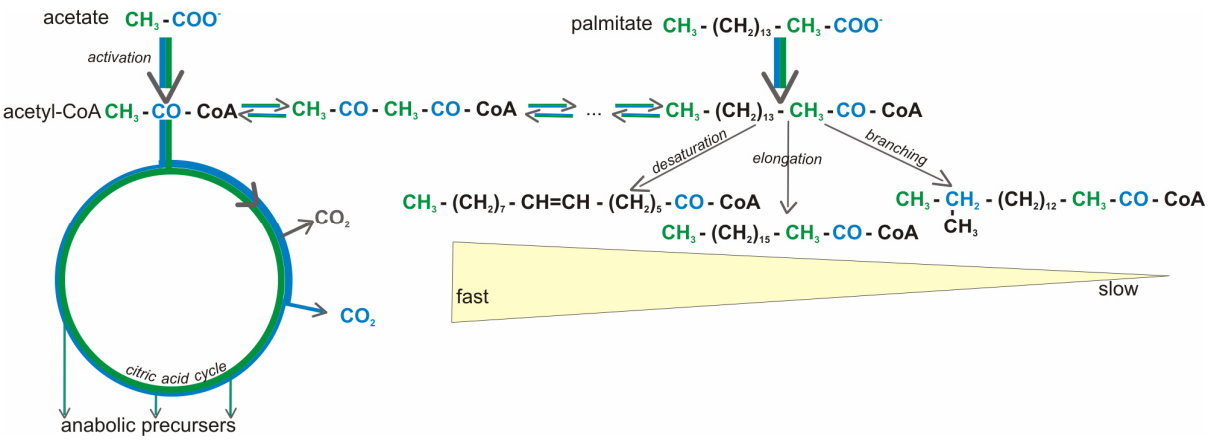

Fig. S7 Metabolic pathways of fatty acid formation from acetate and fatty acid transformations of palmitate in soil.

1.3.3 Identification of specific metabolic pathways

1.3.3.1 Specific pathways of individual members of the microbial community in soils

The investigated compound classes of microbial products are on the one hand specific for a certain biosynthetic pathway within microbial cells. On the other hand, PLFA and the amino sugars are also biomarkers for individual microbial groups in soils. This enables the identification of specific pathways of individual members of the soil microbial community.

Amino sugars enable fungi and bacteria to be distinguished between (Engelking et al., 2007, Glaser et al., 2004): In study 7, bacterial muramic acid showed the higher dynamics of ^{13}C replacement of the cell walls (study 7, Figure 3). In addition the DI of individual C positions changed strongly from day 3 to day 10 for bacterial muramic acid but remained constant for fungal galactosamine (study 7, Figure 2). This reflects a more active maintenance metabolism in bacteria, which caused an increasing incorporation of glucose metabolization fragments within a period of 10 days. Therefore, this study proved for the first time that differences in the C turnover in the slow- and fast-cycling branch of soil food webs can be attributed to metabolic processes (Moore et al., 2005).

A more detailed fingerprint of the microbial community, especially for the prokaryotic groups, can be gained by individual PLFA (MooreKucera and Dick, 2008). Combining statistical grouping of the fatty acids with known fatty acid fingerprints enables functional

microbial groups to be distinguished in soil (studies 3, 6 and 8). The DI revealed for glutamate incorporation into PLFA that two functional microbial groups – gram-positive II and fungi – showed a specific C allocation: Glutamate C-2 was transferred from the citric acid cycle towards fatty acid synthesis. Special reactions (e.g. the glyoxylate bypass) are necessary to avoid the oxidation of glutamate C-2 (Caspi et al., 2012). None of the other microbial groups showed C-2 incorporation into PLFA (see study 3, Figure 4). Consequently, this was the first time that specific pathways of individual members of the soil microbial community could be traced in soils in parallel.

1.3.3.2 Pathways under various concentrations of LMWOS

C allocation within metabolic pathways is strongly affected by environmental conditions. The bioavailability of C sources, especially LMWOS, is one of the main factors controlling the state of the microbial community - from maintenance to growth (Blagodatskaya et al., 2007, Fischer et al., 2010b; Schneckenberger et al., 2008). In study 4, alanine transformations were investigated within an alanine concentration gradient representing the full range of concentrations in soil: from bare soil (0.5 µM) to hot spots (5 mM).

At low alanine concentration, uptake was described by Michaelis-Menten kinetics, whereas with increasing concentration, linear kinetics dominated microbial uptake (Jones and Hodge, 1999). This shift in uptake kinetics revealed that two mechanisms were responsible for the microbial uptake: An unsaturable, unspecific uptake of intact alanine at hot-spot concentrations and specific, active uptake mechanisms at low alanine concentrations. The DI of the non-extractable pool of microbial transformation products (containing macromolecules as well as lipids) reflected additional shifts in the intensity of the alanine metabolism pathways (Figure S8).

A significantly increased incorporation of C-1 under lowest C availability may indicate C starvation pathways e.g. anapleurotic pathways. A convergence of the DI of C-2 and C-3 for highest alanine concentration is characteristic of many anabolic pathways for biomass formation under growth conditions (e.g. lipid biosynthesis from alanine for the formation of new cell membranes: see figures S5 and S7).

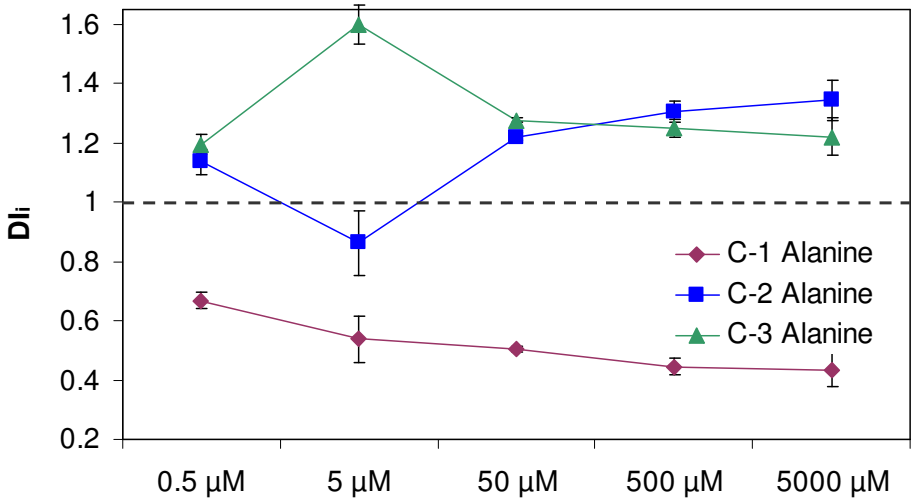

Fig. S8 Concentration-dependent position-specific transformation index DI_i (N=6, ± SEM) of alanine C position incorporated into the not-extractable pool of microbial biomass compounds.

These and further results from study 4 indicate an altered C allocation into individual pathways that is dependent on substrate availability: from anabolic pathways characteristic for C deficiency via maintenance metabolism towards pathways common for growing cells (Figure S9).

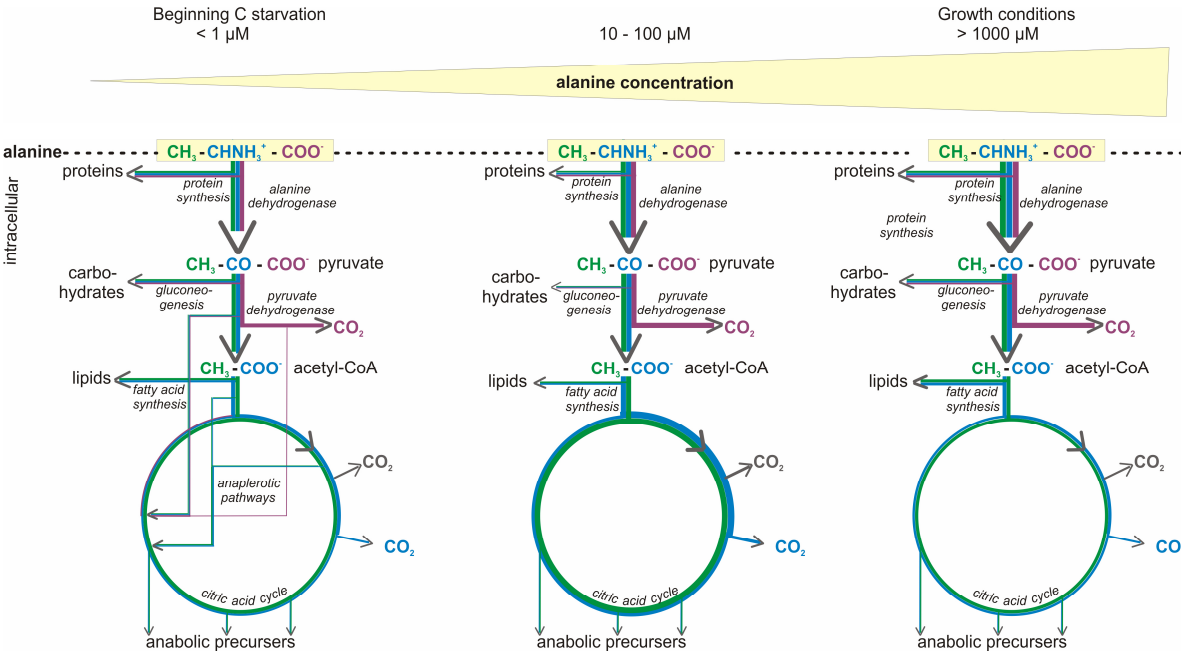

Fig. S9 General biochemical pathways of amino acids metabolization in soil as depending on alanine availability. Line width represents the qualitatively estimated relative shifts of alanine C between certain pathways dependent on the alanine concentration.

1.3.3.3 Pathways of sorbed LMWOS

Sorption is one of the most likely processes causing the long-term stabilization of C in soils (Gonod et al., 2006, Duemig et al., 2012). Nevertheless, a part of the sorbed LMWOS remains bioavailable: Study 5 showed for alanine that at least 20-50% of the mineral-sorbed alanine was microbially metabolized. However, sorption reduced the availability and consequently affected transformation pathways (Jones and Edwards, 1998). In study 5, transformations of position-specifically labeled alanine, sorbed to five sorbents (two iron oxides with different crystalline structure: goethite and hematite; two clay minerals with 2:1 layers – smectite, and 1:1 layers – kaolinite; and active coal) were investigated. Goethite and active coal showed the highest amount of sorbed alanine (~45% of added alanine), and the lowest portion of the sorbed alanine C was microbially utilized (26 and 22%, respectively), whereas clay minerals showed lower sorption (10-26% of added alanine) and a higher portion that was microbially available (30-35%).

The stronger the sorption by the individual sorbent, the lower the microbial utilization was (Jones and Hodge, 1999). The fate of individual molecule positions reflected that, at least for the four mineral phases, alanine was processed by the classical biochemical pathways: deamination, decarboxylation of C-1 by pyruvate dehydrogenase and further oxidation of C-2 and C-3 in the citric acid cycle (Djikstra et al., 2011). However, the intensity of microbial pathways depended on the bioavailability of the sorbed substrate: the less alanine was accessible, the less was oxidized by catabolism and the more alanine C was used for anabolism, i.e. the formation of microbial biomass.

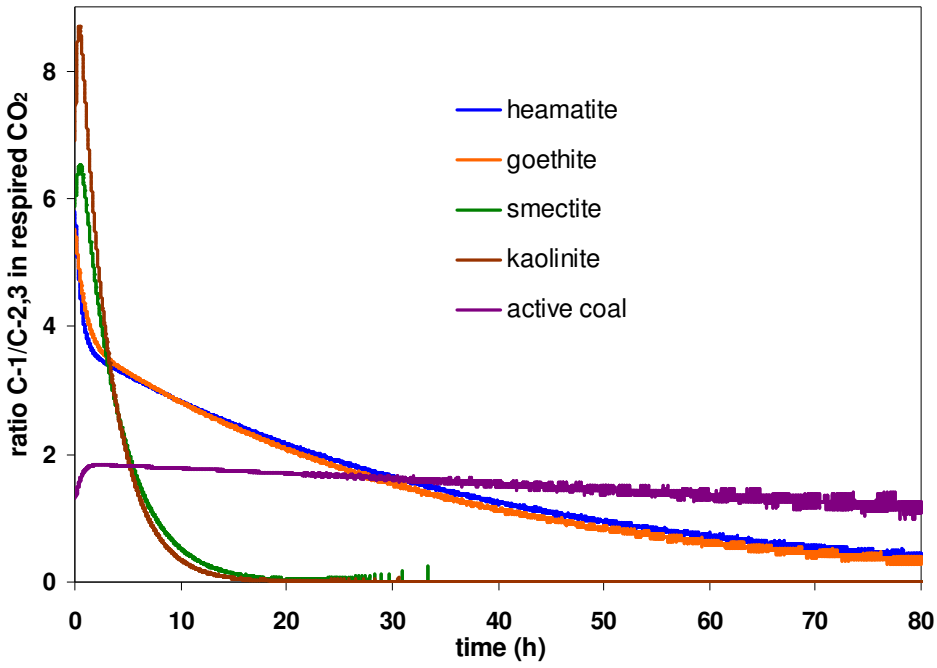

Fig. S10 Ratio of C-1 to (C-2+C-3)/2 respiration of alanine C for the 5 applied sorbents calculated from the fitted, position-specific oxidation rate.

The ratio of C-1 oxidation by pyruvate dehydrogenase versus oxidation of C-2 and C-3 in the citric-acid cycle (Figure S10) depended on the microbial availability of alanine: High availability due to fast desorption of cation-exchangeable bound alanine caused an initial peak in C-1 oxidation by glycolysis for the two clay minerals and an abrupt shift to oxidation via the citric acid cycle. However, low microbial availability of alanine sorbed to iron minerals led to a parallel oxidation of all three positions by glycolysis and citric acid cycle (represented by the C-1/C-2,3 ratio in Figure S10). This slower oxidation rate was associated with an increase in C allocation towards anabolism (Djikstra et al., 2001) (Figure S11).

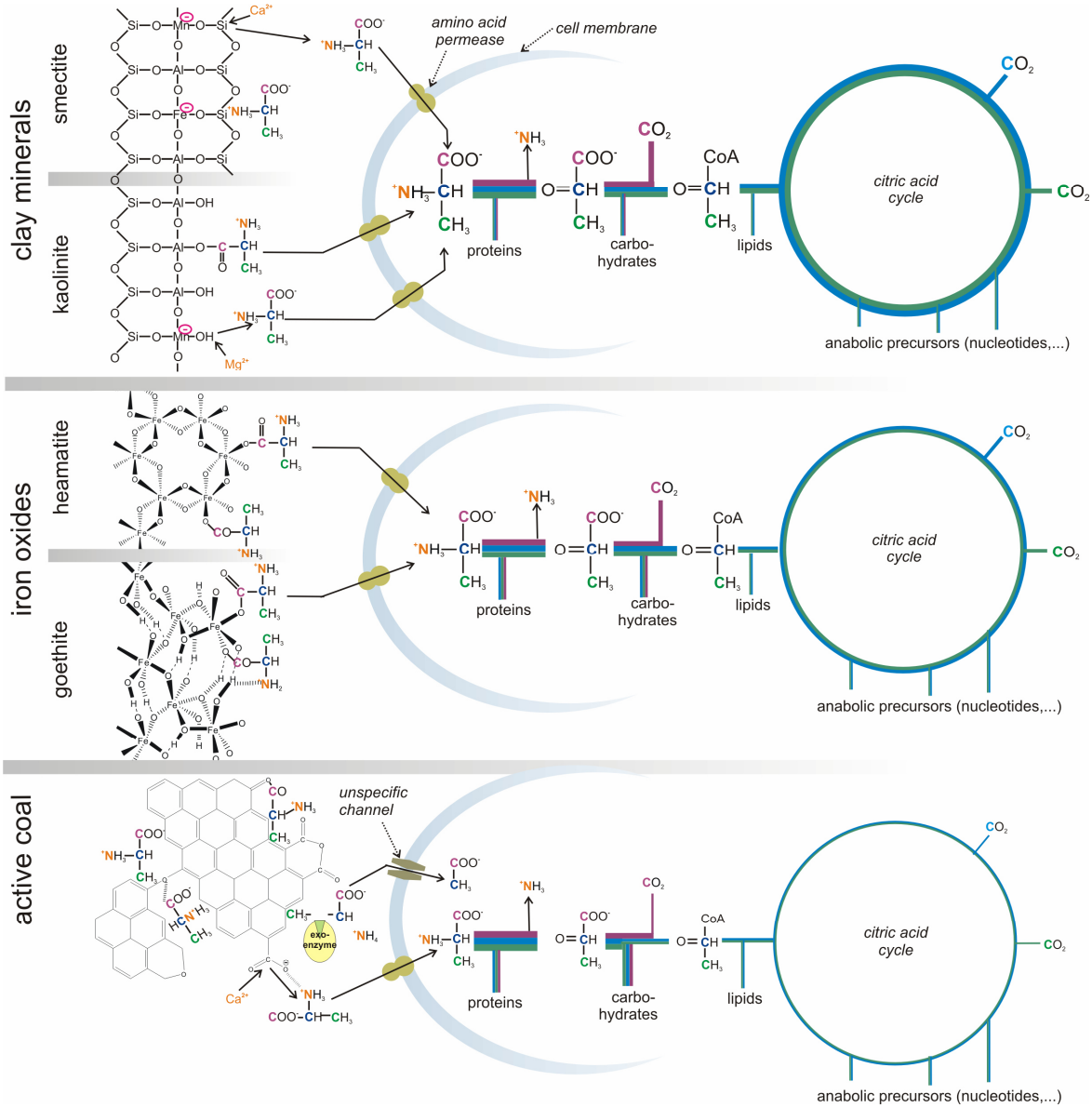

Fig. S11 Metabolic pathways of alanine sorbed on clay minerals (smectite and kaolinite), iron oxides (hamatite and goethite) and active coal. Detailed explanations in text. Various colors show the pathways of C from individual positions of alanine. Line width represents the qualitatively estimated relative shifts in the fate of alanine C positions between certain pathways dependent on the sorbent class.

Alanine sorbed to active coal showed a deviating behavior with preferential stabilization of C-3 and oxidation of C-1 and C-2 (study 5, Figure 3 and 4). This indicates that in addition to basic microbial mechanism, further stabilization and modified transformations of sorbed alanine occured, e.g. by exoenzymatic degradation (Lehmann et al., 2011). Potential desorption processes and pathways are shown in Figure S11. In general, position-specific labeling revealed that the fate of amino acid C in soil is strongly affected by sorption: The stronger the sorption, the more metabolized LMWOS C is allocated into microbial biomass compounds (Figure S11).

1.3.3.4 Extra- versus intracellular transformation pathways

Besides intracellular microbial pathways, extracellular transformations may also play an important role in LMWOS transformations in soils, especially in microhabitats where intact cells have no access (von Luetzow et al., 2006). To distinguish extra- and intracellular transformation pathways, selective inhibition of cellular, energy-dependent pathways was performed in study 4 (Figure S12).

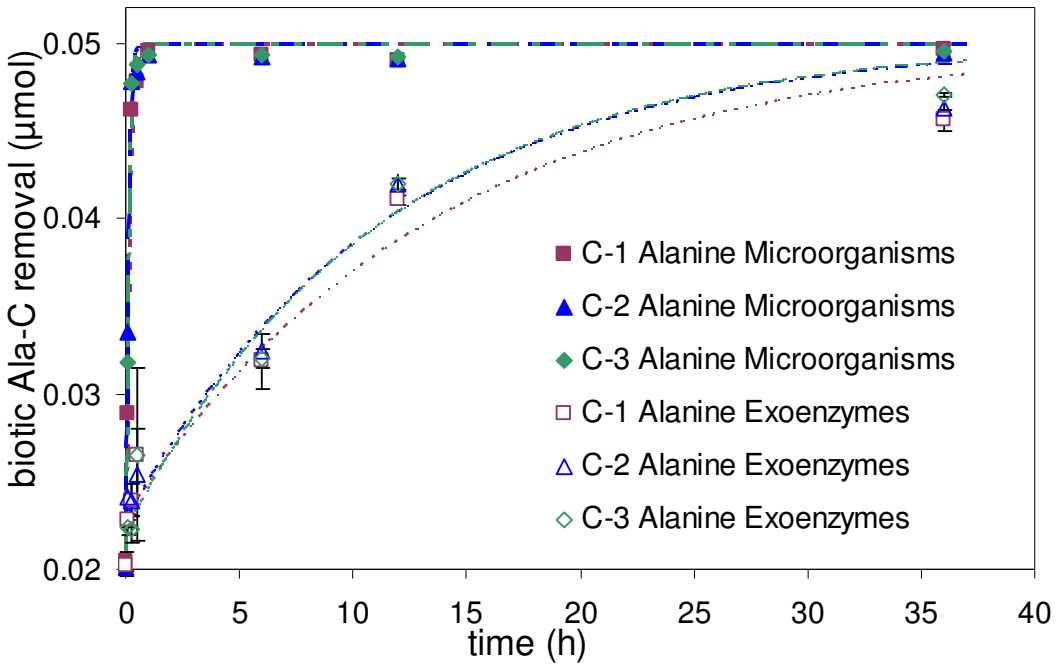

Fig. S12 Removal of alanine from soil solution by extra- and intracellular processes without inhibition (filled symbols, dashed line) and by extracellular transformation in respiration-inhibited treatments (open symbols, dotted line); Experimental points (means ± SEM, N=6) and fitted curves based on an exponential utilization model are presented.

This approach could prove the existence of extracellular transformation of LMWOS in soils: Alanine was decomposed by a stepwise extracellular oxidation starting from the

carboxylic group, presumably by rather unspecific exoenzymes (Hofrichter et al., 1998). However, comparing the kinetics of extra- versus intracellular processes (Figure S12) revealed that cellular uptake of LMWOS always out-competed extracellular transformations, which are quantitatively relevant only at very low alanine concentrations or in specific microhabitats.

1.3.4 Kinetics and ecological relevance of competing sinks for LMWOS

1.3.4.1 Sorption versus microbial utilization

As position-specific labeling enables transformation pathways in soils to be distinguished, it can be used to assess the relevance of individual pathways and their fluxes. Sorption, as an LMWOS sink in soils, can interact and compete with microbial utilization (Fischer et al., 2010b; Kaiser and Kalbitz, 2012).

Study 4 assessed the sorption of alanine in sterilized soil and found that sorption occured as intact molecules and no abiotic cleavage of alanine was detected (in contrast to results of Wang and Huang (2005)). In study 5, the microbial utilization of sorbed alanine was assessed: Desorption, at least from mineral phases, occurred mainly as intact molecules, too. The observed position-specific transformations in both studies were mainly characterized by the microbial metabolism. Therefore, sorption and desorption do not directly contribute to the transformation of LMWOS, but only prevent translocation (Kaiser and Kalbitz, 2012) or microbial transformation. If kinetics of sorption and microbial uptake were compared (study 4), microbial utilization outcompeted sorption in soils.

1.3.4.2 Plant uptake versus microbial utilization

Besides microorganisms, plants are also known to have the ability for LMWOS uptake by their roots (Fischer et al., 1998). The relevance of this process is still in question for many ecosystems due to the effective competition of microorganisms for LMWOS (Hodge et al., 2000; Jones et al., 2005a). The main problem of evaluating the uptake of intact amino acids is methodological constraints: The commonly used dual-isotope labeling approaches coupled with bulk isotope measurements cause an overestimation of the calculated intact amino acid uptake (Sauheitl et al., 2009). Study 9 evaluated the intact uptake of the amino acid alanine by plants using position-specific labeling. Consequently, intact uptake could be distinguished from the uptake of transformation fragments. Position-specific ^{14}C-labeling revealed that a minor portion (less than 1.5% of the applied

amino acids) was taken up intact, whereas the majority of alanine (~98.5%) was used by soil microorganisms (Figure S13). Uptake calculated from uniform labeling reflected an overestimation for the factor 1.2-3 of the quantified intact uptake.

Preferential uptake of C-3 by all three plant species indicated that the uptake of microbial transformation fragments occurred. Previous studies in this thesis revealed that microbial biomass compounds are also characterized by a dominance of alanine C-3 (see study 3 and 4). Consequently, microbial uptake and transformation can produce mineralized N as well as fragments of the C skeleton, which were partially available in the soil solution for root uptake (Jones et al., 2005). Labeling with the N-free LMWOS acetate showed a similar uptake of N-containing and N-free LMWOS (< 2% of the applied ^{14}C). This indicated that plant uptake of LMWOS mainly occurred via passive uptake mechanisms. These passive mechanisms can also account for the unspecific uptake of microbial transformation products. In summary, study 9 suggests that N uptake from organic sources is of minor importance for N nutrition of agricultural plants and even more for the fate of LMWOS in soils (Jones et al., 2005, Hodge et al., 2000).

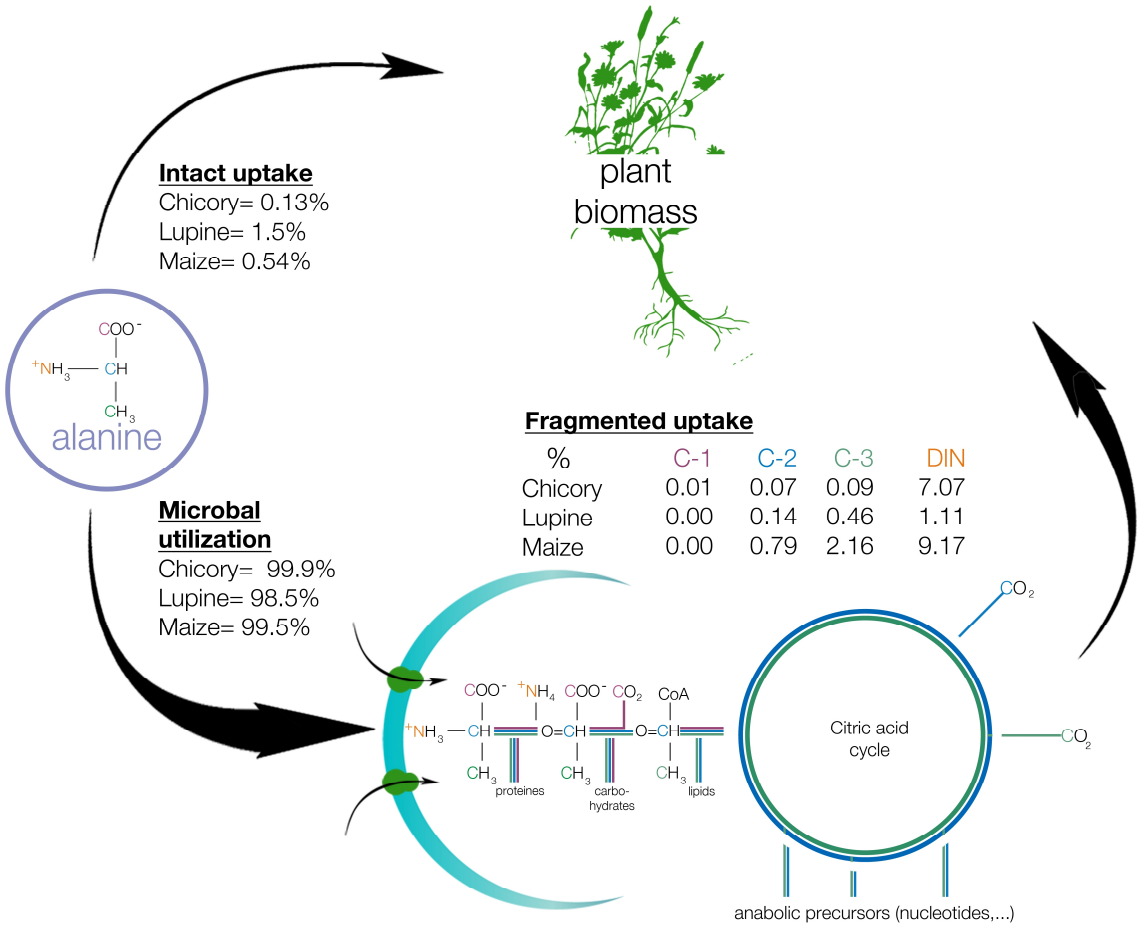

Fig. S13 Illustration of the fate of alanine (numbers represent % of applied tracer: this can either be taken up intact or degraded/mineralized to fragments and subsequently incorporated into microorganisms or plant biomass).

1.4 Conclusions

The high flux of C through the pool of LMWOS clearly defines them as a crucial C pools in the SOC cycle. Many previous studies have analyzed the rates and turnover of LMWOS in soil, but the underlying mechanisms and pathways of C transformation of LMWOS remains unknown. Therefore, these studies were focused on biogeochemical pathways of three main groups of LMWOS in soil: amino acids, monosaccharides and organic acids. Tracing their transformations was achieved by combining for the first time position-specific ^{13}C-labeling with compound-specific isotope analysis (CSIA).

The application of individual LMWOS revealed that entry steps into basic C metabolism account for specifics in the C partitioning between microbial cata- and anabolism: substrates entering citric acid cycle were preferentially mineralized (>80% in 10 days) whereas e.g. monosaccharides entering glycolysis were preferentially allocated to anabolic pathways and incorporated into microbial biomass (less than 70% mineralized in 10 days).

Position-specific ^{13}C- and ^{14}C-labeling provided a unique submolecular approach to reconstruct the main pathways of C transformation in soil and their specifics in individual microhabitats (e.g. at mineral surfaces or at the plant-root interface). The divergence index (DI) was developed and proven to be a valuable tool to compare the position-specific fate of individual substances independent of the used isotopic approach or experimental design used or the pool investigated. ^{13}C incorporation in various microbial compound classes was traced by compound-specific isotope analysis: fatty acids by GC-C-IRMS and amino sugars by IC-O-IRMS. Therefore, a new instrument coupling was applied and purification and measurement methods for soil amino sugar δ^{13}C analysis were established and evaluated.

Basic microbial C metabolism with glycolysis, pyruvate dehydrogenase oxidation and citric acid cycle could be traced in soil under field and laboratory conditions. Oxidizing, catabolic pathways are ongoing in soils in parallel to constructing, anabolic pathways (like gluconeogenesis): for example, up to 55% of the glucose allocated to amino sugar synthesis was not intact glucose but derived from glucose metabolites allocated by gluconeogenesis backflux towards amino sugar formation. Consequently, substrates entering glycolysis are intensively recycled within the cellular C pool, which was shown by a continued decrease of their divergence index.

Specific tracers for individual biosynthetic pathways were identified, which allowed transformations to be followed within these side branches of the basic C metabolism: 1) the pentose phosphate pathway was detected by a combination of hexose and pentose ^{13}C labeling; and 2) turnover within the cellular lipid pool was proven by ^{13}C labeling of

short- (acetate) and long-chain (palmitate) precursors of PLFA. Within 10 days, 65% of the incorporated palmitate was transformed (e.g. by desaturation, elongation or branching) to other fatty acids and the fingerprint of the palmitate ^{13}C-derived fatty acids approached the PLFA pattern of the present microbial community. Knowledge of these fast fatty acid transformations is crucial for the application of fatty acid fingerprints and their isotopic values for palaeoenvironmental reconstructions.

An intensive turnover was not only shown for lipids but also for cell wall polymers. Metabolic recycling activity and turnover was much higher for bacteria than for fungi, which was proven by both biomarker groups – PLFA and amino sugars. Therefore, this thesis experimentally revealed one underlying, mechanistic reason for the previously observed specifics in C turnover of the slow (fungi-based) and the fast (bacteria-based) cycling branch of the soil food web. For the first time, position-specific labeling was coupled with compound-specific isotope analysis of microbial biomarkers. This combination provides a novel opportunity to trace simultaneous, biosynthetic pathways of individual microbial groups in diverse microbial communities of soils.

Furthermore, variations of environmental factors, like substrate concentration, were identified as the main regulatory factors for C allocation within microbial metabolism: Concentration gradients characteristic for soils from C-poor bulk soils to hotspots involved a shift of C allocation within metabolic pathways from C starvation pathways via maintenance pathways towards pathways that are characteristic for cells under growth conditions. Sorption, as a soil-specific process reducing the bioavailability of a substrate, affected microbial metabolism: the stronger a substrate is sorbed, the more of its C is allocated towards anabolism, e.g. is found in the microbial products. Understanding these shifts in metabolic pathways is crucial for the SOC cycle, as C allocation towards anabolism is the prerequisite for the formation and stabilization of microbially-derived SOM.

Three soil-specific processes were traced in parallel with microbial utilization: sorption, exoenzymatic LMWOS utilization and plant uptake. Sorption, as well as desorption, occurred as intact molecules and did not account for LMWOS transformations. Exoenzymes caused a stepwise oxidation of the LMWOS C backbone. However, their kinetics could not compete with microbial uptake systems. Consequently, extracellular transformations can only be relevant in specific soil microhabitats, which are inaccessible for microorganisms. Intact uptake of amino acids by plants was assessed by dual-isotope position-specific ^{13}C- and ^{15}N-labeling. This new approach revealed the overestimation of intact amino acid uptake due to methodological constraints of previous studies and showed that less than 1.5% of the applied amino acids were taken up intact by plants. Consequently, none of the investigated abiotic or biotic processes could compete with

microorganisms for LMWOS utilization and the microbial metabolism determines the fate of LMWOS C in soils.

This thesis established position-specific ^{13}C- and ^{14}C-labeling as a unique tool to trace LMWOS transformation processes in soils. Using this novel approach, the base for detailed mechanistic understandings of microbial LMWOS transformations and subsequent SOM formations were created. Combination of position-specific labeling with dual-isotope labeling and the improvement of CSIA towards the position-specific detection of the isotope label within the newly formed transformation products will be a future task. These techniques will further deepen the understanding of microbial C transformations and their controlling factors and improve prediction as well as manipulation of C allocation and stabilization in soils.

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1.6 Contribution to the included manuscripts and publications

The publications and manuscripts included in this PhD thesis were prepared in cooperation with various coauthors. The coauthors listed in these publications and manuscripts contributed as follows:

Study 1:

Fate of low molecular weight organic substances in an arable soil: from microbial uptake to utilisation and stabilisation

Status at date of thesis submission: Submitted to Soil Biology and Biochemistry;

Status at date of thesis printing: Submitted to Soil Biology and Biochemistry;

Contributors:

Anna Gunina	45%	Accomplishment of experiments, laboratory analysis, data preparation, preparation of the manuscript
Michaela Dippold	40%	Experimental design, accomplishment of the experiments, data preparation, preparation of the manuscript
Bruno Glaser	3%	Discussions on experimental design, suggestions to improve manuscript
Yakov Kuzyakov	12%	Experimental design, discussions on the results, suggestions to improve manuscript

Study 2:

Improved $\delta^{13}C$ analysis of amino sugars in soil by Ion Chromatography - Oxidation - Isotope Ratio Mass Spectrometry

Status at date of thesis submission: In revision at Rapid Communications in Mass Spectrometry;

Status at date of thesis printing: Published in Rapid Communications in Mass Spectrometry;

Contributors:

Michaela Dippold	55%	Establishment of purification method, laboratory analysis, data preparation, preparation of the manuscript
Stefanie Boesel	20%	Establishment of measurement method, suggestions to improve manuscript
Anna Gunina	12%	Laboratory analysis, comments to improve manuscript

Yakov Kuzyakov	3%	Suggestions to improve manuscript
Bruno Glaser	10%	Discussions on method development, suggestions to improve manuscript

Study 3:

Biochemical pathways of amino acids in soil: Assessment by position-specific labeling and ^{13}C-PLFA analysis

Status at date of thesis submission: Accepted in Soil Biology and Biochemistry

Status at date of thesis printing: Published in Soil Biology and Biochemistry

Contributors:

Carolin Apostel	42%	Accomplishment of experiments, laboratory analysis, data preparation, preparation of the manuscript
Michaela Dippold	40%	Experimental design, accomplishment of the experiments, data preparation, suggestions to improve manuscript
Bruno Glaser	3%	Laboratory analysis, suggestions to improve manuscript
Yakov Kuzyakov	15%	Experimental design, discussions on the results, suggestions to improve manuscript

Study 4:

Biogeochemical transformations of amino acids in soil assessed by position-specific labelling

Status at date of thesis submission: Accepted in Plant and Soil

Status at date of thesis printing: Published in Plant and Soil

Contributors:

Michaela Dippold	85%	Experimental design, accomplishment of experiment, data preparation, preparation of the manuscript
Yakov Kuzyakov	15%	Experimental design, discussions on the results, suggestions to improve manuscript

Study 5:

Sorption affects amino acid pathways in soil: Implications from position-specific labeling of alanine

Status at date of thesis submission: Submitted to Soil Biology and Biochemistry

Status at date of thesis printing: Accepted in Soil Biology and Biochemistry

Contributors:

Michaela Dippold	60%	Experimental design, data preparation, preparation of the manuscript
Mikhail Biryukov	25%	Accomplishment of experiment, data preparation,
Yakov Kuzyakov	15%	Experimental design, discussions on the results, suggestions to improve manuscript

Study 6:

Biochemistry of hexose and pentose transformations in soil analyzed by position-specific labeling and ^{13}C-PLFA

Status at date of thesis submission: To be submitted to Soil Biology and Biochemistry

Status at date of thesis printing: Resubmitted to Soil Biology and Biochemistry

Contributors:

Michaela Dippold	45%	Experimental design, accomplishment of experiments, data preparation, preparation of the manuscript
Carolin Apostel	45%	Accomplishment of experiment, data preparation, preparation of the manuscript
Yakov Kuzyakov	10%	Discussions on the results, suggestions to improve manuscript

Study 7:

Metabolic pathways of fungal and bacterial amino sugar formation in soil assessed by position-specific ^{13}C-labeling

Status at date of thesis submission: To be submitted to Biogeochemistry

Status at date of thesis printing: Submitted to Soil Biology and Biochemistry

Contributors:

Michaela Dippold	60%	Experimental design, accomplishment of experiments, data preparation, preparation of the manuscript
Anna Gunina	20%	Laboratory analysis, suggestions to improve manuscript
Stefanie Boesel	5%	Laboratory analysis, data preparation
Bruno Glaser	3%	Suggestions to improve manuscript
Yakov Kuzyakov	12%	Discussions on experimental design, suggestions to improve manuscript

Study 8:

Formation and transformation of fatty acids in soils assessed by position-specific labeling of precursors

Status at date of thesis submission: To be submitted to Organic Geochemistry

Status at date of thesis printing: Submitted to Geochimica et Cosmochimica Acta

Contributors:

Michaela Dippold	85%	Experimental design, accomplishment of experiment, data preparation, preparation of the manuscript
Yakov Kuzyakov	15%	Experimental design, discussions on the results, suggestions to improve the manuscript

Study 9:

Organic N uptake by plants - Reevaluation by position-specific labeling of amino acids

Status at date of thesis submission: Submitted to Soil Biology and Biochemistry

Status at date of thesis printing: Submitted to Journal of Experimental Botany

Contributors:

Daniel Moran	42%	Accomplishment of experiment, data preparation, preparation of the manuscript
Michaela Dippold	42%	Experimental design, accomplishment of experiment, data preparation, preparation of the manuscript
Bruno Glaser	3%	Laboratory analysis, suggestions to improve the manuscript
Yakov Kuzyakov	13%	Experimental Design, Discussions on the results, suggestions to improve the manuscript

2 Publications and Manuscripts

2.1 Study 1: Fate of low molecular weight organic substances in an arable soil: from microbial uptake to utilisation and stabilisation

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Abstract

Microbial uptake and utilization are the main transformation pathways of low molecular weight organic substances (LMWOS) in soil, but detailed of transformations is strongly limited. As various LMWOS classes enter biochemical cycles at different steps, we hypothesize that the percentage of their carbon (C) used by microbial biomass and consequently stabilization in soil is different.

Representatives of the three main groups of LMWOS: amino acids (alanine, glutamate), sugars (glucose, ribose) and carboxylic acids (acetate, palmitate) – were applied at naturally-occurring concentrations into a loamy arable Luvisol in a field experiment. Incorporation of ^{13}C from these LMWOS into microbial biomass (MB) and into phospholipid fatty acids (PLFAs) was investigated 3 d and 10 d after application. The microbial utilization of LMWOS for cell membrane construction was estimated by replacement of PLFA-C with ^{13}C.

Mineralization of LMWOS to CO_2 comprised 20–65% of the initially applied ^{13}C, whereas ^{13}C incorporation into MB amounted to 10–24% at day 3 and was reduced to 1–15% on day 10. Maximal incorporation of ^{13}C into MB was observed from sugars and minimal from amino acids. Strong differences in microbial utilization between LMWOS were observed mainly at day 10. Thus, despite similar initial rapid uptake by microorganisms, further metabolism within microbial cells accounts for the specific fate of C from various LMWOS in soils.

^{13}C from each LMWOS was incorporated into each PLFA. This reflects the ubiquitous utilization of all LMWOS by all functional microbial groups. The preferential incorporation of palmitate into PLFAs reflects its role as a direct precursor for fatty acids. Higher ^{13}C incorporation from alanine and glucose into PLFAs compared to glutamate, ribose and acetate reflects the preferential use of glycolysis-derived substances in the fatty acids synthesis.

Gram-negative bacteria (16:1ω7c and 18:1ω7c) were the most abundant and active in LMWOS utilization. Their high activity corresponds to a high demand for anabolic products, e.g. to dominance of pentose-phosphate pathway, i.e. incorporation of ribose-C into PLFAs. The ^{13}C incorporation from sugars and amino acids in filamentous microorganisms was lower than in all procaryotic groups. However, for carboxylic acids, the incorporation was in the same range (0.1 – 0.2% of the applied carboxylic acid C) as that of gram-positive bacteria. This may reflect the dominance of fungi and other filamentous microorganisms for utilization of acidic and complex organics.

Thus, we showed the divergence of C pathways from LMWOS over the 10 days, despite their similar initial uptake by microorganisms. Consequently, stabilization of C in

soil is mainly connected not with its initial microbial uptake, but with its incorporation into microbial compounds of various stability.

Keywords: low molecular weight organic substances, ^{13}C-labelling, monosaccharides, amino acids, carboxylic acids, compound-specific isotope analysis, PLFAs turnover, soil microorganisms

2.1.1 Introduction

Low molecular weight organic substances (LMWOS) comprise 5–10% of dissolved organic carbon (DOC) in soils (Ryan et al., 2001) and are products of rhizodeposition, above and belowground litter and microbial residue degradation.

Microbial removal of LMWOS from solution in the upper soil horizons appears within minutes (Jones et al., 2004), whereas the half-life of C from LMWOS is much longer, from several hours to months or even decades (van Hees et al., 2005). This occurs due to rapid microbial uptake und further utilisation of LMWOS within the microbial biomass, which out-compete processes of physicochemical sorption of LMWOS at mineral surfaces and their leaching from the soil profile, probably by orders of magnitude (Fischer et al., 2010). Due to the strong link LMWOS dynamics with microbial utilization, the fate of LMWOS should be investigated at natural applied amounts, to avoid any changes in microbial response strategy.

The main compound classes within the LMWOS are amino acids, sugars (mainly monosaccharides) and carboxylic acids (Fischer et al., 2010). Amino acids represent the largest pool of N in soils, mainly bound in proteins. About 30% of N obtained after acid hydrolysis from the protein pool (Stevenson, 1982) and a large portion of N released from soil organic matter (SOM) by enzymes are amino acids (Barraclough, 1997). Amino acid-C half-lives are between 3–45 days and do not strongly differ between field and laboratory conditions (Glanville et al., 2012). The great variability in amino acid utilisation reported in the literature is a consequence of the diversity of metabolic pathways within microbial cells (Apostel et al., 2013) and also can be depend on activity of microorganisms (Jones et al., 2005).

Numerous studies have reported that carbohydrates are the most abundant substance class, amounting for 5–25% of soil organic matter (SOM) (Benzingpurdie, 1980; Cheshire, 1979). Glucose is the most abundant carbohydrate derived either from the decomposition of plant residues (Derrien et al., 2006) or from root exudates (Derrien et al., 2004; Fischer and Kuzyakov, 2010). Half-life of glucose-derived C comprises around 15 days in field conditions (Glanville et al., 2012). On average, 60% of the added glucose is incorporated into cellular compounds (Fischer et al., 2010) Despite glucose supposed to be a ubiquitous substrate, which can be used by nearly all microorganisms (Macura and Kubatova, 1973), specifics of its utilisation in soil is still a topic of discussion (Reischke et al., 2014).

The third most abundant class of LMWOS in soils is carboxylic acids. 80–90% of C from the applied carboxylic acids were decomposed during the first 7 days and only 10–20% of C were incorporated into microbial biomass (MB) (Unteregelsbacher et al., 2012;

van Hees et al., 2002). The utilisation of carboxylic acids is substrate-dependent: acetate has a lower mineralization capacity than citrate and oxalate (van Hees et al., 2002) and citrate can be degraded faster than malate and oxalate (Strom et al., 2001). At the intramolecular level, –COOH groups can be oxidized to CO_2 very rapidly, whereas CH_3-groups are preferentially used for biosynthesis (Dippold and Kuzyakov, 2013; Fischer and Kuzyakov, 2010). Thus, differences in carboxylic acid utilisation can be attributed to their various roles in cell metabolism as well as to their differences in chemical structure.

Studies that simultaneously compare the fate of amino acids, sugars and carboxylic acids are not numerously reported in literature. In most cases studies either consider the microbial utilisation of LMWOS by the entire MB (Glanville et al., 2012; Rousk and Baath, 2011) or focus on contribution of various microbial groups to LMWOS utilization (Apostel et al., 2013; Rinnan and Bååth, 2009; Rinnan et al., 2013).

Information concerning the evaluation of the contribution of functional microbial groups to LMWOS utilization can be obtained by coupling of ^{13}C or ^{14}C labeling with analysis of microbial biomarkers such as amino sugars (Amelung et al., 2001; Engelking et al., 2007; Glaser et al., 2004), phospholipids-derived fatty acids (Frostegard et al., 2011; Zelles, 1997) or DNA-based methods (Ibekwe et al., 2002, Radejewski et al. 2003). Coupling PLFA analysis with ^{13}C-labelling has shown that gram-negative (G-) bacteria are more active in the utilisation of plant C (low or high molecular weight) than gram-positive (G+) bacteria, even if the latter group has a higher PLFA content in soil (Garcia-Pausas and Paterson, 2011; Waldrop and Firestone, 2004). Fungi contribute less to the utilisation of plant-derived C than bacteria (Waldrop and Firestone, 2004). In contrast, the use of ^{13}C pulse-labelling of plants to trace ^{13}C in PLFAs has shown that either fungi (Butler et al., 2003) or G- bacteria (Tian et al., 2013) are the most active in rhizodeposits consumer. Incorporation of ^{13}C from labelled straw into PLFAs has shown that fatty acids such as 16:0; 18:1w9, 18:2w6,9 were more ^{13}C-enriched, whereas other 16:1w5 or 10Me17:0 fatty acids contained negligible amounts of ^{13}C (Williams et al., 2006). Consequently, members of the microbial community are differentially involved in the assimilation of litter- or root-derived C (Williams et al., 2006) and the activity of individual microbial groups appears to depend on the quality of substrate and on environmental conditions such as soil type, season and climatic conditions (Bray et al., 2012). Thus, general principles of LMWOS utilisation by individual groups of bacteria and fungi still remain open.

The second factor controlling LMWOS fate is microbial metabolism: various classes of LMWOS enter different pathways and consequently are utilised differently. Sugars are mainly used directly by the basic glycolysis pathway (Caspi et al., 2008; Keseler et al., 2009), carboxylic acids enter from side branches of the citric acid cycle (Caspi et al.,

2008; Keseler et al., 2009), and amino acids enter glycolysis or the citric acid cycle from individual side branches at different steps (Apostel et al., 2013; Knowles et al., 2010). Thus, we assume that universal substances such as sugars, entering glycolysis directly, will be metabolised very rapidly in comparison to carboxylic acids and amino acids entering the citric acid cycle. However, glycolysis also enables entry into many anabolic pathways, i.e., we hypothesise that sugars are used more for anabolism than carboxylic acids, which enter the oxidising citric acid cycle and can be directly metabolised for energy production. The highest diversity in pathways can be expected for amino acids, because they enter basic metabolism at various steps (Apostel et al., 2013). Since carboxylic acid utilisation is substrate-controlled, we expect divergence in the utilisation of short- and long-chain acids. Because three classes of LMWOS enter metabolic cycles at various points, we hypothesise that their role in the synthesis of cell components such as PLFAs should be different.

Thus, the overall aim of this study was to estimate the short-term transformation of representatives of three main classes of LMWOS: monosaccharides (glucose and ribose), carboxylic acids (acetate and palmitate) and amino acids (alanine and glutamate) under field conditions, coupling ^{13}C substrate labelling with the analysis of specific cell PLFA biomarkers.

2.1.2 Material and Methods

2.1.2.1 Experimental design

The field experiment was carried out at an agricultural field trial in Hohenpölz (49°54' N, 11°08'E, at 500 m a.s.l.). The mean annual temperature was +7°C and mean annual precipitation was 870 mm. The site is cultivated by a rotation of triticale, wheat and barley. The soil is an arable loamy haplic Luvisol (IUSS Working group WRB, 2007) and had the following characteristics: pH 6.6, total C content 1.5%, C/N 10.7, CEC 13 $cmol_C\ kg^{-1}$, clay content 22%.

In August 2010, following harvest of the triticale and spudding of the soil, columns were inserted to a depth of 10 cm and six, ^{13}C uniformly-labelled substances: alanine, glutamate, glucose, ribose, sodium acetate and palmitate were injected into separate columns. The amounts of applied tracer were: alanine 96.3, glutamate 91.6, glucose 93.4, ribose 91.8, acetate 95.8 and palmitate 49.5 µmol $^{13}C\ column^{-1}$. The amount of added C was kept as low as possible and constant for all columns, including the controls, where similar amounts of non-labelled C was applied (0.40–0.77 µg C g $soil^{-1}$). Each column contained 1.5 kg soil. The field experiment had a randomised block design with four

blocks, which represented four field replicates. Preventing rainfall by using a protective roof excluded leaching through the columns for the 10 d of the experiment. Due to the absence of leaching losses and negligible uptake by plants, we assumed that all losses of ^{13}C from soil are connected to LMWOS mineralisation to CO_2. After day three and day 10, separate soil columns were destructively sampled. The soil was removed from the column, weighed and the water content was determined in a subsample. Each soil sample was sieved to 2 mm and divided into two portions. One was cooled (+5 C) for MB analysis and another was stored frozen (-20ºC) until PLFA analysis.

2.1.2.2 Bulk soil $\delta^{13}C$ analysis

The soil for the $\delta^{13}C$ analysis was freeze-dried, milled and $\delta^{13}C$ values of bulk SOM were determined using a Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit coupled via a ConFlo III interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). The amount of incorporated ^{13}C $[C]_{soil}$ in the soil from applied $[C]_{AS}$ was calculated based on the mixing model (Equations 1 and 2), where the C content of the background $[C]_{BG}$ in Equation 1 was substituted according to Equation 2.

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C-BG} + [C]_{AS} \cdot r_{AS} \quad (1)$$

$$[C]_{soil} = [C]_{BG} + [C]_{AS} \quad (2)$$

with:

$[C]_{soil/BG/AS}$	C content of sample / background / applied substances	$(mol \cdot g_{soil}^{-1})$
$r_{soil/BG/AS}$	^{13}C atom% of sample / background / applied substances	(at%)

2.1.2.3 Microbial biomass

Extractable microbial biomass carbon (EMBC) was determined by the fumigation-extraction technique in fresh soil shortly after sampling. Briefly, 15 g fresh soil was placed into glass vials, which were exposed to chloroform for 120 h. After complete removal of chloroform, the EMBC was extracted from the soil with 45 mL 0.05 M K_2SO_4. Organic C was measured with a high-temperature combustion TOC-analyser (Analyser multi N/C 2100, Analytik Jena, Germany) and the EMBC was calculated as the difference between organic C in fumigated and unfumigated samples without further correction factors. After analysis of the organic C concentration, the liquid samples were freeze-dried and $\delta^{13}C$ values of 40 µg subsamples were determined using EA-IRMS (described above). The amount of ^{13}C in fumigated and unfumigated samples was calculated by the mixing

model (Equations 1 and 2). Enrichment of ^{13}C in the EMBC was calculated from the difference of these values.

2.1.2.4 Phospholipid fatty acid analysis

PLFAs extraction and purification

Analysis of PLFAs was performed using the liquid-liquid extraction method of Frostegard et al. (1991) with some modifications. Firstly, 25 µg 19:0-phospholipid (dinonadecanoylglycerol-phosphatidylcholine) was added to each sample as an initial internal standard to calculate the recovery of the phospholipid extraction and purification (Frostegård et al., 1991). Extraction of 6 g soil was performed twice, firstly with 18 mL and secondly with 6 mL of a one-phase mixture of chloroform, methanol and 0.15 M citric acid (1:2:0.8 v/v/v). Six mL chloroform and 6 mL 0.15 M citric acid were added to the extract to generate a two-phase solution and the sample was shaken. The lower phase was then separated and an additional liquid-liquid extraction was performed with 12 mL chloroform. The combined chloroform phase was reduced to 0.5 mL and the phospholipids were separated from the neutral and glycolipids using a solid phase extraction method with activated Silica gel (Silica gel Merck, particle size 0.063–0.200 mm). After transfer to the column, the first fraction (neutral lipids) was eluted with 5 mL chloroform and the second fraction (glycolipids) with 20 mL acetone and the PLFAs were obtained by a four-fold elution with 5 mL methanol. The methanol phase was reduced to 0.5 mL and dried under nitrogen flow.

For the alkaline saponification, 0.5 mL 0.5 M NaOH in dried MeOH (Sigma-Aldrich, assay ≥99.9%) was added and samples were heated to 100°C for 10 min. The free fatty acids were methylated with 0.75 mL BF_3 in methanol (10%, 1.3 M, Fluka) for 15 min at 80°C. For hydrolysing the excess BF_3, 0.5 mL saturated NaCl solution was added. Fatty acid methyl esters (FAMEs) were extracted three times with 1 mL hexane by liquid-liquid extraction. Combined hexane aliquots were dried under N_2, and re-dissolved in 185 µL toluene with the addition of 15 µL of a second internal standard (IS2) (13:0 FAME at 1 mg/mL) (Knapp, 1979).

PLFAs quantification on GC-MS

All PLFA samples were analysed using a Hewlett Packard 5890 gas chromatograph coupled to a mass-selective detector 5971A. A 25 m HP-1 methylpolysiloxane column (internal diameter 0.25 mm, film thickness 0.25 µm) was used. A single 1 µL injection was analysed with an initial temperature of 80ºC, which was then ramped to 164ºC at 10ºC min^{-1}, then to 230ºC at 0.7ºC min^{-1} and finally to 300ºC at 10ºC min^{-1} at a constant

flow rate of 2.4 mL min^{-1}. Peaks were integrated and the ratio to IS2 was calculated for each peak per chromatogram. Substances were quantified using a calibration curve, which was constructed using 29 standard substances as external standards at six different concentrations (see Table 1, supplementary material). All peaks per sample were corrected for the recovery of the first internal standard.

Analysis of $\delta^{13}C$ on GC-C-IRMS

The $^{13}C/^{12}C$ isotope ratios of the single fatty acids were determined by IRMS Delta PlusTM (Thermo Finnigan, Bremen, Germany) coupled to a gas chromatograph (GC; Trace GC 2000, Thermo Finnigan) via a GC-II/III-combustion interface. A 15 m HP-1 methylpolysiloxane column coupled with a 30 m HP-5 (5% Phenyl)-methylpolysiloxane column (both had an internal diameter of 0.25 mm and a film thickness of 0.25 µm) were used. A single 1.5 µL injection was analysed with an initial temperature of 80ºC, which was then ramped to 180ºC at 7ºC min^{-1}, then to 185ºC at 0.3ºC/min followed by holding for 3 min, then to 204ºC at 0.5ºC min^{-1} and holding for 1.5 min, then to 300ºC at 15ºC min^{-1} and holding for 10 min and finally to 80ºC at 50ºC min^{-1} at a constant flow rate of 2 mL min^{-1}. Detailed information about the instrumental set-up is described in (Sauheitl et al., 2005). Online referencing of $\delta^{13}C$ values was performed by the injection of several reference gas pulses directly into the IRMS during measurement (Glaser and Amelung, 2002). Measured $\delta^{13}C$ values of the PLFAs were corrected for the effect of derivative C in analogy to Glaser and Amelung (2002) and referenced on Pee Dee Belemnite by external standards. The enrichment of ^{13}C in single PLFAs was calculated in analogy to bulk soil and MB according to Equations 1 and 2, following a two-pool dilution model (Gearing et al., 1991).

2.1.2.5 Calculations and statistical analysis

All soil, EMB and PLFAs data were tested with nested, mixed effect ANOVA with block as a random factor. Significant differences between individual data points were tested with the HSD *post-hoc* test for unequal N at a 95% significance level. For the repetitive measurements of $\delta^{13}C$ values, a Nalimov outlier test with significance levels of 95% (when four replicates were available) or 99% (when three replicates were available) was performed. PLFAs were classified into corresponding microbial groups by a factor analysis with a principal component extraction method (for factor loadings of the PLFA fingerprint, see Supplementary material, Table 2.). We excluded ubiquitous fatty acids (i.e. unsaturated, straight-chain fatty acids) from the factor analysis and those which were at the detection limit. The classified data were compared with the literature for single fatty

acids, to ascertain functional groups of the microorganism (Zelles, 1997). Incorporation of ^{13}C into individual fatty acids was summed to create incorporation of individual microbial groups. Mixed effect ANOVA with block as a random factor was performed to test for significant differences in ^{13}C incorporation depending on the factors time point and individual substance for each microbial group. Significant differences for individual data points were assessed using the HSD *post-hoc* test for unequal N.

2.1.3 Results

2.1.3.1 Microbial community structure

Grouping of PLFAs occurred by combining the factor analysis of the PLFA contents with results from the literature for PLFA fingerprints of taxonomical microbial groups (Zelles, 1997). Characteristic G+ bacteria fatty acids (i:15:0, , i16:0, i17:0, a17:0, i14:0, a14:0) and G- bacterial fatty acids (16:1w9c, 18:1w7c, 18:1w9c, a15:0) were loaded on different factors and thus, various groups of G+ and G- PLFAs were defined; a15:0 was used to characterise G- bacteria, because it was loaded in one factor with 18:1w9 and also found to be G- by Zelles (1997). 18:1w9c was characterized as G- fatty acid, 1) because it loaded together with bacterial fatty acid and 2) because of it was also used as bacterial biomarker in many other soils where the low content of fungal biomass is expected (Frostegard et al., 2011).

Actinomycetes were characterised by 10Me16:0 and 10Me18:0 (Drenovsky et al., 2004; Fierer et al., 2003; McMahon et al., 2005) , fungi by 18:2w6,9 (Drenovsky et al., 2004; Fierer et al., 2003; McMahon et al., 2005); 16:1w5c cannot be interpreted specifically as it was used for Vesicular Arbuscular Mycorrhiza (VAM) (Frostegard and Baath, 1996; Frostegard et al., 1993; Nordby et al., 1981) but for characterizing G- bacteria (Olsson, 1999). 20:4w6c was used for protozoa (Fierer et al., 2003).

Absolute PLFAs contents (Table 1) showed that the PLFAs fingerprint was dominated by bacterial fatty acids, with a dominance of G- bacteria; G+ bacteria and actinomycete fatty acids were a minor proportion of the total bacterial PLFAs content.

As the community structure was not affected by substrate addition (Table 1), the LMWOS were used by the same microbial community in each treatment. Thus, the differences in LMWOS use can be attributed solely to individual pathways of the substrates or to specific use by individual microbial groups.

Table 1 Absolute and relative abundance (absolute in µg per g and relative in % of total FAs) of the fatty acids of the microbial groups, classified by factor analysis (factor loadings see Supplementary Table 2).

Microbial groups	Abbr.	Fatty acids (FAs)	Fas content (µg g soil-^{1})		Relative abundance FAs (%)	
			day3	day10	day3	day10
Gram negative 1	G-	16:1w7c+ 18:1w7c	9.06±0.77	9.14±0.75	20.08±0.53	20.46±0.89
Gram negative 2	G-	18:1w9c+a15:0	5.96±0.54	6.12±0.43	13.25±0.44	14.07±0.63
Gram positive 1	G+	i16:0+ i17:0+ a17:0	3.26±0.23	2.35±0.2	7.58±0.53	5.21±0.31
Gram positive 2	G+	i15:0	3.38±0.35	3.24±0.3	7.46±0.41	7.06±0.26
Gram positive 3	G+	i14:0+a14:0	0.49±0.11	0.62±0.15	1.04±0.15	1.19±0.23
Actinomycetes	Ac	10Me16:0+10Me18:0	3.72±0.31	3.6±0.26	8.34±0.34	7.96±0.3
VAM	VAM	16:1w5c	1.85±0.16	2.06±0.18	4.11±0.15	4.62±0.19
Fungi	F	18:2w6,9	1.3±0.09	1.31±0.1	3.32±0.12	3.14±0.23
Protozoa	prot	20:4w6c	0.6±0.09	0.53±0.05	1.65±0.19	1.51±0.17
Stress1	Str1	cy17:0	5.01±0.66	5.39±0.52	11.05±1.11	11.71±0.86
Stress2	Str2	cy19:0	1.2±0.08	1.29±0.08	2.81±0.1	2.92±0.11

2.1.3.2 Microbial utilisation of LMWOS

The three classes of LMWOS were utilised differently (Fig. 1); between 55–65% of ^{13}C from both amino acids alanine and glutamate were mineralised. The proportion of ^{13}C from alanine incorporated into EMB decreased from day three to day 10, but this decrease was not significant. In contrast, ^{13}C from glutamate contained in the MB decreased during one week by about eight-fold ($p < 0.05$).

Mineralization of monosaccharides was lower than that of amino acids: 65–80% of the applied tracer was still present in the soil after 10 days. Incorporation of ^{13}C from glucose into EMB was slightly higher than that of ^{13}C-ribose on day three (Fig. 1).

Mineralization of carboxylic acids was about 50% at day 10 and thus, was comparable to that of amino acids. Incorporation of ^{13}C into EMB was higher for acetate than for palmitate, whereas incorporation into PLFAs was highest for palmitate (Fig. 1).

The proportion of ^{13}C from all substances in EMB on day three was in the range from 10–24% of applied ^{13}C and was similar for all substances except glucose and palmitate. In contrast, more ^{13}C from monosaccharides remained in EMB on day 10 compared to amino acids and carboxylic acids. This reflects the universal role of sugars as a C source for microorganisms.

Only 1–3% of the applied ^{13}C from the five LMWOS (except palmitate) was used for the synthesis of cell membrane components, evaluated by ^{13}C in total PLFA. Palmitate-C was the only exception: more than half of its incorporation into EMB was recovered in the PLFAs after 3 days and half on day 10 (Fig. 1). Although PLFAs comprised only about 5% of the EMBC, they incorporated a comparatively high percentage of LMWOS-C, namely, 10% of the microbially used C.

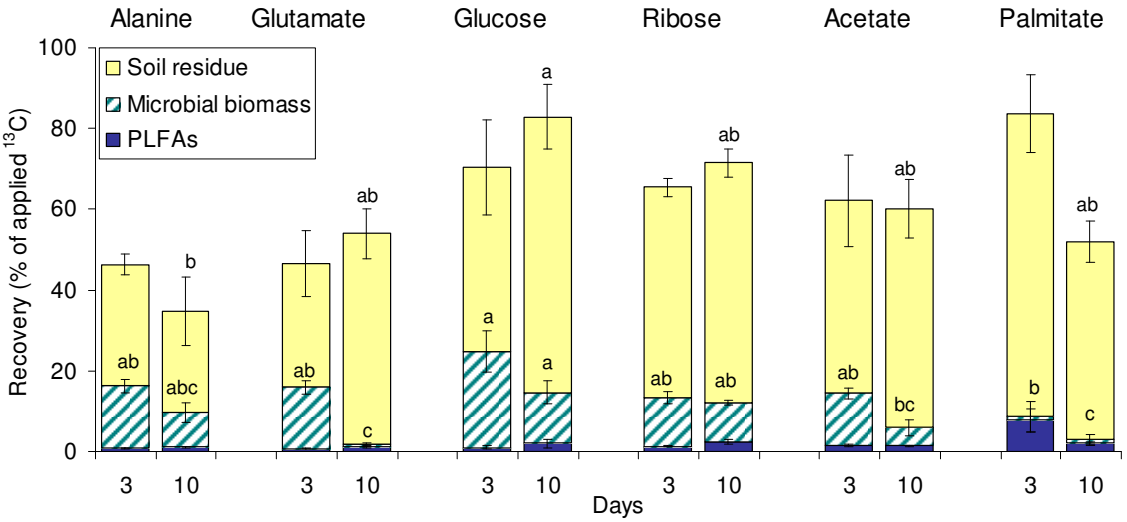

Fig. 1 *^{13}C recovery (in % of applied ^{13}C) from six LMWOS in soil, microbial biomass and PLFAs, 3 and 10 days after application. Significant differences ($p < 0.05$) for ^{13}C in soil were observed only on day 10 and are shown by letters above error bars.*

2.1.3.3 Utilisation of LMWOS by functional microbial groups

Bacterial groups played a greater role than eukaryotes in the utilisation of amino acids (Fig. 2, top). On day three, G- and G+ bacteria were the most active groups in incorporating ^{13}C from alanine and glutamate. Among the potentially eukaryotic PLFA, 16:1w5 had highest amino acid ^{13}C incorporation. Actinomycetes, G-1 group bacteria and 16:1w5 PLFA showed an absolute increase in ^{13}C incorporation from day 3 to day 10 for alanine and glutamate 13C incorporation. Furthermore, none of these PLFAs showed a higher abundance at day 10 compared to day three, i.e. the increase was not reasoned in an increase of this microbial group.

In general, alanine-C was preferred over glutamate-C for PLFAs synthesis, but for the majority of microbial groups, this difference was not significant. The G- group 2 and G+ group 1 bacteria and fungi showed similar ^{13}C incorporation on day three for both amino acids. In contrast, alanine ^{13}C replacement at day 10 was significantly higher than glutamate for each of these groups (Fig. 2, bottom).

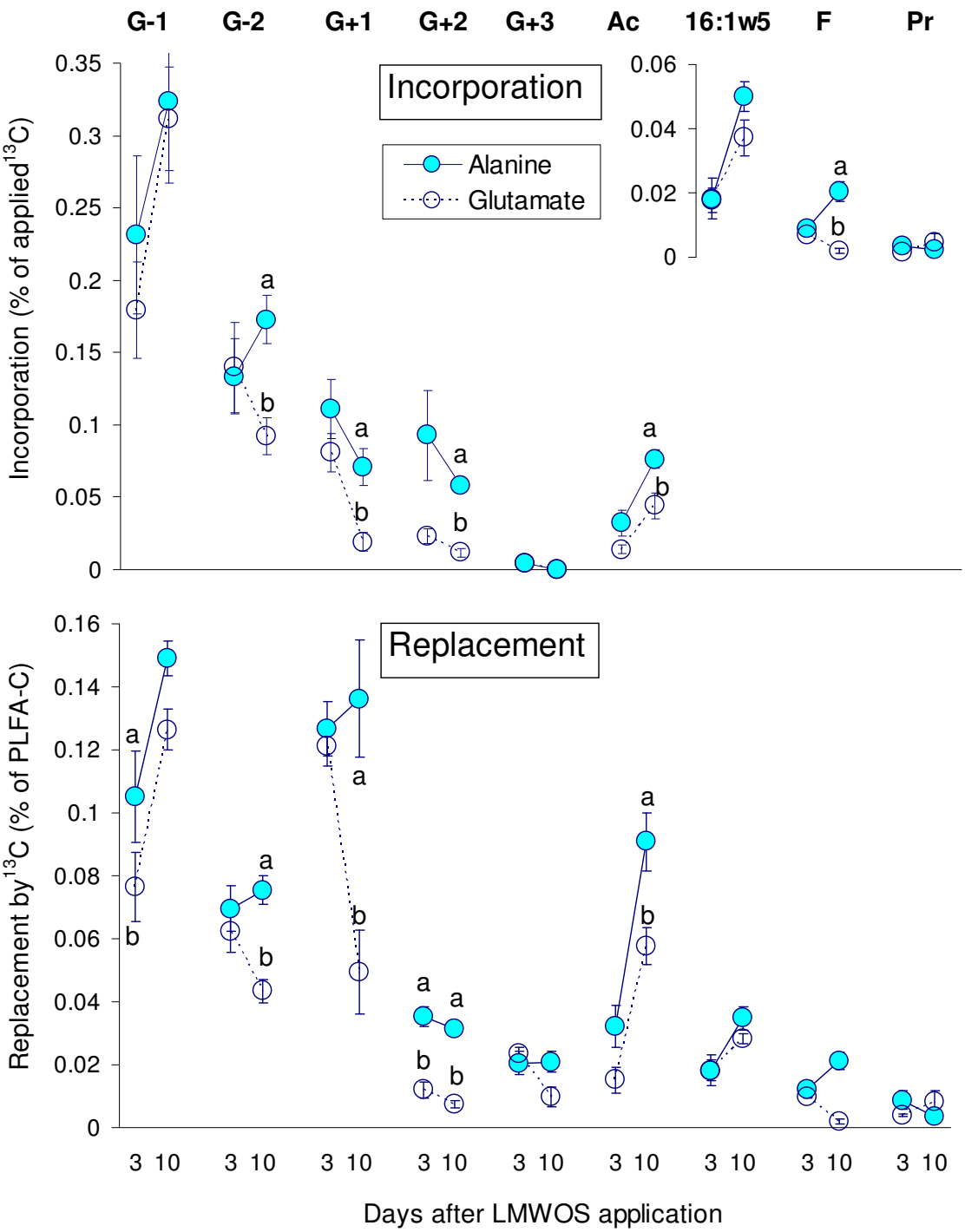

Fig. 2 *^{13}C incorporation from both amino acids (in % of applied ^{13}C) into PLFAs (top) and percent of ^{13}C replacement (in % of PLFA-C) (bottom) of microbial groups 3 and 10 days after alanine and glutamate application. Letters reflect significant differences between alanine and glutamate uptake into microbial groups.*

Utilisation of ^{13}C from sugars for PLFAs formation showed different trends in bacterial and fungal groups and much higher absolute ^{13}C incorporation compared to amino acids (Fig. 3, top). Between 0.01 and 0.70% of initially applied sugars ^{13}C was found in various taxonomic groups after three days, whereas only 0.001–0.25% was recovered

from amino acids. The incorporation of ^{13}C from sugars into all microbial groups increased or remained constant between days three and 10. All bacterial species used glucose-C more efficiently than ribose-C except G- group 1, which preferred ribose. Among the filamentous microorganisms, fungi did not differ from 16:1w5 in glucose-^{13}C incorporation into PLFAs, but fungi used more ^{13}C from ribose than 16:1w5. In general, the microbial specialisation for individual monosaccharides as building blocks for PLFAs was visible within bacterial but not within eukaryotic groups.

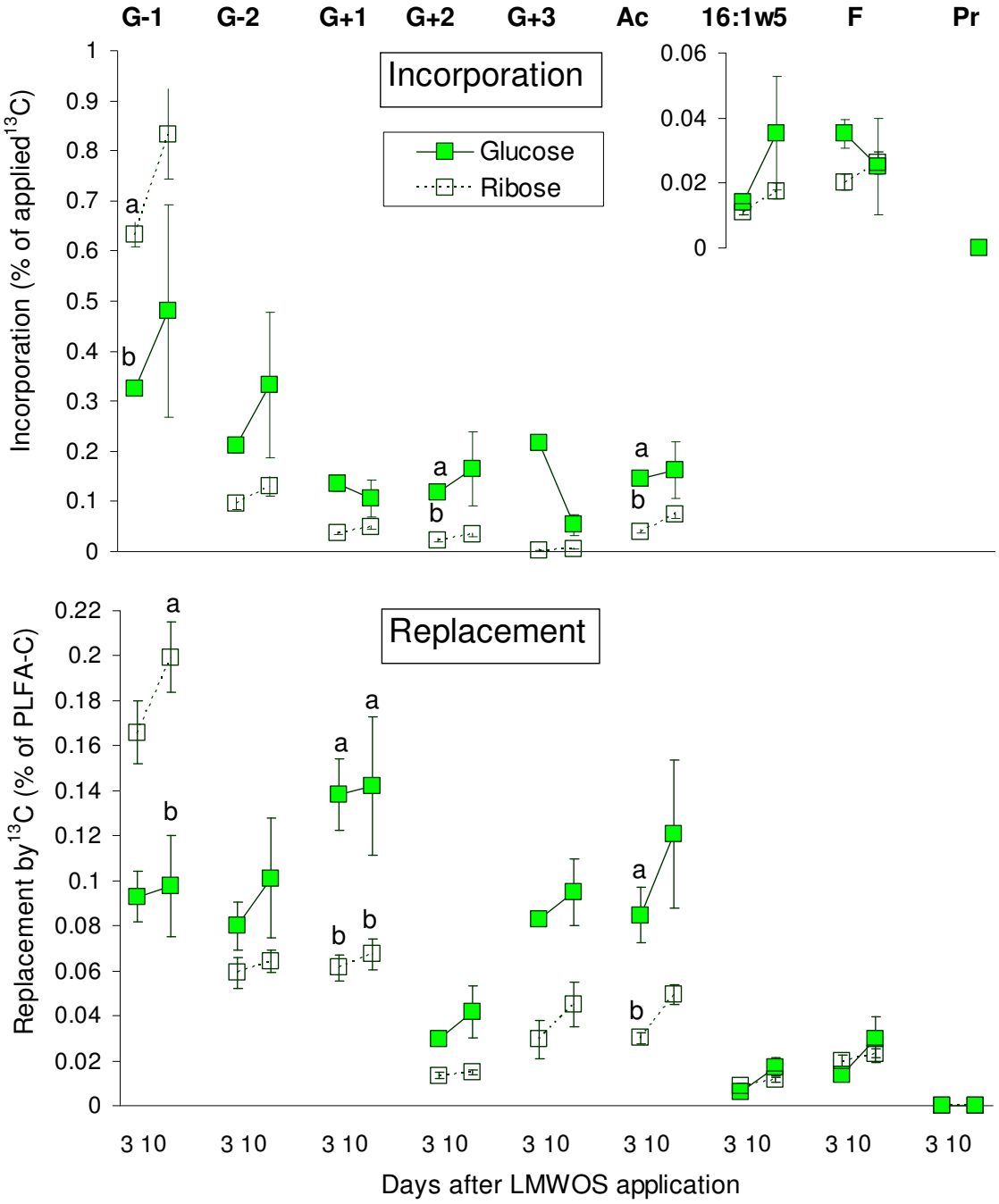

Fig. 3 *^{13}C incorporation from both monosaccharides (in % of applied ^{13}C) into PLFAs (top) and percent of ^{13}C replacement (in % of PLFA-C) (bottom) of microbial groups 3 and 10 days after glucose and ribose application. Letters reflect significant differences between glucose and ribose uptake into microbial groups*

Incorporation of ^{13}C from both carboxylic acids into PLFAs of G- bacteria group 1 was higher than from other LMWOS (Fig. 4, top). Other bacterial groups used ^{13}C from carboxylic acids less efficiently than ^{13}C from sugars and amino acids for PLFAs synthesis. Filamentous microorganisms (actinomycetes and fungi, but also 16:1w5) exceeded the prokaryotic groups of G+ bacteria in incorporation of ^{13}C from the most complex substrate, palmitate, into PLFAs.

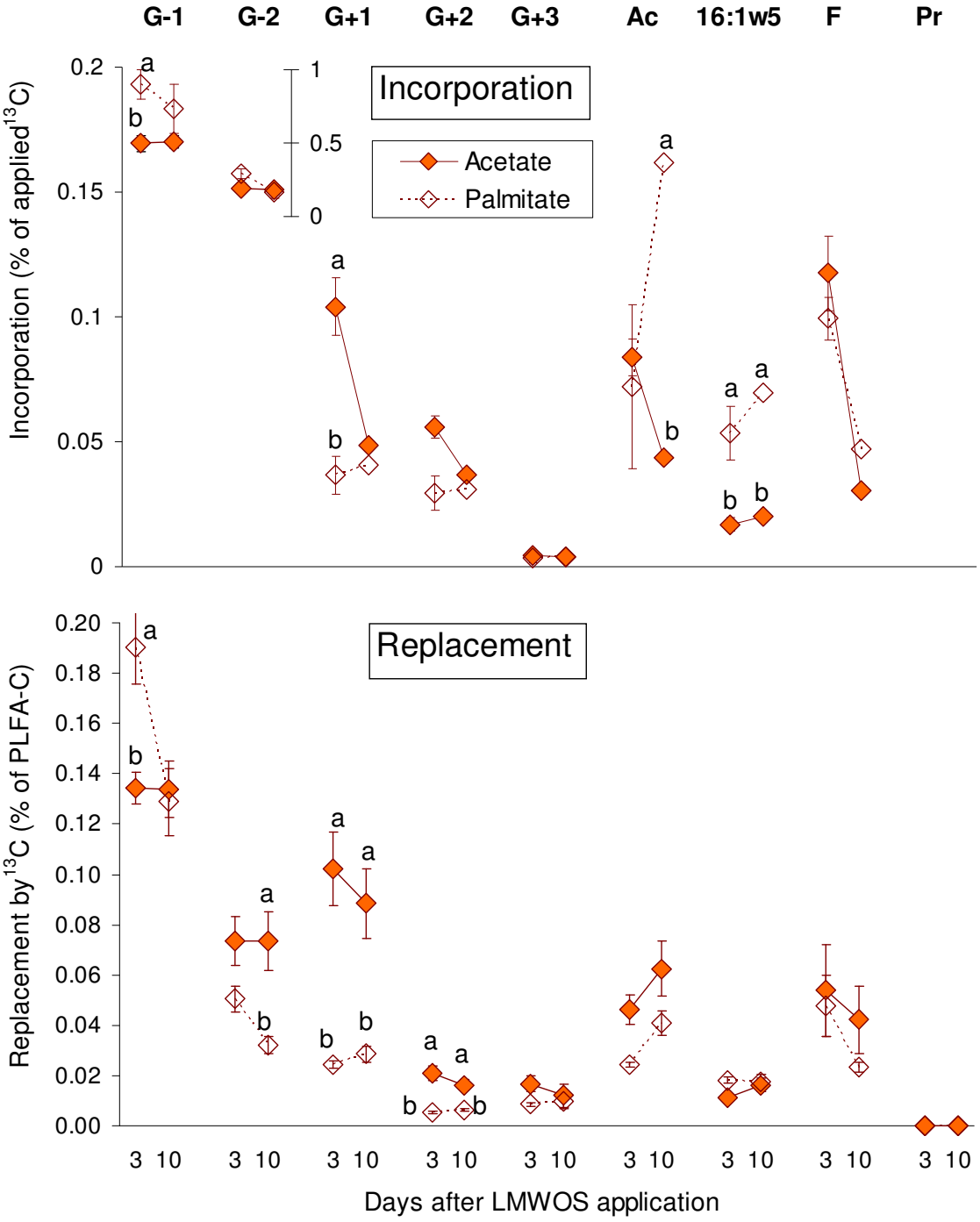

Fig. 4 *^{13}C incorporation from both carboxylic acids (in % of applied ^{13}C) into PLFAs (top) and percent of ^{13}C replacement (in % of PLFA-C) (bottom) of microbial groups 3 and 10 days after acetate and palmitate application. Letters reflect significant differences between acetate and palmitate uptake into microbial groups.*

In general, incorporation of LMWOS-C in bacterial species was higher than that in eukaryotes.

2.1.4 Discussion

2.1.4.1 Incorporation of LMWOS into SOM and microbial biomass

Amino acids

The mineralization of alanine and glutamate in our experiment was similar to literature data (Jones et al., 2005) and less than 50% of applied ^{13}C remained in the soil after 10 days. The half-life of alanine and glutamate-derived C reported for field conditions was 18 and 3 days, respectively (Glanville et al., 2012). This is much longer than in our experiment and we observed a similar mineralization of glutamate and alanine C within 10 days. These contrasting results might be attributable to differences in total microbial activity or community structure (Jones et al., 2005) in the studied soils as well as due to methodological differences. Similar ^{13}C amounts from glutamate and alanine remaining in the soil on day 10 reflected a similar mineralization of these amino acids and corresponds to similar microbial decomposition of differently charged amino acids (Jones and Hodge, 1999).

The high amount of ^{13}C incorporated into the EMB pool at day three (Fig. 1) corresponded with the rapid and efficient uptake of free amino acids as intact molecules (Dippold and Kuzyakov, 2013; Geisseler et al., 2010; Jones and Hodge, 1999). After uptake, amino acids can either be oxidised for energy production, directly incorporated into proteins (Geisseler et al., 2010) or used in other metabolic pathways (Fig. 5) (Dippold and Kuzyakov, 2013; Knowles et al., 2010). The incorporation of alanine into EMB was higher than for glutamate on day 10, showing the more rapid mineralisation of glutamate C. Similarly, glutamate was utilised more rapidly than glycine and lysine over a broad concentration range (Jones and Hodge, 1999). This corresponds to the different entry point of these amino acids into metabolism (Knowles et al., 2010). Alanine enters the basic cellular metabolism at the connecting step between glycolysis and the citric acid cycle (Apostel et al., 2013; Caspi et al., 2008). Thus, it is easily distributed throughout all anabolic pathways for the synthesis of cell components, e.g., glyconeogenesis, protein synthesis, fatty acid synthesis and ribonucleotide synthesis (Fig. 5). In contrast, glutamate directly enters the citric acid cycle as oxoglutarate (Knowles et al., 2010). This demands energy for glyconeogenesis and therefore, fatty acid synthesis pathways will not be used if other more appropriate substrates are available. Thus, alanine C is preferentially incorporated into the more stable components of microbial cells – the cell walls and

the membranes – compared to glutamate. In contrast, glutamate plays a central role in the amino acid cycle, and oxoglutarate produced from glutamate by transamination will be rapidly decompose to CO_2 (Vinolas et al., 2001).

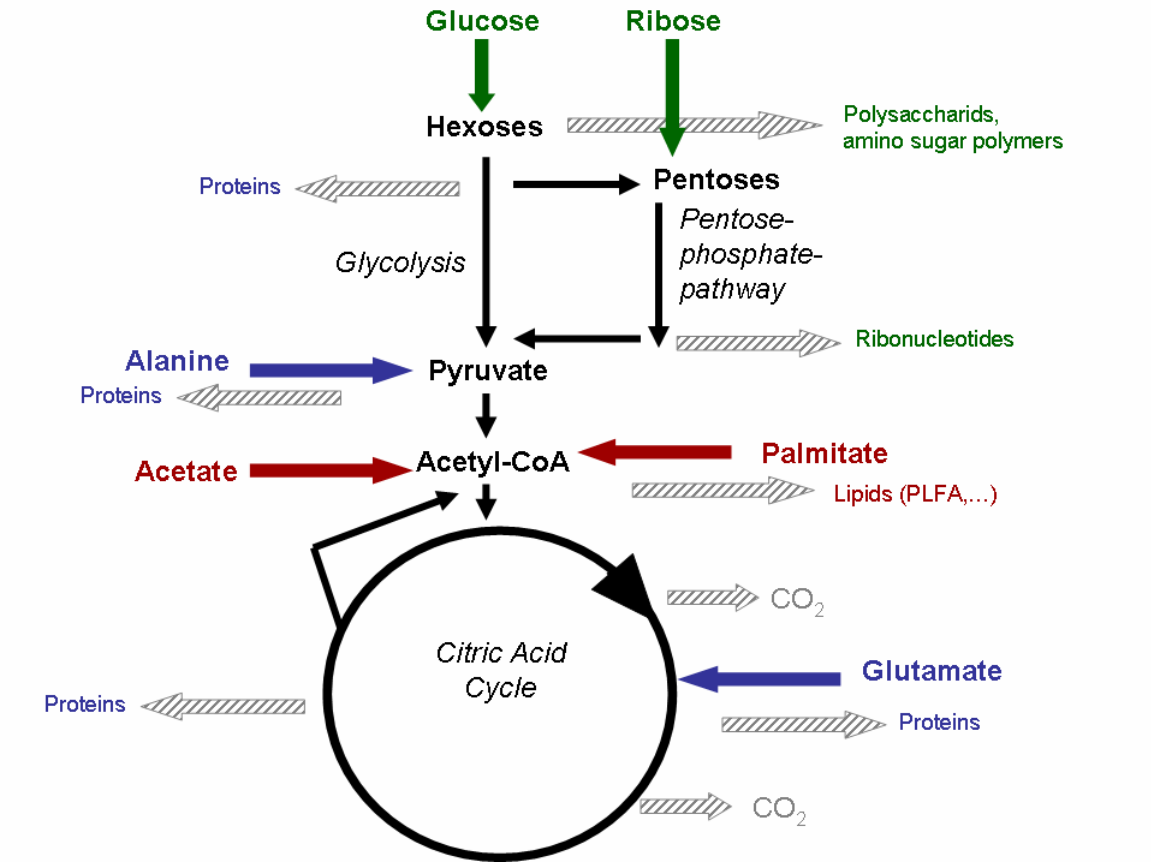

Fig. 5 Primary metabolic pathways of the six representatives of three LMWOS classes (amino acids (blue), sugars (green) and carboxylic acids (red)). Thick arrows reflect the entering points of LMWOS in the metabolic pathways; black fine arrows show the basic C metabolism and shaded arrows reflect anabolic pathways for formation of cellular compounds.

Sugars

The half-life of glucose-C in our experiment (25% mineralized within 10 days) is within the range of previous studies: Glanville et al. (2012) reported a decomposition of 50% of glucose-C after 20 days, Saggar et al. (1999) measured a glucose-C decomposition of 51–66% within 35 days and Schneckenberger et al. (2008) observed a mineralisation of 26–44% of ^{14}C from glucose within 22 days. The incorporation of a significant proportion of applied 13C from sugars into EMB in our experiment is in agreement with the model of short-term glucose utilisation (Nguyen and Guckert, 2001). In this model, glucose taken up from solution is initially allocated to an intermediate pool and thereafter can be respired or used as a structural component. Thus, due to the demand for cellular products, glucose C will preferentially be transferred to anabolic pathways rather than be oxidised for energy production.

The lower mineralisation and incorporation of pentose compared to hexose on day three corresponds with its slower uptake rate, as it is a less common soil monosaccharide than glucose. The metabolisation of pentoses occurs mainly via the pentose-phosphate-pathway, leading to incorporation into various cell components such as DNA or other ribonucleotides (Fig. 5). Both phenomena explain the lower utilisation of ribose compared to glucose. Preferential incorporation of ribose into biosynthetic ribonucleotide products has yet to be proven by substance-specific analysis, e.g., by stable isotope probing of DNA (Radajewski et al., 2000). Indeed, ribose mineralisation and incorporation into the EMB on day 10 were nearly the same as for glucose. This confirms that the hexose and pentose pathways are closely linked and that C from both monosaccharides is transferred within these pathways towards biosynthetic pathways according to the C demand of cells.

Carboxylic acids

Similar to amino acids and monosaccharides, the rapid uptake of acetate outcompetes its physicochemical sorption in soils (Fischer et al., 2010). Within the microbial biomass, acetate C can be subjected to “arrest metabolism” and stored in cells before use (Fischer and Kuzyakov, 2010). Acetate can also be transformed into carbohydrates, amino acids (Sorensen and Paul, 1971) and other cell components and thus is fixed in diverse microbial products. Incorporation of acetate into EMB was less than that of sugars, which confirms that acetate was used for respiration (ca. 80–90%) rather than for new cell biomass production (Jones and Edwards, 1998; van Hees et al., 2002). This occurs due to the oxidation of a high proportion of acetate C into the citric acid cycle. Furthermore, acetate must be activated prior to incorporation into the key metabolic pathways (van Hees et al., 2002). The transformation of acetate to anabolic products is thus unfavorable, as long as microorganisms have access to more freely available substrates. An exception to this pathway, however, is fatty acid synthesis, where acetate is a direct precursor.

Palmitate is an anion of a short-chain fatty acid, the most dominant fatty acid in bacteria and fungi (Lawlor et al., 2000) and is a precursor for the synthesis of more complex fatty acids. Due to its high molecular weight and long aliphatic chain, we hypothesised that its decomposition is much slower than the decomposition of short chain carboxylic acids such as acetate. However, this hypothesis was not confirmed in our experiments. Previous studies showed that the degradation of palmitate was more rapid than that of similar or longer fatty acids (Moucawi et al., 1981). It was estimated that 41% of oleic and 31% of stearic acids were decomposed within four weeks (Moucawi et al., 1981), whereas 50% of palmitate decomposed within 10 days (Fig. 1).

In contrast to net mineralization, the incorporation of ^{13}C from palmitate into EMB was the lowest (Fig. 1). This might be due to hydrophobic interactions of palmitate with SOM that led to the lower uptake into microbial biomass compared to other LMWOS. However, if taken up, it was preferentially incorporated in PLFAs, but not used for the synthesis of other microbial compounds. Incorporation into PLFAs was higher than for any other LMWOS, which is in accordance with their direct precursor role for PLFA formation.

In general, our results reflect that the uptake and utilisation of six LMWOS within 3 days was quite similar and comparable with the literature (Glanville et al., 2012). As far as substance-specific differences in incorporation into EMB were the most visible only at day 10 (Supplementary Table 3.), when the total amount of incorporated C decreased, the long-term fate of LMWOS-C in soils are caused by the metabolic pathways of LMWOS classes within microbial cells and not by rapid LMWOS uptake during the first few days. Therefore, we can consider the medium-term divergence of C depending on its initial form that entered the soil.

2.1.4.2 Microbial community composition

The constant composition of PLFAs (Table 1) after the addition of very low amount of LMWOS-C shows that microbial community structure was under steady-state conditions (Blagodatskaya et al., 2007, , 2009). This corresponds to other studies with similar amounts of applied C (Brant et al., 2006) and leads to ^{13}C incorporation into microbial biomass and individual microbial groups reflected the typical utilization of these substances under natural soil conditions – i.e. a microorganisms under maintenance metabolism.

The main classes of decomposers for the six substances were G- and G+ bacteria. G- bacteria are very common in the rhizosphere, which reflects their preference for LMWOS common in rhizosphere hotspots. In contrast, G+ bacteria are abundant in bulk soil (Soderberg et al., 2004).

The soil environment of this study, with aerobic conditions, a neutral soil pH as well as the above- and belowground litter remaining after the harvest, provide optimum conditions for the development of actinomycetes, which is assumed to be important for the primary degradation of recalcitrant SOM (McCarthy and Williams, 1992). In contrast, present environmental conditions and loadings of small amounts of complex substrates did not supported fungal growth (Reischke et al., 2014), which explains their low abundance as well as their low activity in LMWOS utilization in our experiment.

We detected relevant amounts of 16:1w5 fatty acid, which can be used to characterized the VAM fungi or gram-negative bacteria (Olsson, 1999; Zelles, 1997). Results of the factor analysis do not attribute the 16:1w5 to the group 1 or group 2 of G- bacteria, moreover they were loaded up similarly with fungi. Second the VAM are usually abundant in soils, because they form a symbiotic relationship with up to 80% of land plants (Madan et al., 2002). Third 16:1w5 behaved similarly with fungi in utilization of investigated LMWOS (especially for carboxylic acids). All these factors support the interpretation that 16:1w5 reflect VAM in this soil. For ensuring the interpretation of 16:1w5 as VAM fatty acid, the simultaneous analysis of 16:1w5 in PLFAs and neutral lipids should be done. As far as we didn't measure this in our experiment, the interpretation of 16:1w5 as VAM is not assured.

2.1.4.3 Incorporation of LMWOS into PLFAs

Amino acids

The observed dominant role of bacteria in amino acid utilisation is in agreement with previous studies, which found that the relative incorporation of ^{13}C from glutamate (the added amount was 50 µg C g^{-1} soil) into bacteria was high, whereas incorporation into fungi was significantly lower (Brant et al., 2006; Rinnan and Bååth, 2009). Actinomycetes utilised amino acids in a forest soil, similar to G+ bacteria (Brant et al., 2006). Our results with agricultural soil support those of Brant et al. (2006) and confirm the theory that amino acid turnover is mainly controlled by microbial activity and not by microbial community structure (Jones et al., 2005).

We demonstrated a preferred incorporation of alanine than glutamate into PLFAs and a higher replacement of PLFA-C by alanine ^{13}C than glutamate ^{13}C (Fig. 2, bottom). Despite a similar uptake of alanine and glutamate into EMB, their contrasting incorporation into PLFAs shows differences in intracellular metabolisation: alanine C is directly converted to acetyl-CoA, the direct precursor of fatty acids, whereas complex energy-consuming pathways are needed to transform glutamate C into acetyl-CoA (Fig. 5). In addition, the formation of acetyl-CoA from alanine causes a loss of one-third of its C backbone compared to a three-fifths loss of C from glutamate if converted to acetyl-CoA (Apostel et al., 2013). This also contributes to the lower incorporation of glutamate-C into PLFAs. Thus, our results confirm those of previous studies on metabolic tracing, showing that intracellular metabolisation is the master process that determines the fate of amino acid C in soils (Apostel et al., 2013; Dippold and Kuzyakov, 2013; Knowles et al., 2010).

Sugars

The preference of bacteria for glucose utilization compared to fungi, corresponds with the dominance of bacteria within the soil microbial community, but can also be attributed to the more efficient uptake of LMWOS by bacteria (Moore et al., 2005) especially if low concentrations of LMWOS are applied (Reischke et al., 2014). This was revealed previously by a higher relative glucose incorporation into bacteria (Brant et al., 2006). The preferential incorporation of sucrose into bacterial fatty acids (16:1ω7 and 18:1ω7) was also reported by Nottingham et al. (2009), who noted the importance of the 16:1w7 biomarker in the control of priming effects. Thus, G- bacteria (corresponding to our G-1 group) represent a group whose growth is based on easily available substrates and which are the most competitive for LMWOS in many ecosystems (Treonis et al., 2004). Hence, the majority of studies show that bacteria are the most relevant group for the uptake and degradation of easily available substrates e.g., following the initial stage of litter decomposition, whereas fungi decompose more complex substrates that remain at later stages (Moore-Kucera and Dick, 2008). However, studies based on nuclear magnetic resonance showed the significant utilization of ^{13}C from glucose for the formation of unsaturated triacylglycerols, typical storage metabolites of eukaryotes (Lundberg et al., 2001). Based on these results, it has been suggested that fungi are the most active organisms in glucose degradation. Interpretations in our study are based on membrane lipids – a substance class whose structure, function and biosynthetic pathways are similar between many prokaryotes and eukaryotes. Thus, a comparison of the utilization pattern is probably more reliable if functional and biosynthetically comparable compounds are included (Rinnan and Bååth, 2009).

The uptake pattern of ribose was relatively similar to that of glucose (Fig. 3, top), with predominant utilization by G- bacteria. This primary incorporation of pentose by G- bacteria was also characterised by 13C-xylose utilization (Waldrop and Firestone, 2004) a similar structure and thus presumably a similar uptake and metabolism. The high percentage incorporation of ribose ^{13}C into EMB compared to the relatively small amount of ^{13}C detected in PLFAs can be explained by the use of ribose for the synthesis of other cell polymers. After modification in the pentose-phosphate pathway and phosphorylation, ribose is likely to become a subunit for ribonucleotides and less used for fatty acid biosynthesis (Fig. 5). Ribonucleotides are extracted after chloroform fumigation and this can explain high ^{13}C incorporation in the microbial pool. Only G-1, the most active group in LMWOS utilization, incorporated high amounts of ribose into PLFAs, i.e. pentose-phosphate intermediates. This accounts for high intracellular turnover of this most active microbial group.

Carboxylic acids

Acetate is a ubiquitous substrate in soil: it is the main product of lipid degradation, the main substance of plant litter anaerobic decomposition (Reith et al., 2002), present at high concentrations in slurry (Laughlin et al., 2009) and is known as direct precursor role for the formation of fatty acids.

The amount of acetate incorporated into membranes of G- bacteria 1 (16:1w7c and 18:1w7c) was 5-fold higher than for most of the other PLFAs (Fig. 4, top). Similar to the other LMWOS, this might be a result of their higher abundance within the microbial community and their rapid uptake of LMWOS. A similar high recovery of 13C from acetate in 16:1w7c and 18:1w7c PLFAs was reported in experiments with anoxic brackish sediment (Boschker et al., 2001). Our experiment with well-aerated agricultural soil showed that the high competitiveness of these G- bacteria for acetate does not depend on the oxygen supply. In addition, experiments with sediment and groundwater samples showed that only few genera were involved in acetate degradation (Pombo et al., 2005). The 16:1w7c PLFA has been suggested as a biomarker for acetate-oxidising sulphate-reducing bacteria. This anaerobic degradation can only occur in O_2-deficient microhabitats such as aggregate cores and is very unlikely to play a relevant role in freshly tilled soil.

Fatty acids that characterise G+ bacteria (such as i15:0, i16:0, i17:0, a17:0) were also enriched, but to a much lower degree than G- bacterial fatty acids (Fig. 4, top). Similar results were obtained with anoxic brackish sediments, where 10Me16:0, cy17:0, i15:0 and a15:0 PLFAs were enriched in ^{13}C from acetate and were related to sulphate-reducing bacteria (Boschker et al., 2001). Carboxylic acids were the only substrate class where fungal uptake and incorporation could compete with those of prokaryotic, G+ groups. Thus, although fungi are less competitive for the most LMWOS, they prefer acidic substrates within the LMWOS (Rinnan and Bååth, 2009). This correlates with their preference for acid soil conditions, where acidic (non-neutralised), more complex substrates, dominate (Haider, 1996).

A comparison of palmitate and acetate utilization in soils is important because acetate is a direct microbial precursor for palmitate synthesis. There are three pathways for the incorporation of palmitate into phospholipids: 1) partial step-by-step degradation of C2-units without total breakdown of palmitate can occur and subsequently, only parts of the molecule are used for further biosynthesis (Rhead et al., 1971); 2) the resynthesis pathway includes the complete degradation of the molecule to acetyl-CoA and the following synthesis of new fatty acids by a series of enzymatic reactions (Rhead et al., 1971); 3) the alternative is the utilization of palmitate directly without further transformation, because it is the most abundant fatty acids in microorganisms and might be slightly modified by elongation or desaturation. According to the first pathway, palmitate should have a

similar behaviour to acetate at least concerning the relative fate of ^{13}C-PLFA/^{13}C-EMB ratios. Indeed, the ^{13}C-PLFA/^{13}C-EMB ratio was the highest for palmitate than for any other investigated LMWOS. This indicates at least partial use of palmitate without breakdown for PLFA synthesis (the third pathway), whereas complete or partial breakdown pathways have a lower importance.

Comparable to the other LMWOS, there was a higher incorporation of ^{13}C from palmitate in bacteria than in fungi (Fig. 4, top) and the most enriched were G- bacterial PLFAs (Fig. 4, top). This shows the high activity and cellular turnover of this bacterial group (Fig. 4, bottom). Notably, palmitate ^{13}C incorporation into actinomycetes (10Me16:0, 10Me18:0) was higher than acetate ^{13}C. Comparison of single cell G+ bacteria with their corresponding filamentous microorganisms, the actinomycetes, shows the preference of osmotrophic groups for highly water-soluble, simple substrates. In contrast, filamentous organisms respond slower, but with a similar incorporation of less water-soluble, more complex carboxylic acids, such as palmitate. Thus, a lack of mobility and defined filamentous organisation of microorganisms leads to a lowered competitiveness for simple LMWOS and a slow turnover within microbial biomass (Rousk and Baath, 2007). In contrast, if the focus is set on long-term C uptake, filamentous organisms show a higher incorporation of C from complex, less-available substrates (Brant et al., 2006).

2.1.5 Conclusion

This study compared and revealed the role of three classes of LMWOS – amino acids, sugars and carboxylic acids in short-term microbial utilisation in soil. The similar LMWOS uptake at day 3 but differences in microbial incorporation at day 10 reflect that instead of initial uptake, the intracellular metabolization accounted for the observed differences in LMWOS fate in soils.

Amino acids were taken up by soil microorganisms in similar amounts on day 3, but much less glutamate than alanine remained in EMB on day 10. This reflects that substrates with direct incorporation into the oxidising citric acid cycle, such as glutamate, are preferentially oxidised for energy production compared to alanine, which enters glycolysis. The high and rapid glucose uptake by microbial biomass is connected with the fact that glucose is the most abundant sugar in the soils and ribose is taken up more slowly. More sugar ^{13}C was incorporated into MB than from amino acids and carboxylic acids, which reflects the preference of glycolysis substrates for anabolic utilization compared to catabolism. Higher amounts of ^{13}C from acetate were incorporated into EMB than palmitate. For carboxylic acids, the ^{13}C in EMB declined by a factor of two from day 3 to day

10, also reflecting the preferred catabolic oxidation of substances entering the citric acid cycle.

0.8 - 2% of the initial applied ^{13}C were used for the formation of cell membranes i.e., for total PLFAs with no differences between amino acids, sugars and acetate. 8% of ^{13}C from palmitate detected in PLFAs was a result of its direct use as a precursor for PLFA formation.

The PLFAs of individual microbial groups showed bacteria (especially G-) were highly competitive for LMWOS uptake. The contribution of fungi to LMWOS-C utilization was less than that of bacteria, due to the low amount of fungi as well as their low competitiveness for water-soluble, easily available and easily degradable substances. Only in utilization of acidic substrates like acetate or palmitate fungi can compete with some bacterial groups. In general, more complex substrates such as palmitate are preferred by filamentous microorganisms. Thus, metabolisation and C partitioning within microbial cells between catabolism and anabolism affect the fate of individual LMWOS in soil. This can be attributed to their entering steps of basic C metabolism and consequently, to their individual metabolic pathways.

Further studies on the metabolic pathways of LMWOS, based on tools such as position-specific (Dijkstra et al., 2011; Dippold and Kuzyakov, 2013; Apostel et al., 2013), and multiple-isotope labeling (Fokin et al., 1994, (Knowles et al., 2010) are necessary. Studies on the long-term fate of LMWOS-C in soil should focus on microbial metabolism and products formed from this highly available C source in soil.

Acknowledgements

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG KU 1184 19/1). Thanks are extended to MolTer and DAAD, which provided a fellowship for A. Gunina. The authors are grateful to Stefanie Bösel, a technical staff member of the Department of Soil Biochemistry, Institute of Agricultural and Nutritional Science, Martin-Luther University Halle-Wittenberg for performing the bulk isotope measurements.

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Supplementary Data

Supplementary Table A1: Fatty acids in the external standard

FA-type	Name	Common name	Abbreviation	Retention
Saturated	Tetradecanoic acid	Myristic acid	14:00	150
	Pentadecanoic acid	-	15:00	355
	Hexadecanoic acid	Palmitic acid	16:00	630
	Heptadecanoic acid	Margaric acid	17:00	1010
	Octadecanioc acid	Stearic acid	18:00	1520
	Eicosanoic acid	Arachidic acid	20:00	2930
Branched	11-Methyltridecanoic acid	Anteisomyristic acid	a14:0	100
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	90
	12-Methyltetradecanoic acid	12-Methylmyristic acid	a15:0	290
	13-Methyltetradecanoic acid	13-Methylmyristic acid	i15:0	270
	13-Methylpentadecanoic acid	Anteisopalmitic acid	a16:0	545
	14-Methylpentadecanoic acid	Isopalmitic acid	i16:0	525
	14-Methylhexadecanoic acid	14-Methylpalmitic acid	a17:0	890
	15-Methylhexadecan acid	15-Methylpalmitic acid	i17:0	860
Cyclo-propane	cis-9,10-Methylenhexadecanioc acid	cis-9,10-Methylpalmitic acid	cy17:0	940
	cis-9,10-Methylenoctadecanoic acid	Dihydrosterculic acid	cy19:0	2025
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic acid	10Me16:0	780
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	1745
Mono-unsaturated	9-Tetradecaeoic acid	Myristoleic acid	14:1w5c	130
	cis-11-Hexadecenoic acid	-	16:1w5c	595
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1w7c	570
	cis-Octadecenoic acid	Cis-Vaccenic acid	18:1w7c	1400
	cis-9-Octadecenoic acid	Oleic acid	18:1w9c	1375
	11-Eicosenoic acid	Eicosenoic acid	20:1w9c	2700
Poly-unsaturated	cis,cis-9,12-Octadecadienoic	Linoleic acid	18:2w6,9	1320
	6,9,12-Octadecatrienoic acid	g-linolenic acid	18:3w6,9,12	1355
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4w6	2320

*for 45 m (30 m DB-5 MS + 15 m DB-1 MS) ± 0.5 m column lengths, deviations of ± 15 s possible

Supplementary Table A1: Results of factor analysis: Factor loadings and grouping of fatty acids derived from factor loadings and PLFAs literature.

Fas	Factor 1	Factor 2	Factor 3	Factor 4	Microbial group
i16:0	**-0.83**	-0.03	0.04	0.09	Gram positive 1
i17:0	**-0.91**	0.20	0.05	-0.27	
a17:0	**-0.91**	-0.04	0.08	-0.26	
10Me16:0	**-0.89**	0.11	-0.11	0.01	Actinomycetes
10Me18:0	**-0.79**	0.09	0.46	-0.21	
16:1w7c	0.28	**-0.81**	0.05	0.06	Gram negative 1
18:1w7c	0.47	**-0.72**	0.21	-0.05	
i15:0	0.10	**-0.75**	-0.13	0.30	Gram positive 2
16:1w5c	-0.18	**0.63**	-0.46	-0.10	VAM
18:2w6,9	0.11	**0.71**	0.45	-0.21	Fungi
20:4w6c	0.36	**0.69**	-0.39	-0.24	Protozoa
a15:0	-0.17	0.21	**-0.81**	0.39	Gram negative 2
18:1w9c	0.50	-0.20	**-0.65**	-0.34	
i14:0	0.15	-0.35	0.07	**0.83**	Gram positive 3
a14:0	0.19	-0.11	-0.03	**0.93**	

Supplementary Table A3: Nested ANOVA between classes of LMWOS and single substances nested in class of LMWOS for soil, microbial biomass and PLFAs. Degrees of freedom (df), values (F) and significance level (p) are shown for the two time points.

		Soil			MB			PLFAs		
Day	Factor	dF	F	p	dF	F	p	dF	F	p
3	Intercept	1	130.0	*	1	443.5	*	1	6.5	n.s.
	Class	2	4.2	n.s.	2	3.3	n.s.	2	3.0	n.s.
	Substance (class)	3	0.8	n.s.	3	1.4	n.s.	3	2.6	n.s.
10	Intercept	1	528.0	*	1	403.9	*	1	169.1	*
	Class	2	11.8	*	2	17.8	*	2	1.2	n.s.
	Substance (class)	3	1.8	n.s.	3	7.6	*	3	1.2	n.s.

2.2 Study 2: Improved $\delta^{13}C$ analysis of amino sugars in soil by Ion Chromatography – Oxidation – Isotope Ratio Mass Spectrometry

SHORT TITLE: Amino sugar $\delta^{13}C$ analysis by Ion Chromatography - Isotope Ratio Mass Spectrometry

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Abstract

Rationale: Amino sugars build up microbial cell walls and are important compounds of soil organic matter. To evaluate their sources and turnover, $\delta^{13}C$ analysis of soil-derived amino sugars by liquid chromatography was recently suggested. However, amino sugar $\delta^{13}C$ determination remains challenging due to 1) a strong matrix effect, 2) CO_2-binding by alkaline eluents, and 3) strongly different chromatographic behavior and concentrations of basic and acidic amino sugars. To overcome these difficulties we established an ion chromatography-oxidation-isotope ratio mass spectrometry method to improve and facilitate soil amino sugar analysis.

Method: After acid hydrolysis of soil samples, the extract was purified from salts and additional components impeding chromatographic resolution. Amino sugar concentrations and $\delta^{13}C$ values were analyzed by coupling an ion chromatograph to an isotope ratio mass spectrometer. The accuracy and precision of quantification and $\delta^{13}C$ determination were assessed.

Results: Internal standards enabled correction for losses during analysis, with a relative standard deviation < 6%. The higher magnitude peaks of basic compared to acidic amino sugars required an amount-dependent correction of $\delta^{13}C$ values. This correction allowed to decrease the accuracy of $\delta^{13}C$ determination of < 1.5‰ and their precision of < 0.5‰ for basic and acidic amino sugars in a single run.

Conclusion: This method enables parallel quantification and $\delta^{13}C$ determination of basic and acidic amino sugars in a single chromatogram due to the advantages of coupling an ion chromatograph to the isotope ratio mass spectrometer. Small adjustments of sample amount and injection volume are necessary to optimize precision and accuracy for individual soils.

Keywords: compound-specific isotope ratio mass spectrometry, amino sugars, soil organic matter, microbial biomarker analysis, ion chromatography, IC-O-IRMS coupling.

2.2.1 Introduction

The great relevance of microbial compounds within soil organic matter (SOM) became evident within the last decade. Microbial cell wall compounds seem to be the most relevant microbial-derived compound class within slow cycling SOM, as they 1) are highly polymeric substances (Amelung, 2003) and 2) stabilized by interaction with soil surfaces (Amelung et al., 2001; Miltner et al., 2011). Thus, an increasing interest arose to investigate their turnover and accumulation in soils (Miltner et al., 2011). Beside its contribution to the soil organic C (SOC) pool, amino sugars are - together with proteins – the compound classes linking the C and N cycles in soil and contribute significantly to the soil organic N (Amelung, 2003). In addition, amino sugars provide information about the microbial community structure. Bacterial cell walls consist of peptidoglycan – a polymer of N-acetylmuramic acid and N-acetylglucosamine whereas fungal cell walls consist of chitin, a N-acetylglucosamine polymer (Engelking et al., 2007; Glaser et al., 2004). The origin of mannosamine and galactosamine, additional amino sugars found in hydrolysis extracts of soils, are still debated.

In contrast to cell membrane compounds like phospholipids, which turn over rapidly in soils (Rethemeyer et al., 2004), amino sugars are more stable. Contribution of living biomass versus necromass in soils (Glaser et al., 2004) or fungal and bacterial biomass (Joergensen and Wichern, 2008) as well as reliable and generally accepted results on their turnover time in soils are still rare (Amelung et al., 2008; Glaser, 2005) as no methods for ^{14}C measurements of amino sugars, neither in their natural abundance nor ^{14}C-labeled, have been reported in the literature to our knowledge. Recent approaches have focused on determinations of $\delta^{13}C$ or $\delta^{15}N$ values of amino sugars. These studies started from the quantification of amino sugars by gas chromatography (Guerrant and Moss, 1984; He et al., 2006; Zhang and Amelung, 1996) and continued with gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) (Glaser and Gross, 2005). However, $\delta^{13}C$-determination by GC-C-IRMS has aggravating shortcomings (Decock et al., 2009): ^{13}C fractionation occurs during measurement; the resulting offset and amount dependence of the isotope signal can in part be corrected by the use of external standard (Glaser and Amelung, 2002; Schmitt et al., 2003). However, the greater the amount of introduced derivative C compared to C atoms of interest, the larger the error in the $\delta^{13}C$ determination that still remains after applying correction functions (Decock et al., 2009; Gross and Glaser, 2004).

As amino sugars are water-soluble low molecular weight organic substances, they can also be quantified by high performance liquid chromatography (HPLC) (Appuhn et al., 2004; Indorf et al., 2011). Therefore, current methodological developments have fo-

cused on the establishment of liquid chromatography-oxidation-isotope ratio mass spectrometry (LC-O-IRMS) methods (Krummen et al., 2004) for $\delta^{13}C$ measurement of amino sugars (Bode et al., 2009), which have already revealed its high potential for application in soil science (Bai et al., 2013; Bode et al., 2013; Indorf et al., 2012).

Many LC-O-IRMS methods and in particular the amino sugar method are not routinely used. Conventional liquid chromatographs are constructed for organic eluents and problems occur if continuously used with strong acids or bases. However, performing LC-O-IRMS analysis for $\delta^{13}C$ determination does not allow any organic eluents i.e. organic C. Hence, liquid chromatography is restricted to ion exchange columns which implies the use of salt solutions or acids and bases as eluents (Basler and Dyckmans, in press; Bode et al., 2009). Thus, metallic ions can be dissolved from stainless steel pumps or capillaries and salt crystallization occurs within the system (Bode et al., 2009; Rinne et al., 2012). This causes a loss in the performance of the columns as well as blockages of the system. To prevent such problems, time and money consuming purging steps have to be implemented between sample measurements (Bode et al., 2009; Rinne et al., 2012). In addition, any contamination by HCO_3^- has to be avoided for $\delta^{13}C$ determination as HCO_3^- increases C background (i.e. baseline) and it will influence the $\delta^{13}C$ value of the analytes. However, liquid chromatographs are *per se* not constructed to avoid gas diffusion into the system. Thus, pre-degassing of eluents have to be performed to enable carbonate-free chromatography – especially if bases are used as eluents. In addition, basic amino sugars (glucosamine, galactosamine and mannosamine) show greatly different chromatographic behavior than the acidic muramic acid. Thus, a high gradient with the eluents has to be driven, leading to strong elution of the matrix, especially for soils (Bode et al., 2009). In addition, concentrations of muramic acid are ten to hundred times lower than those of basic amino sugars. This hampers quantification due to the limited linear range of the detectors as well as $\delta^{13}C$ determination due to a limited range of peak area with reproducible results. Therefore, current methods use a double measurement with different chromatographies to first measure muramic acid and afterwards basic amino sugars (Bode et al., 2009). This double measurement as well as the additional effort required for solvent-free HPLC methods renders routine measurement of $\delta^{13}C$ values of amino sugars nearly impossible.

The aim of this study was to establish an ion chromatography–oxidation–isotope ratio mass spectrometry (IC-O-IRMS) method for quantification and $\delta^{13}C$ determination of soil-derived amino sugars. We hypothesized that using an ion chromatograph would strongly facilitate IRMS measurement of many biomarkers, as some basic requirements like carbonate-free measurement or metal-free systems are already fulfilled by the instrument. In addition, we intended to optimize amino sugar purification to reduce cationic

contamination and matrix peaks originating from soil. The aim was to provide a method enabling a routine application of $\delta^{13}C$ amino sugar measurements, which are crucial regarding the increasing interest in microbial contributions to stable SOM.

2.2.2 Material and Methods

2.2.2.1 Soil

Topsoil (0-10 cm) from the Ap horizon of a silt loamy haplic Luvisol (WRB, 2006) was collected from a long-term cultivated field in Bavaria (49.907 N, 11.152 E, 501 m. a. s. l, mean annual temperature 6-7 °C, mean annual precipitation 874 mm). The soil had a pH_{KCl} of 4.88 and pH_{H2O} of 6.49, TOC and TN content were 1.77% and 0.19%, respectively, and potential cation exchange capacity was 13.6 $cmol_c$ kg^{-1}. Field fresh soil was sieved to 2 mm and all roots were removed with tweezers. Soil was then freeze dried, ball milled and 500 mg of the resulting powder were used for each hydrolysis.

2.2.2.2 Chemicals, reagents and external and internal standards

All chemicals for hydrolysis and purification were obtained from Sigma-Aldrich (St. Louis, MO, USA) with a minimum grade of “pro analysis” (>99.0% purity). For ion chromatography, a 50-52%, ultra-pure NaOH solution was purchased from Sigma Aldrich (St. Louis, MO, USA). $NaNO_3$-solution (0.01 M) was produced from metal-free sodium nitrate, puratronic (99.999% purity, Alfa Aesar, Karlsruhe, Germany). For oxidation, a 0.26 M sodium persulfate solution and 10% phosphoric acid solutions were used (Sigma Aldrich, St. Louis, MO, USA).

Methylglucamine p. a. (5 mg mL^{-1}) and fructose p.a. (1 mg mL^{-1}) (Sigma Aldrich, Louis, MO, USA) were used as the first and second internal standards (IS1 and IS2), respectively. Stock solutions for external standards contained methylglucamine, glucosamine, mannosamine and galactosamine at concentrations of 5, 14, 1.5 and 20 mg l^{-1} (Sigma Aldrich, Louis, MO, USA) and muramic acid (Toronto Research Chemicals Inc., Toronto, Canada) at 7.5 mg l^{-1}. The IAEA-calibrated $\delta^{13}C$ value of each external standard was determined by repeated Elemental Analyzer-Isotope Ratio Mass Spectrometry (Flash 2000 HT Plus Elemental Analyzer and Delta V Advantage Isotope Ratio Mass Spectrometer, both from Thermo-Fisher, Bremen, Germany) measurement of these substances and calibrated against certified standards of the International Atomic Energy Agency IAEA (IAEA-CH6: -10.4‰, IAEA-CH7 -31.8‰ and USGS41 37.8‰) versus Pee Dee Belemnite (PDB).

2.2.2.3 Soil hydrolysis and ion removal

Soil hydrolysis and ion removal were performed according to Zhang and Amelung (1996), which was optimized for $\delta^{13}C$ determination by Glaser and Gross (2005). Briefly, hydrolysis was performed with 10 mL of 6 M HCl at 105 °C for 8 h. The filtrate extract was dried completely and redissolved in 20 mL H_2O. One hundred microliters of the IS1 methylglucamine (i.e. 50 µg) were then added. The pH was adjusted to 6.6-6.8 with 0.6 M KOH and precipitated iron was removed by centrifugation (4000 rpm for 15 min). After freeze-drying the residue was redissolved in 5 mL of dry methanol and salt precipitates were removed by centrifugation (4000 rpm for 10 min). The supernatant was dried under a gentle stream of N_2 and stored frozen until column purification.

2.2.2.4 Purification by cation exchange column

Liquid chromatography requires a column purification to remove hydrolysable non-cationic compounds like monosaccharides and carboxylic acids from the extract. A cation exchange column (AG 50W-X8 Resin, H^+ form, mesh size 100-200, Biorad, Munich, Germany) was used as suggested by Indorf et al. (2013): a thin layer of clean glass wool was installed under 4 cm of cation exchange resin in the glass column (inner diameter: 0.8 cm). Resin was filled in by rinsing with ~10 mL of 0.1 M HCl solution to ensure the H^+ form of the sorbent, covered with a thin layer of glass wool and preconditioned with 5 mL of water. Dried extracts were redissolved in ~1 mL of water with one drop of 0.1 M HCl to ensure the cationic form of muramic acid. After transferring the sample onto the column, neutral and anionic compounds were eluted with 8 mL water. The cationic fraction containing the amino sugars was eluted by 15 mL 0.5 M HCl, freeze-dried and transferred with 5 mL of dry methanol. After evaporation of the methanol by a gentle stream of dried N_2, the sample can be stored frozen (-20 °C) for at least one month. For subsequent measurement, the samples were re-dissolved in 200 µL water with the addition of 50 µL of IS2 solution and measured within 24 hours after re-dissolving.

2.2.2.5 Development of the measurement by IC-O-IRMS

All measurements were performed by a Dionex ICS-5000 SP ion chromatography system coupled by an LC IsoLink to a Delta V Advantage Isotope Ratio Mass Spectrometer (Supplementary Figure 1) (all components from Thermo-Fisher, Bremen, Germany). Chromatographic conditions were modified and optimized with the aim of reaching baseline separation and a resolution factor Rs greater than 1.

$$Rs = \frac{t_2 - t_1}{0.5 \cdot (w_2 + w_1)}$$ equation 1

t_1 and t_2 are the retention times of two neighboring peaks and w represents their respective peak width at the tangents baseline (Figure 1).

Nine microliters of the water-dissolved sample or external standard were injected via a 25 µL injection loop and the injection time was defined as 0 sec. Chromatography was performed by a CarboPac^{TM} PA 20 analytical anion exchange column (3 x 150 mm, 6.5 µm) which was preceded by a PA 20 guard column (Bode et al., 2009) (both from Dionex, Amsterdam, The Netherlands). The elution sequence contained a preconditioning before injection (15 min with 200 mM NaOH and 10 min with 8 mM NaOH). Elution sequence lasted for 35 min in total and was performed at constant temperature of 30 °C and a flow rate of 0.4 ml min^{-1}. 8 mM NaOH was increased after 11 min to 8 mM NaOH with a pulse of 2.5 mM $NaNO_3$ until 15th minute. Then $NaNO_3$ was decreased and NaOH concentration increased for final 20 min of chromatogram (details in Supplementary Table 1).

We measured external standards at four concentrations (e.g. 50, 100, 175 and 250 µL of the stock solution) at least once before and once after a sample batch. A sample batch consisted of 4–6 samples, each measured 4 times. A sample batch was always measured once in its entirety and then repeated three times.

Integration was performed by Isodat 3.0 (Thermo Fisher Scientific, Bremen, Germany) with the following parameters: start slope 1 mV/s, end slope 2 mV/s, peak min 50 mV, peak resolution 50% and an individual background.

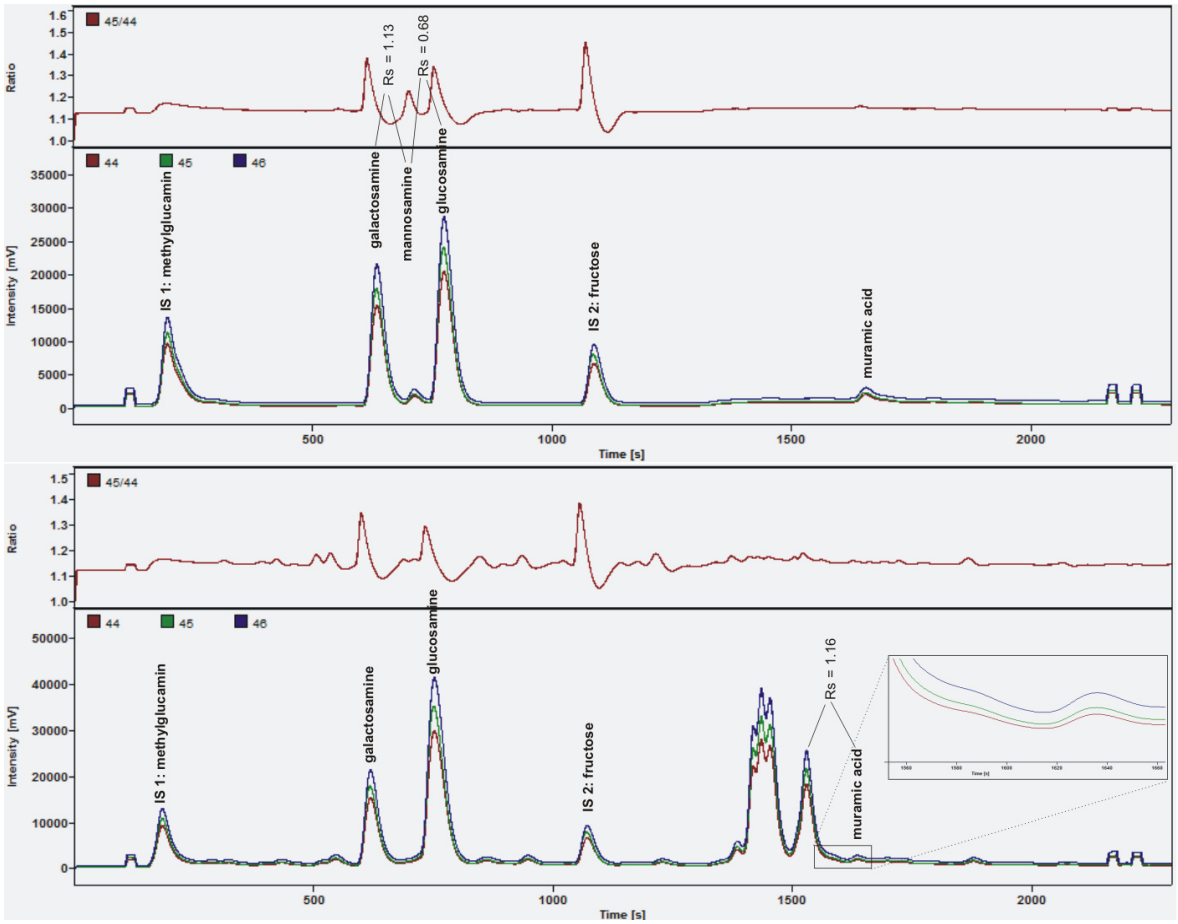

Fig. 1 Chromatogram of external standard (top) and un-spiked sample (down). First and second internal standards as well as basic amino sugars (galactosamine, mannosamine and glucosamine) and acidic muramic acid are marked. Peak resolution Rs is included for the triplett of basic amino sugars in the upper chromatogram of the external standard and Rs for muramic acid and its preceeding matrix peak is shown in the chromatogram of the sample.

2.2.2.6 Evaluation of amino sugar quantification via IC-O-IRMS

To validate the method by standard addition, the standard mixture serving as external standards was added to the hydrolysis extracts. The amounts of substance added were in the range of 0, 1.3, 1.7, 2.1 and 3 times of the expected concentrations.

The data from the standard addition experiment were statistically evaluated according to Birk et al. (2012): For each substance (including IS1) a linear regression was fitted by the method of least squares to the measured amounts as a function of the added amounts per sample (Supplementary Figure 2). The y-intercept represented the fitted amount of substance in soil and the slope gave the mean recovery of a substance. The significance of regression was tested and Steven's Runs Test was performed to identify deviations from linearity. Significant differences between recoveries were detected by covariance analysis (ANCOVA) of the slopes. All regression parameters were calculated

with GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). Relative Standard Deviation (RSD) was calculated from the standard addition experiment according to Birk et al. (2012)

$$RSD = \sqrt{\frac{\sum_{i=1}^{i=n}\left(X_i - \overline{X}\right)^2}{(n-1)\cdot \overline{X}^2}} \cdot 100 \qquad \text{equation 2}$$

whereas Xi represents the difference between the quantified amount of a substance (corrected for recovery) and the spiked amount of the standard added to each of the n samples and $\overline{X}$ is the mean of these differences.

Limit of Detection (LoD) and Limit of Quantification (LoQ) were calculated based on the signal-to-noise ratio: for LoD the signal-to-noise has to exceed 3:1 and for LoQ a value of 10:1 is necessary.

2.2.2.7 Evaluation of $\delta^{13}C$ determination via IC-O-IRMS

First, measured $\delta^{13}C$ values were drift corrected based on the reference gas drift according to GC-C-IRMS methods (Apostel et al., 2013). Thereafter, correction for offset and amount dependence were performed (2002): We tested for linear, exponential and partial linear amount dependence by fitting the following functions to the measured data $at\%_{corrected}$:

a) Linear: $at\%_{corrected}(A_i) = a \cdot A_i + b$

b) Exponential: $at\%_{corrected}(A_i) = c \cdot \exp(A_i) + d$ equation 3

c) No amount dependence: $at\%_{corrected}(A_i) = b$

In these correction functions a, b, c and d are parameters fitted to the plot of measured at% values against peak area A_i (Figure 2). The function with the best fit was used to correct the measured $at\%_{measured}$ values of the sample dependent on the peak area. The difference between the amount-dependent correction value $at\%_{corrected}(A_i)$ and the measured and calibrated value of the substance $at\%_{EA}$ was subtracted from the measured value to gain the PDB-calibrated ^{13}C enrichment ($at\%_{sample}$):

$$at\%_{sample} = at\%_{measured} - (at\%_{EA} - at\%_{corrected}(A_i)) \qquad \text{equation 4}$$

We corrected each substance and sample batch individually by the correction function that described best the behavior of the external standards. All corrections and calculations were done in at% to avoid errors due to the nonlinearity of $\delta^{13}C$ values.

The accuracy of IC-O-IRMS determination of $\delta^{13}C$ values was assessed from the standard addition experiment by using the mixing model to calculate back to the original values of the spiked substances (Bode et al., 2009):

$$\delta^{13}C_{Sample} = \frac{N_{soil} \cdot \delta^{13}C(soil)_{fitted} - N_{Std} \cdot \delta^{13}C(Std)_{fitted}}{N_{soil} + N_{Std}} \qquad \text{equation 5}$$

$\delta^{13}C_{Sample}$ reflects the PDB-calibrated, measured $\delta^{13}C$ value (derived from $at\%_{sample}$ in equation 4), N_{soil} is the quantified amount of amino sugar in the soil and N_{Std} is the amount of standard added (% of total amino sugar per vial). Using a nonlinear fit based on least square regression algorithm $\delta^{13}C(soil)_{fitted}$ and $\delta^{13}C(Std)_{fitted}$ were calculated by Statistica 6.0 (StatSoft Inc, Tulsa USA).

Precision was determined 1) as the measured standard error of the four measurement replications of un-spiked soil and 2) as an area-dependent function for the standard error of $\delta^{13}C(soil)_{sample}$ gained by Gaussian error propagation of the standard errors of each term contributing to equation 4 (2009) (see Supplementary Equation 1),

Isotopic LoQ was defined as the milligrams of amino sugar per vial needed to reach a standard error $\sigma_{final}(A_i)$ less than 0.5‰ according to this equation.

2.2.3 Results and Discussion

2.2.3.1 Chromatography

Measurement of both basic and acidic amino sugars was possible in a single run (Figure 1). Methylglucamine showed only low retention by the column and was followed by the triplet of the basic amino sugars. Peak resolution Rs within the triplet is shown in Figure 1 for an external standard. If a soil contains a large amount of mannosamine, peak separation between mannosamine and glucosamine might not be complete at baseline. Whether the $\delta^{13}C$ value of mannosamine is influenced by glucosamine in this case has to be evaluated for soils with a higher mannosamine content. Mannosamine was not detected in this soil (Figure 1), even when GC-MS was applied to achieve a lower detection limit (data not presented). Mannosamine values reported in the literature are also very low (Amelung, 2003; Glaser and Gross, 2005; Glaser et al., 2004; Zhang and Amelung, 1996).

We tested several substances from a broad spectrum of monosaccharides and uronic acids, both substance classes that didn't elute in the amino sugar fraction. We chose fructose as IS2 as it is in the middle of our chromatogram and no matrix peak is close to it. However, the second internal standard can be exchanged if soils with other

matrix peaks will be investigated. Muramic acid was the last peak eluting from the PA 20 column and needed a nitrate pulse as a pusher to become mobile on the column (Bode et al., 2009). Muramic acid occurred in samples directly after a large matrix peak. Although the matrix peak was much higher than muramic acid, it did not tail into the muramic acid peak and the resolution between the peaks was sufficient (Figure 1).

Column performance was maintained only by pre-purging (Supplementary Table 1) and no further purging steps in between samples were needed. Carryover from sample to sample, as described previously for LC-O-IRMS (Bode et al., 2009), could not be detected in any blank. Thus, sample run time and purging time is strongly reduced compared to previous methods (Bode et al., 2009). This can be attributed to the smaller amounts of metal ions and carbonates accumulating on the column due to the advantages of IC compared to liquid chromatography.

2.2.3.2 Recovery, linearity, precision and detection and quantification limits

Evaluation of the quantification by standard addition revealed linearity over a wide range of concentrations (Supplementary Figure 2): R^2 was higher than 0.99, the slope was, significantly, not zero and Steven's Runs Test revealed no deviation from linearity (Table 1). As we exceeded soil concentrations by a factor of 3, we conclude that the linear range for quantification by IRMS is sufficient to cover the range of naturally occurring amino sugar concentrations in soil (even for soils having much higher SOC contents like chernozems), especially if the used amount of soil is adapted to the SOC content. In particular, quantification of muramic acid and glucosamine, which occur in soils in wildly different concentrations, is possible in one run irrespective of the soil type.

Calculated recoveries ranged from 57 to 68%. ANCOVA revealed that recoveries of the first internal standard and basic amino sugars as well as muramic acid did not differ significantly. Thus, correcting the dataset with the recoveries gained by IS1 sufficiently corrects for the loss of the other analytes during analysis. Recoveries were slightly less than those observed by Bode et al. (2009), which can mainly be attributed to the additional column purification step included here. However, this column purification improved peak shape and decreased chromatographic noise and thus enhanced LoD and LoQ. Correcting the amounts of analytes by the IS1 recovery will compensate for these losses. However, if strongly different soil types are compared in one study, recovery of amino sugars should be checked for these particular soils before analysis to ensure similar recoveries irrespective of matrix type.

The precision of quantification was calculated by the RSD. Whereas basic amino sugars revealed precisions <2.3%, the precision for muramic acid was less, at 6.7% (Ta-

ble 1). This can be attributed to the small amount of muramic acid, which is close to the limit of quantification. However, all RSDs were in an acceptable range and precise quantification of amino sugars was possible by IC-O-IRMS.

LoD ranged from 0.001 to 0.02 and LoQ from 0.02 to 0.07 mg per vial depending on the noise surrounding the peaks. Thus, detection is possible even at low concentrations. However, for accurate detection (especially of low concentrated muramic acid) the injection volume or amount of hydrolyzed soil used should be adapted to the respective soil.

To conclude, quantification of basic and acidic amino sugars is possible in a single run. However, depending on the ratio of glucosamine to muramic acid, an adjustment in sample amount or injection volume may be needed to reach optimum precision. Dilution of the final sample or adaptation of the injection volume may be necessary to obtain muramic acid concentrations above LoQ and at the same time glucosamine concentrations that are still in the linear range of quantification.

Table 1 Recovery (%), relative standard deviation (RSD) and parameters of regression analysis as well as detection (LoD) and quantification limits (LoQ) for the quantification of amino sugars assessed from the standard addition experiment.

Substance	Recovery (%)	RSD (%)	R^2	$p_{slope \neq 0}$	$p_{RunsTest}$	LOD	LOQ
methylglucamine	67.88 ± 2.41	n.d.	0.996	< 0.001	≥ 0.05	0.006	0.019
galactosamine	56.89 ± 1.99	3.23	0.996	< 0.001	≥ 0.05	0.005	0.066
glucosamine	58.37 ± 4.84	2.92	0.986	< 0.001	≥ 0.05	0.001	0.021
muramic acid	65.49 ± 1.92	5.94	0.997	< 0.001	≥ 0.05	0.018	0.057

2.2.3.3 3.3 Amount dependence and correction factors of $\delta^{13}C$ values

The external standards measured parallel to each batch of samples were used to adapt the amount dependence and offset correction. For each compound a decrease in at%^{13}C with increasing area following a linear equation was observed (Figure 2). However, the function with the best fit (equation 3) changed between individual measurement batches and between days. Therefore, 1) measurement of external standards in the concentration range of the samples and 2) individual correction functions derived from these external standards per sample batch are obligatory in order to achieve reliable determination of $\delta^{13}C$ values. This has also been observed in other compound-specific isotope studies (Glaser and Amelung, 2002; Gross and Glaser, 2004; Schmitt et al., 2003; Zech and Glaser, 2009).

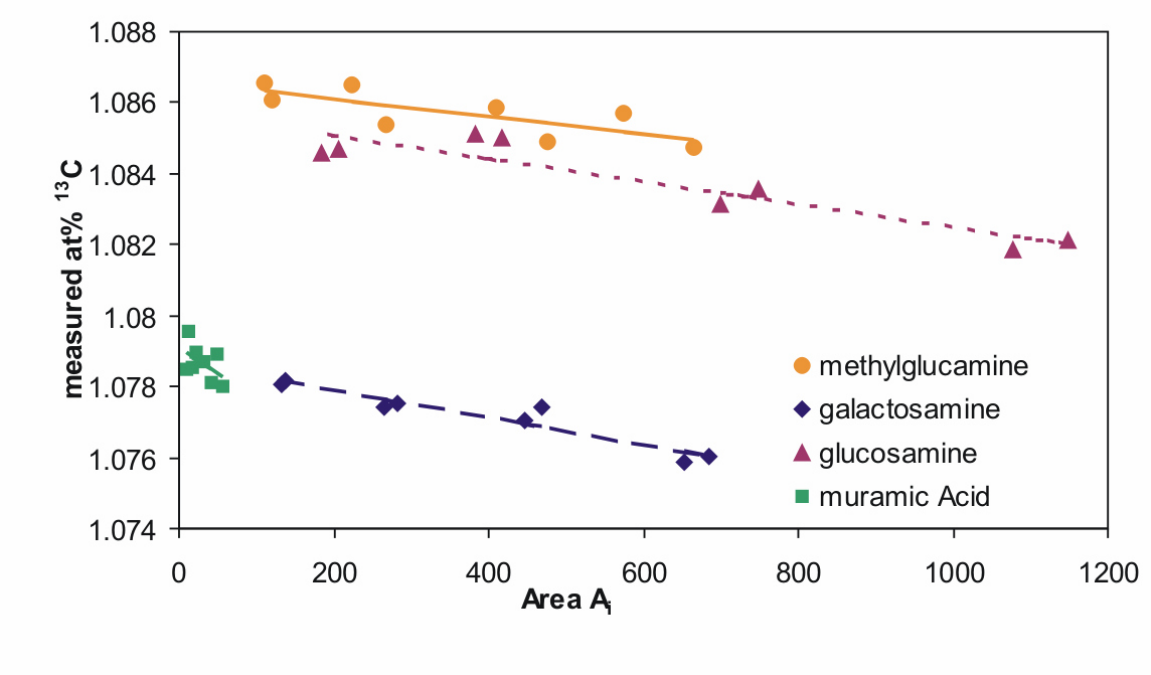

Fig. 2 Linear functions adapted to the ^{13}C values of the external standard line to correct for amount dependency of ^{13}C values.

2.2.3.4 3.4 Accuracy, precision and isotopic LoQ of $\delta^{13}C$ determination

Accuracy was assessed by comparing the fitted $\delta^{13}C$ values (from equation 5, illustraded in Supplementary Figure 3) to the measured $\delta^{13}C$ values (Table 2). Deviations in the corrected $\delta^{13}C$ $(Std)_{fitted}$ of the added standard from those measured by EA-IRMS were less than 1‰ for the basic amino sugars but higher for muramic acid (~1.5‰). These deviations from the true $\delta^{13}C$ value were slightly higher than those observed by Bode et al (2009), who also had the greatest deviation for muramic acid. This can be attributed to the small amount of muramic acid present and the fact that the $\delta^{13}C(Std)$ value of the standard spiked to the sample was quite close to the $\delta^{13}C$ value of soil muramic acid , which leads to a high uncertainty in the estimation of the fitted parameters. Choosing a soil with higher $\delta^{13}C(soil)$ (e.g. by input of C4 plants) would presumably reveal higher and more realistic accuracies for muramic acid. However, fitted values for $\delta^{13}C(soil)$ deviated by less than 0.5‰ from those of direct measurement of non-spiked soil, reflecting that the determination of amino sugar $\delta^{13}C$ under non-spiked conditions is reliable.

Table 2 Comparison of $\delta^{13}C(Std)_{EA\text{-}IRMS}$ (EA-IRMS PeeDeeBe calibrated $\delta^{13}C$ value of standard substances spiked to the sample) and $\delta^{13}C(Std)_{IC\text{-}O\text{-}IRMS}$ (fitted $\delta^{13}C$ value of the spiked standards from the mixing model of the standard addition method) reflecting the accuracy of IC-O-IRMS measurement. Fitted $\delta^{13}C$ values for soil from mixing model ($\delta^{13}C(soil)_{calculated}$) and real measurement of un-spiked soil $\delta^{13}C(soil)_{IC\text{-}O\text{-}IRMS}$ are also presented. Precision is shown 1) by the standard deviation of the measurement repetitions and 2) by calculating the area dependent standard deviation according to equation 6 for the measured peak area. Isotopic LoQ reflects the minimum amount per vial needed to receive a standard error of the measurement repetitions lower than 0.5‰. Gal=galactosamin, Glc=glucosamine and MurA=muramic acid

Substance	$\delta^{13}C(Std)_{EA\text{-}IRMS}$	$\delta^{13}C(Std)_{fitted}$	$\delta^{13}C(soil)_{fitted}$	$\delta^{13}C(soil)_{IC\text{-}O\text{-}IRMS}$	$\sigma(\delta^{13}C(soil)_{measured})$	$\sigma(\delta^{13}C(soil)_{final})$	isotopic LoQ (mg/vial)
Gal	-28.42‰	-27.60 ± 0.14‰	-24.58 ± 0.12‰	-25.02‰	0.26‰	0.64‰	0.237
Glc	-22.58‰	-21.65 ± 0.25‰	-26.64 ± 0.10‰	-26.87‰	0.20‰	0.87‰	0.250
MurA	-20.54‰	-19.04 ± 0.06‰	-19.51 ± 0.09‰	-19.65‰	0.08‰	0.51‰	0.048

The standard error of the four measurement replications was calculated for all samples of the standard addition line ($\sigma_{IC\text{-}O\text{-}IRMS}(A_i)$) (Table 2). The area dependence of this standard error $\sigma_{IC\text{-}O\text{-}IRMS}(A_i)$ followed a parabolic function for the basic amino sugars (Supplementary Figure 4), which resulted from the broad range of areas covered by the standard addition approach: With decreasing peak area a loss in precision occurs due to approaching the isotopic detection limit, i.e. the error $\sigma_{IC\text{-}O\text{-}IRMS}(A_i)$ increases. Increasing peak area can lead to an overload of the system with subsequent imprecise isotope determination. In contrast, muramic acid showed a linear decrease in the standard error with increasing areas reflecting that even in highest spiked samples were far from overload conditions. The error of the amount dependence (derived from the external standards) $\sigma_{correction}(A_i)$ showed a similar dependency on area than the sample-derived error: $\sigma_{correction}(A_i)$ of basic amino sugars had a parabolic area dependence whereas $\sigma_{correction}(A_i)$ of muramic acid showed linear behaviour by the same reasons as in the samples (Supplementary Figure 4). These functions were used to sum up the amount-dependent standard error $\sigma_{final}(A_i)$ (Figure 3). For the basic amino sugars, the standard error followed a function close to a parabolic function and there was a broad range of areas enabling reliable determination of the $\delta^{13}C$. For muramic acid, showing this function showed a sharp increase in standard deviation if areas became too small. Sample preparation should be optimized to reach the isotopic LoQ, i.e. to have >0.048 mg muramic acid in the final lyophilized sample. If the amount is less, either the volume the sample is finally dissolved in has to be decreased or the injection volume has to be increased to achieve a sufficient peak area of muramic acid. The agricultural soil used for this method evaluation had a relative high portion of bacteria compared to fungi. In soils with strong preference of fungal growth e.g. podzols the amount of muramic acid may be too low, to reach the needed

LoQ without having an overload in the glucosamine peak. Under such special conditions a double measurement with high concentrated sample for determination of muramic acid $\delta^{13}C$ values and diluted concentration for determination of glucosamine $\delta^{13}C$ values might be necessary.

Average amino sugar $\delta^{13}C$ values differ for ~0.1 to 1.1‰ within the basic (Bode et al., 2009; Bode et al., 2013) and for more than 3-5‰ between the basic and acidic amino sugars (Bode et al., 2009; Bode et al., 2013; Glaser, 1999) and differ for around 7‰ from bulk SOC (Glaser and Gross, 2005). The achieved accuracies of individual amino sugars enable to distinguish amino sugar from their C sources even under natural abundance conditions. Resulting precisions (0.5‰) are lower than differences between basic and acidic amino sugars and consequently enables to identify microbial group specifics in amino sugar formation (e.g. specifics in the used substrates or the fractionations in biochemical formation pathways). Especially in experiments leading to a higher $\delta^{13}C$ differences in amino sugars like C3 to C4 C source changes (Indorf et al., 2012), FACE experiments (Glaser and Gross, 2005) or application of labeled substrates (Bode et al., 2013) this method can fully distinguish C sources and individualities in cell wall formation of fungi and bacteria.

In summary, this method enables a combined determination of $\delta^{13}C$ values of amino sugars for the majority of soils. However, adjustments to new sample types are necessary to identify the optimum amount of sample to hydrolyze or the final volume to inject so that the optimum range for accuracy and precision of the $\delta^{13}C$ values are met.

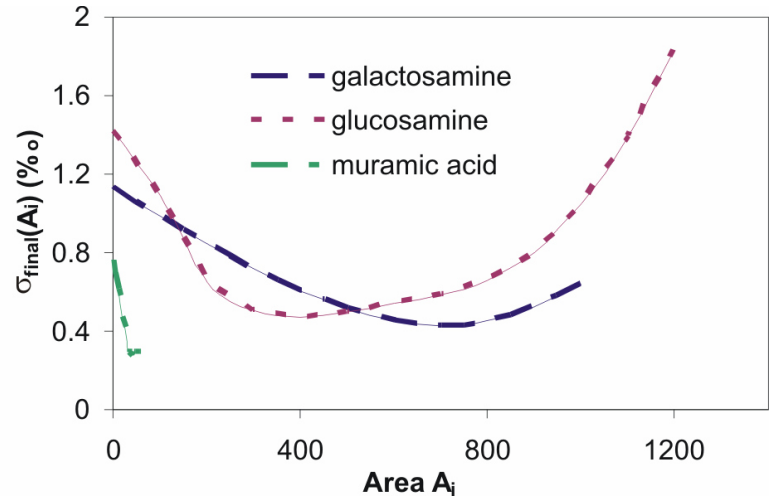

Fig. 3 Amount-dependent function for estimation of standard error of $\delta^{13}C$ ($\delta_{final}(Ai)$ determination calculated according to equation 6.

2.2.3.5 Advantages of IC-O-IRMS

Many previous studies reported severe problems with LC-O-IRMS, e.g. the impossibility of measuring muramic acid in non-spiked samples due to very low peak areas or the requirement for time-consuming purging steps to maintain performance of the PA 20 column (Bode et al., 2009). The absence of these issues in the currently proposed method can mainly be attributed to the advantages of IC over HPLC. Ion chromatographs are free of metals: all elements that are in contact with sample or eluents are made from polyether ether keton (peek). Thus, metal contamination can originate only from the sample. However, our method contains iron and salt precipitation steps, removing all (potentially column destroying) cations. This not only reduces measurement time but also reduces costs as, e.g., in-line high pressure filters protecting the column from colloids and metal ions are not needed. Even after 600 injections, no decrease in performance of the PA 20 column was detected and the pre-column did not need to be exchanged.

In addition, the CO_2-tight construction of Ion Chromatographs is a great advantage of $\delta^{13}C$ determination as no shifts in the $\delta^{13}C$ value due to increasing carbonate background occurred. Therefore, even CO_2-binding eluents, like NaOH, do not cause problems for chromatography and isotope ratio mass spectrometry. In addition, Ion Chromatographs are routinely equipped with a degasser, which keeps the eluents and oxidizing reagents of the Isolink CO_2-free. Thus, although acquisition costs may be higher, the improved performance, higher sample throughput and lower follow-up costs reflect the clear advantages of ion chromatographs for improving LC-O-IRMS.

2.2.4 Conclusions

Amino sugars are important biomarkers for research on bacterial and fungal contribution to SOM. This new method enables parallel quantification and $\delta^{13}C$ determination of the most frequent amino sugars in soils and thus sets the preconditions for wider adoption of $\delta^{13}C$ amino sugar determination in soil science.

The combination of iron and salt removal from gas chromatography protocols with purification via cation exchange resins adapted from liquid chromatography methods proved to be an optimal sample preparation for ion chromatography including chromatographic separation, system stability and longevity of system components. In addition, using ion chromatograph sets clear advantages over HPLCs as metal and carbonate exclusion from the system avoids column contamination as well as disturbance of $\delta^{13}C$ determination by a carbonate background.

These improvements over previous methods enabled parallel quantification and $\delta^{13}C$ determination of high-concentrated basic amino sugars and low-concentrated muramic acid. Recoveries ranged from 57 to 66% and could be corrected by using methylglucamine as the first internal standard. The quantification limit of muramic acid, the compound with the lowest concentration, was around 0.05 mg per vial for quantification and for isotope measurement. When muramic acid exceeded these values, glucosamine, the most concentrated compound, was still in a linear range for quantification and $\delta^{13}C$ measurement. The accuracy of IC-O-IRMS was better than 1‰ for basic amino sugars and better than 1.5‰ for muramic acid compared to calibrated EA-IRMS values. Precision was amount-dependent and less than 0.5‰ over a comparatively broad range of areas. However, the dependence on the matrix and the ratio of muramic acid to glucosamine in individual samples necessitates adjustment in soil amount or injection volume to achieve the optimal accuracy and precision of $\delta^{13}C$.

The quality of the quantification and $\delta^{13}C$ determination as well as sample throughput of this method should enable this method to be used routinly in soil science. The advantages of IC-O-IRMS compared to HPLC-O-IRMS are evident and might also bring advantages for analysis of other biomarkers.

Acknowledgments

We thank the DFG for financing the IC-O-IRMS instrument and the project DFG KU 1184 19/1.

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Supplementary Data

Figure Supplementary A1: Scheme of the instrument coupling: Ion Chromatograph is shown on the left side with pump, autosampler and detector-chromatography compartment. Connection to isolink occurs via a peek capillary with interposed colloid filter. Scheme of LC Isolink is adapted from Krummen et al. (2004).

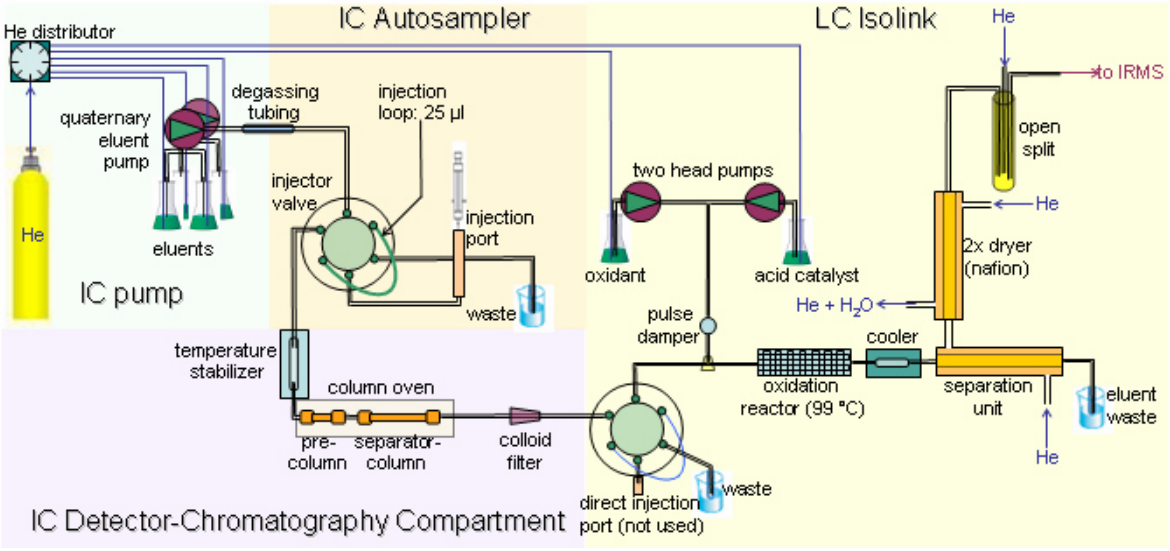
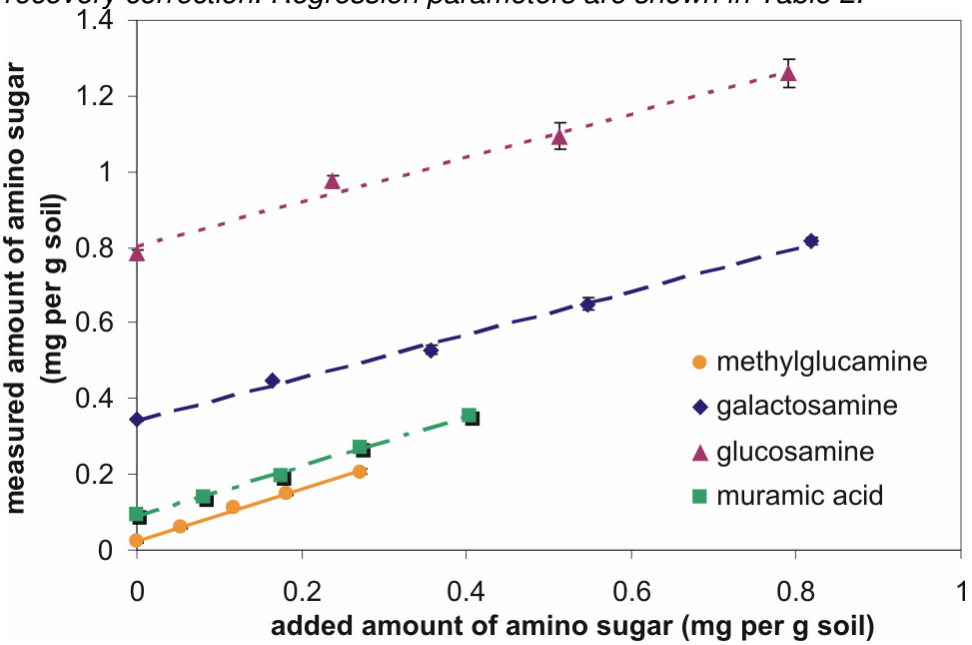


Figure Supplementary A2: Standard addition line of the quantified amino sugars: quantified amount per g soil is plotted against the amount of spiked amino sugar. Slope represents recovery of the individual analytes and y-axis gap represents soil content without recovery correction. Regression parameters are shown in Table 2.



Supplementary Figure A3: measured $\delta^{13}C$ values of spiked samples are plotted against the percent of peak area, which is derived from the added standard: y-intercept of the fitted linear regression reflects the fitted value of soil whereas $\delta^{13}C$-value at 100% standard reflects the $\delta^{13}C$ value of the added standard substance

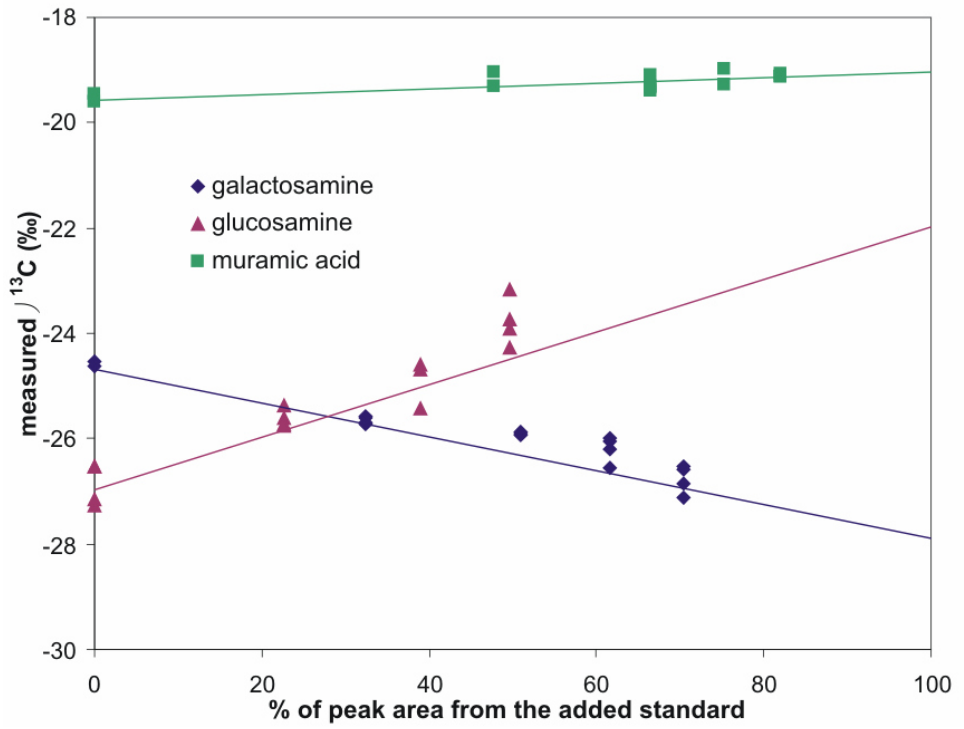
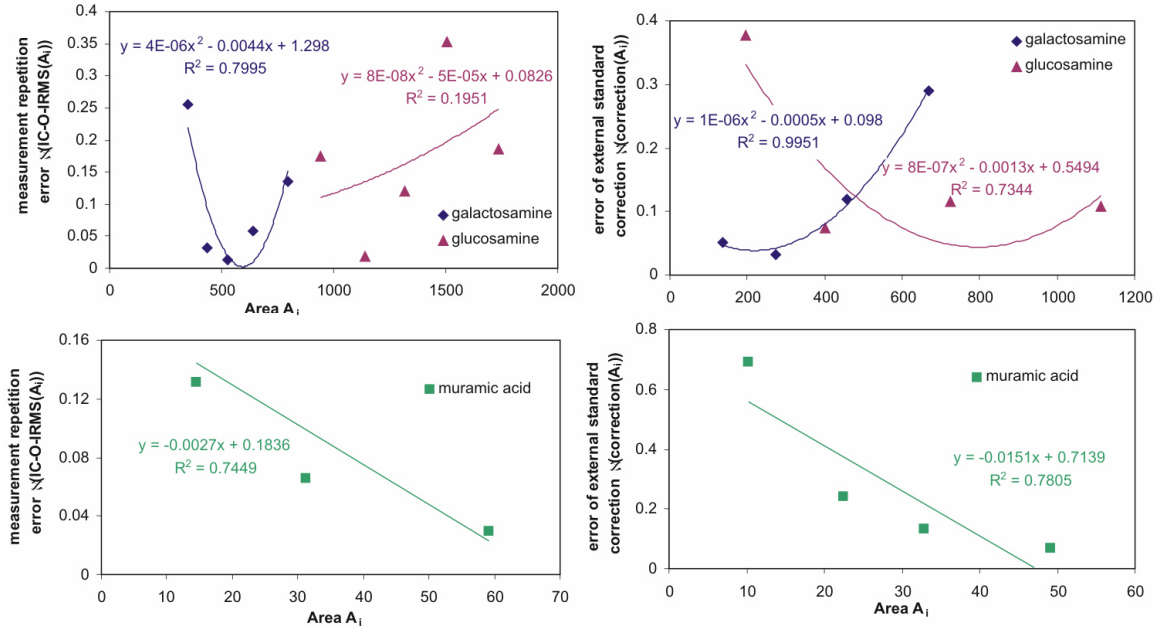


Figure Supplementary A4: area-dependant error terms of equation 6: left side shows the standard error of the measurement repetition of soil samples and right side shows the area-dependant error of the calibration/correction function from the external standard line



Supplementary Table A1: Solvent gradient and flow conditions of the IC-O-IRMS system

time	20 mM NaOH	200 mM Na-OH	H_2O	0.01 M $NaNO_3$	flow (ml min^{-1})
-25 min	0%	100%	0%	0%	0.400
-10 min	8%	0%	92%	0%	0.325
11 min	40%	0%	35%	25%	0.400
15 min	45%	0%	45%	10%	0.400
18 min	25%	25%	50%	0%	0.380
35 min			standby		

2.3 Study 3: Biochemical pathways of amino acids in soil: Evaluation by position-specific labeling and ^{13}C-PLFA analysis

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Abstract

Microbial utilization is a key transformation process of soil organic matter (SOM). For the first time, position-specific ^{13}C labeling was combined with compound-specific ^{13}C-PLFA analysis to trace metabolites of two amino acids in microbial groups and to reconstruct detailed biochemical pathways. Short-term transformation was assessed by applying position-specifically ^{13}C labeled alanine and glutamate to soil in a field experiment. Microbial utilization of the amino acids' functional groups was quantified by ^{13}C incorporation in total microbial biomass and in distinct microbial groups classified by ^{13}C-PLFA.

Loss from PLFAs was fastest for the highly oxidized carboxyl group of both amino acids, whereas the reduced C positions, e.g. $C_{3\text{-}5}$, were preferentially incorporated into microorganisms and their PLFAs. The incorporation of C from alanines' C_2 position into the cell membrane of gram negative bacteria was higher by more than one order of magnitude than into all other microbial groups. Whereas C_2 of alanine was still bound to C_3 at day 3, the C_2 and C_3 positions were partially split at day 10. In contrast, the C_2 of glutamate was lost faster from PLFAs of all microbial groups. The divergence index, which reflects relative incorporation of one position to the incorporation of C from all positions in a molecule, revealed that discrimination between positions is highest in the initial reactions and decreases with time.

Reconstruction of microbial transformation pathways showed that the C_2 position of alanine is lost faster than its C_3 position regardless of whether the molecule is used ana- or catabolically. Glutamate C_2 is incorporated into PLFAs only by two out of eight microbial groups (fungi and part of gram positive prokaryotes). Its incorporation in PLFA can only be explained by either the utilization of the glyoxolate bypass or the transformation of glutamate into aspartate prior to being fed into the citric acid cycle. During these pathways, no C is lost as CO_2 but neither is energy produced, making them typical C deficiency pathways. Glutamate is therefore a promising metabolic tracer in regard to ecophysiology of cells and therefore changing environmental conditions.

Analyzing the fate of individual C atoms by position-specific labeling allows insight into the mechanisms and kinetics of microbial utilization by various microbial groups. This approach will strongly improve our understanding of soil C fluxes.

Key words: metabolite tracing, transformation pathways, stable isotope applications, microbial community structure and functions, compound-specific isotope analysis

2.3.1 Introduction

Soil organic carbon (SOC) plays a major role in the global carbon (C) cycle. The estimated soil organic C stocks of 1462 - 1548 Pg (Batjes, 1996) are about twice as high as in the atmosphere and three times as high as in the vegetation (IPCC, 2000). Soils can function both as a sink and a source for C, depending on climate, vegetation and management (Van Miegroet and Jandl, 2007; Vesterdal et al., 2012). Therefore it is important to understand processes that lead to C release from or sequestration in soil.

The main input of C into soils is via plant litter or rhizodeposition (Rasse et al., 2005). Litter is composed of macromolecules such as cellulose, hemicellulose, lignin or proteins (Crawford et al., 1977; Sorensen, 1975); rhizodeposition also contains those macromolecules as well as low molecular weight organic substances (LMWOS) (Farrar et al., 2001). Traditionally, the chemical properties of some of these macromolecules were thought to prevent soil biota from digesting them. This so-called “recalcitrance” should lead to an enrichment of those molecules in soil. Newer studies, however, have shown contrary results (Crawford et al., 1977; Grandy and Neff, 2008; Jones and Darrah, 1994; Marschner et al., 2008). In their review, Schmidt et al. (2011) combined data from 20 field experiments with durations of up to 23 years. In such experiments, presumably stable macromolecules have shown turnover rates well below that of bulk soil. In contrast, the supposedly labile products of their decomposition, such as amino acids, sugars and other LMWOS, can persist in soil for years or even decades. As the initial quality of the OM seems to have a minor effect on its persistence in soil, future research is called upon to identify the mechanisms that stabilize SOC, especially from LMWOS.

Growing evidence points to microbial incorporation and transformation as key factors in stabilizing soil organic carbon (SOC) (Koegel-Knabner, 2002). This could explain the lower-than-expected stability of macromolecules: prior to being incorporated into microbial biomass, they are split by exoenzymes and only then are the emerging LMWOS further processed (Cadisch and Giller, 1996; Kuzyakov et al., 2009). After being taken up by microorganisms, LMWOS are partly degraded and transformed to CO_2 and are thus lost from the soil C pool; the other part is transformed into microbial biomass, which is more stable to decomposition even after cell death (Six et al., 2006). Nonetheless, resource utilization by microbial groups differs with respect to uptake preferences and speed (Treonis et al., 2004) and, more importantly, stability of microbial products. Residues from gram positive bacteria can have twice the mean residence time of that from gram negative bacteria (Schmidt et al., 2011). Learning more about the means of SOC degradation and sequestration therefore requires techniques that yield information on: i) microbial groups present in the soil and ii) their metabolic processes.

To achieve the first goal, phospholipid fatty acid (PLFA) analysis has been established. It is based on the production of different PLFAs for cellular membranes by different morphological groups of microorganisms (Zelles, 1999; Zelles et al., 1995). To gain a better understanding of microbial transformations of organic matter by those microorganisms, ^{13}C- or ^{14}C-labeled substances have been applied to soil and traced in various pools (SOM, microbial biomass) and, if possible, in the released CO_2 (Evershed et al., 2006; Kuzyakov, 1997; Treonis et al., 2004). The shortcoming of uniformly labeled substances is that they do not allow distinction of individual positions of a molecule: e.g. if one third of uniformly labeled alanine is incorporated into a pool, one does not know whether the molecule has been split and all of one position was incorporated, while the other two have been mineralized or whether one third of all molecules was incorporated without having been split. By use of uniformly labeled substances only total incorporation respectively degradation of the molecule can be assumed. Nonetheless, in regard to the different oxidation states of C in organic molecules, a preferential incorporation of some positions and degradation of others is conceivable (Dijkstra et al., 2011a; Fischer and Kuzyakov, 2010b). This hypothesis cannot be investigated with uniformly labeled substances. Instead, position-specific labeling (^{13}C or ^{14}C) should be applied.

We used two model substances in our study: the amino acids alanine and glutamic acid. Amino acids are a source for both C and N and, as such, represent an important link between C and N cycles. The two LMWOS are also very abundant in soil; alanine accounts for about 15% and glutamic acid 10% of the amino acids recovered in DOC (Fischer et al., 2007). They are also important components of root exudates (Fischer et al., 2010a).

We tested the following hypotheses:

I. Functional groups in the amino acids are utilized differently:
    a. carboxyl C is lost fastest from soil,
    b. C with lower oxidation states is preferably incorporated in microorganisms.
II. Individual microbial groups incorporate different amounts of amino acid C in their PLFA:
    a. uptake and incorporation is highest for single cell groups,
    b. filamentous groups incorporate more C from lower oxidized positions than prokaryotic, single-cell organisms.
III. The fate of individual C positions can be used for metabolic tracing, i.e. to identify different metabolic pathways among microbial groups:
    a. the ratio of ^{13}C in PLFAs and in total microbial biomass will differ among the microbial groups, reflecting C transfer to fatty acid syntheses pathways,

b. the preference for the incorporation of C from individual amino acid positions will differ between the microbial groups, reflecting their metabolic pathways.

2.3.2 Material and Methods

2.3.2.1 Field experiment

Preliminary to this study, a number of short laboratory experiments with position-specifically ^{14}C-labeled LMWOS were conducted (Fischer & Kuzyakov 2010, Dippold & Kuzyakov (in press)). With a field experiment, we tested whether ^{13}C enrichment in various microbial and soil pools would allow metabolic tracing.

Sampling site

The experimental site is located on an agriculturally used loamy Luvisol in northern Bavaria (49°54' northern latitude; 11°08' eastern longitude, 500 a.s.l.). The last crop was *Triticale;* before application of the tracers, all above-ground biomass was removed. The mean annual temperature in the region is 7 °C, mean annual precipitation 874 mm. The soil had a pH_{KCl} of 4.88, a pH_{H_2O} of 6.49, TOC content of 1.77% and TN content of 0.19%. CEC was 13 $cmol_C\ kg^{-1}$.

Experiment design

The 12 × 12 m field was divided into four quadrants to allow four replications. PVC-tubes with a diameter of 10 cm and height of 13 cm were installed 10 cm deep in the soil, resulting in a soil sample weight of about 1 kg for each tube. To ease application, the soil inside each column was pierced with five wooden rods 5 days prior to applying the amino acid. A multipette (Eppendorf, Hamburg, Germany) was used to apply 10 ml tracer-solution per column with concentrations of ^{13}C labeled amino acids according to Table 1. A 7-cm-long needle with lateral holes enabled homogeneous lateral distribution. Leaching was avoided by only injecting solution in the upper 2/3 of the column and blocking rainfall by installing a roof above the plot. In each of the quadrants and per each of both sampling times, alanine and glutamic acid were applied once as 1) non-labeled background (not shown in Table 1), 2) uniformly ^{13}C-labeled and 3) as two and three position-specifically ^{13}C-labeled isotopomeres of alanine and glutamic acid, respectively (see Table 1). The distribution of substances in each block was chosen randomly. The ^{13}C-content and excess ^{13}C atom-% of the residual glutamic acid molecules were calculated

by subtracting measured values for the first two positions from results for the whole molecule.

Table 1 Concentrations of amino acid solutions for soil labeling

	Ala−^{13}COOH[a]	Ala−^{13}CNH$_2$[a]	Ala−^{13}CH$_3$[a]	Ala−U[b]	Glu−^{13}COOH[a]	Glu−^{13}CNH$_2$[c]	Glu−U[b]
C concentration (μmol ml^{-1})	65.5	65.3	65.2	65	65.1	68.7	65
C concentration (μg g soil^{-1})	0.59	0.59	0.58	0.58	0.58	0.62	0.58
Atom% ^{13}C	13.2	13.1	13.1	13.9	13.1	7.1	12.8

[a] Biotrend, Köln, Germany.
[b] Cambridge Isotope Laboratories, Andover, MA, USA.
[c] Campro Scientific GmbH, Berlin, Germany.

Sampling and sample preparation

Soil was sampled 3 and 10 days after labeling. After 3 days, we expected total uptake of the amino acids (3 days corresponds to approximately 10 mean residence times for the amino acids). After 10 days, we expected incorporation of ^{13}C into PLFAs but yet no degradation of the enriched PLFAs. Both times, complete columns from one set (background, uniformly and position-specifically labeled) of four replications were dug out and the height of the soil inside the column was noted to calculate its volume. Afterward, the soil was transferred into a plastic bag and weighed; a subset was sieved to 2 mm for further analysis and stored at -20 °C for PLFA-extraction and at 5 °C for chloroform-fumigation extraction.

2.3.2.2 Analytical methods

Bulk soil measurements

For the analysis of bulk soil C content and δ^{13}C-values, the samples were freeze dried, ground in a ball mill and 5 - 6 mg per sample were filled into 5 x 12 mm tin capsules (IVA, Meerbusch, Germany). The samples were measured on the Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit with a ConFlo III interface (Thermo-Fischer, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). Uptake of ^{13}C from the applied amino acids into the soil was calculated according to the mixing model (Eq. 1 and 2), where the C content of the background in Eq. 1 was substituted according to Eq. 2.

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C-BG} + [C]_{appAA} \cdot r_{appAA} \quad (3)$$

$$[C]_{soil} = [C]_{BG} + [C]_{appAA} \quad (4)$$

with:

$[C]_{soil/BG/appAA}$ C content of sample / background / applied amino acid (mol · g_{soil}^{-1})

$r_{soil/BG/appAA}$ ^{13}C atom%-excess of sample / background / applied amino acid (at%)

Chloroform fumigation extraction

To determine microbial C and its $\delta^{13}C$ values, two subsets of 15 g of soil were taken from each sample. One sample was directly extracted as described below; the other was first fumigated with chloroform for 5 days in an exsiccator to lyse microbial cells.

The samples were extracted twice with 22.5 ml of 0.05 M K_2SO_4. They were shaken on a horizontal shaker, 1 h on the first, 0.5 h on the second extraction. After shaking, the samples were centrifuged (10 min, 2000 rpm) and the supernatant was filtered (Rotilab® round filters, type 15A, cellulose, membrane 70 mm).

The carbon content of the K_2SO_4 extracts was measured on the TOC analyser multi C/N® 2000 (Analytik Jena, Jena, Germany). For $\delta^{13}C$ measurements, all of the remaining extracts (approx. 43 ml) were freeze-dried. A subsample of the freeze-dried crystals was transferred to 5 x 12 mm tin vessels (IVA, Meerbusch, Germany) and then measured on the Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit with a ConFlo III interface (Thermo-Fischer, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). ^{13}C uptake into the microbial biomass was calculated according to the mixing model (Eq. 1 and 2).

PLFA-Analysis

Phospholipids were extracted and purified by a modified method of Frostegard et al. (1991). Modifications included using 6 g of soil for extraction and eluting polar lipids four times instead of once with 5 ml of water-free methanol. Before extraction, 25 µl of a 1 M solution of the internal standard 1 (IS 1) phosphatidylcholin-dinonadecanoic acid were added. For measurements on a GC, the fatty acids were saponified to free fatty acids and derivatized into fatty acid methyl esters (FAME) following the description by Knapp (1979). Before transferring the samples to autosampler vials, 15 µl of the internal standard 2 (IS 2) tridecanoic acid methyl ester were added. External standards consisting of the 27 fatty acids given in Supplementary Table 1 and internal standard 1 were prepared with total fatty acid contents of 1, 4.5, 9, 18, 24 and 30 µg, respectively, and derivatized and measured together with the samples.

FAME-contents were measured on a GC-MS (GC 5890 with MS 5971A, Agilent, Waldbronn, Germany) with a 30 m DB1-MS column, in the selected ion mode. The rela-

tion between the area of each FAME and the area of the IS 2 was calculated and quantified by a linear regression calculated from the six external standards. The recovery rate for every sample was determined based on the area of the initially added 25 µg of IS 1, and applied against the quantified masses of the FAMEs.

$\delta^{13}C$-values were analyzed on a GC-C-IRMS; consisting of the autosampler unit AS 2000, the Trace GC 2000 by ThermoQuest, the combustion unit Combustion Interface III and the isotope-ratio mass spectrometer DeltaPlus (Thermo Finnigan, Bremen, Germany). Volumes of 1.5 µl were injected into a liner (Type TQ(CE) 3 mm ID TAPER) at a liner temperature of 250 °C, with a splitless time of 1 min. Gas chromatography was accomplished with a combination of two capillary columns: a 30 m DB5-MS and a 15 m DB1-MS (both: internal diameter 0.25 mm, film thickness 0.25 µm; Agilent); a constant He-flux (99.996% pure) of 2 ml · min-1 and the temperature program presented in Supplementary Table 2. CO2 reference gas (99.995 % pure) was injected for 20 s into the detector four times throughout the measurement to identify any detection drift. The δ13C of the second reference gas peak was defined as -40‰ and all other δ13C values were calculated by comparison. $\delta^{13}C$ of all PLFA samples was measured four times.

The chromatograms were evaluated with ISODAT NT 2.0. The $\delta^{13}C$- value in ‰ was computed from the output in the isotopic ratio 13C/12C.

To correct for any drift during measurements, linear regressions were calculated from reference gas peaks two and three, and three and four. Eq. 3 was applied to the $\delta^{13}C$ value of FAMEs that were detected before reference gas peak three; Eq. 4 was applied to those that were detected after reference gas peak three.

$$C_{FAME-DK}(at\%) = C_{FAME-0}(at\%) - (m_{RG} \cdot (t_{FAME} - t_{RG})) \quad (3)$$

$$C_{FAME-DK}(at\%) = C_{FAME-0}(at\%) - (m_{RG} \cdot (t_{FAME} - t_{RG})) - \Delta C_{RG}(at\%) \quad (4)$$

with:	$C_{FAME-DK}$(at%)	drift-corrected ^{13}C amount of the FAME	(at%)
	C_{FAME-0}(at%)	measured ^{13}C amount of the FAME	(at%)
	m_{RG}	slope of regression between the reference gas peaks enveloping the FAME	(s^{-1})
	t_{FAME}	retention time of FAME	(s)
	t_{RG}	retention time of reference gas peak prior to FAME	(s)
	ΔC_{RG}(at%)	difference between reference gas peaks three and two	(at%)

To correct for amount-dependent ^{13}C isotopic fractionation during measurements (Schmitt et al., 2003) and for the addition of C during derivatization, linear and logarithmic regressions of the external standards $\delta^{13}C$-values to their area were calculated. If both regressions were significant, that with the higher significance was applied. As the $\delta^{13}C$-value for the derivatizating agents was unknown, the correction was performed according to Glaser and Amelung (2002a) (Eq. 5).

$$C_{FS}(at\%) = \frac{N(C)_{FAME}}{N(C)_{FS}} \cdot (C_{FAME-DK}(at\%) - (m_{lin/\ln} \cdot A_{FAME} + t_{lin/\ln})) + C_{EA-FS}(at\%) \quad (5)$$

with:	C_{FS}(at%)	corrected ^{13}C amount of the fatty acid	[at%]
	C_{FAME}(at%)	drift-corrected ^{13}C amount of the FAME	[at%]
	$m_{lin/ln}$	slope of linear/logarithmic regression	[at% · Vs^{-1}]
	$t_{lin/ln}$	y-intercept of linear/logarithmic regression	[at%]
	A_{FAME}	area of FAME	[Vs]
	$N(C)_{FAME}$	number of C atoms in FAME	
	$N(C)_{FS}$	number of C atoms in fatty acid	
	C_{EA-FS}(at%)	measured ^{13}C-value of fatty acid	[at%]

2.3.2.3 Divergence Index

Discrimination of C from individual positions in one molecule during uptake and/or utilization was assessed. The extent of discrimination between pools, microbial groups and at two sampling times was compared as well. For both of these tasks, the differences in absolute uptake into C pools or microbial groups had to be relativized. Therefore, the divergence index (DI) was defined:

$$DI_i = \frac{n \cdot C_i}{\sum_1^n C_i} \quad (6)$$

with:	n	number of C atoms in molecule	
	C_i	relative incorporation of tracer C	[mol · mol^{-1}]

As required, the DI can be calculated with relative incorporation of tracer per bulk soil, microbial biomass, single PLFA or ΣPLFA of microbial groups. The DI compares the calculated actual incorporation of C from each position with the mean C incorporation from all positions. This can be understood as the result the experiments would have had if uniformly labeled tracers had been used. A DI of 1 would indicate no discrimination

between the positions; values above 1 indicate preferential incorporation, values below 1 show preferential degradation.

2.3.2.4 Statistical analysis

For the repetitive measurements of d13C-values, a Nalomov outlier test with significance levels of 95% (when four repetitions were available) or 99% (when three repetitions were available) was performed. PLFAs were classified into corresponding microbial groups by a factor analysis of C contents of the entire dataset. Fatty acids with a loading of more than 0.5 (absolute value) on the same factor were categorized with regard to previous studies (Zelles, 1999; Zelles et al., 1995). All the data presented in this study were tested with a one-way analysis of variance (ANOVA); significances were determined with the Tukey Honest Significance Difference (Tukey HSD) post-hoc test with a significance level of 99.5%. All positions were tested for significant differences between recoveries in soil, microbial biomass and PLFAs. For every microbial group and soil pool, the difference in DI for the six position-specifically labeled positions was also tested for significance. All statistical tests were accomplished with R version 2.9.0 (17.04.2009).

2.3.3 Results

2.3.3.1 Incorporation of uniformly labeled amino acids

The C content in the soil was 1230 µmol · g^{-1} (Table 2), which corresponds to 15.0 mg C · g^{-1} soil. Of this C, 3.5% is contained in microbial biomass, and 0.01% in the sum of PLFA (Σ-PLFA). The incorporation of uniformly ^{13}C-labeled alanine and glutamic acid into soil and microbial biomass decreased between days 3 and 10. The recovery in Σ-PLFA remained stable or even increased. Recovery of applied alanine and glutamic acid C in soil decreased by about half between days 3 and 10. Recovery from applied glutamic acid in microbial biomass decreased by nearly 90% between days 3 and 10 (Table 2).

Table 2 Total C content and ^{13}C incorporation of uniformly labeled amino acids into soil, microbial biomass and sum of PLFA (Σ-PLFA).

		Soil	Microbial biomass	Σ-PLFA
Total C stock (µmol g^{-1})	Day	1229 ± 240	44.0 ± 5.2	0.146 ± 0.058
Incorporation of applied	3	39.5 ± 9.1	31.6 ± 5.0	2.0 ± 0.7
Ala C (%)	10	12.8 ± 1.9	17.5 ± 6.5	3.1 ± 0.4
Incorporation of applied	3	42.4 ± 8.6	35.8 ± 6.0	1.8 ± 0.5
Glu C (%)	10	12.7 ± 4.3	3.9 ± 2.4	1.9 ± 0.6

2.3.3.2 Incorporation of position-specifically labeled amino acids

With the tool of position-specific labeling, we were able to trace C from individual positions of alanine and glutamic acid into different soil C pools. On day 3 (Fig. 1, top), a clear discrimination against the carboxyl C of both amino acids and glutamic acids amino-bound position in soil, microbial biomass and Σ-PLFA is evident. On day 10 (Fig. 1, bottom), the recovery of the carboxyl C in soil remained stable, while the recovery of all other positions in soil decreased by up to 60% of applied ^{13}C. This results in an equal recovery of all positions in soil on day 10. In microbial biomass and Σ-PLFA the recovery of ^{13}C from both carboxyl groups and glutamic acids amino bound position on day 10 was still lower than the ^{13}C recovery from other positions of both amino acids. In microbial biomass, the ^{13}C recovery of glutamic acids amino-bound position decreased by 35%. The same amount of ^{13}C from glutamic acid's positions was recovered in Σ-PLFA on both days. Fig. 1 shows that on day 10, all C from the C_2 and C_3 positions of alanine in soil was located in the microbial biomass. The carboxyl C from alanine and glutamic acid, however, was stabilized in soil by other mechanisms.

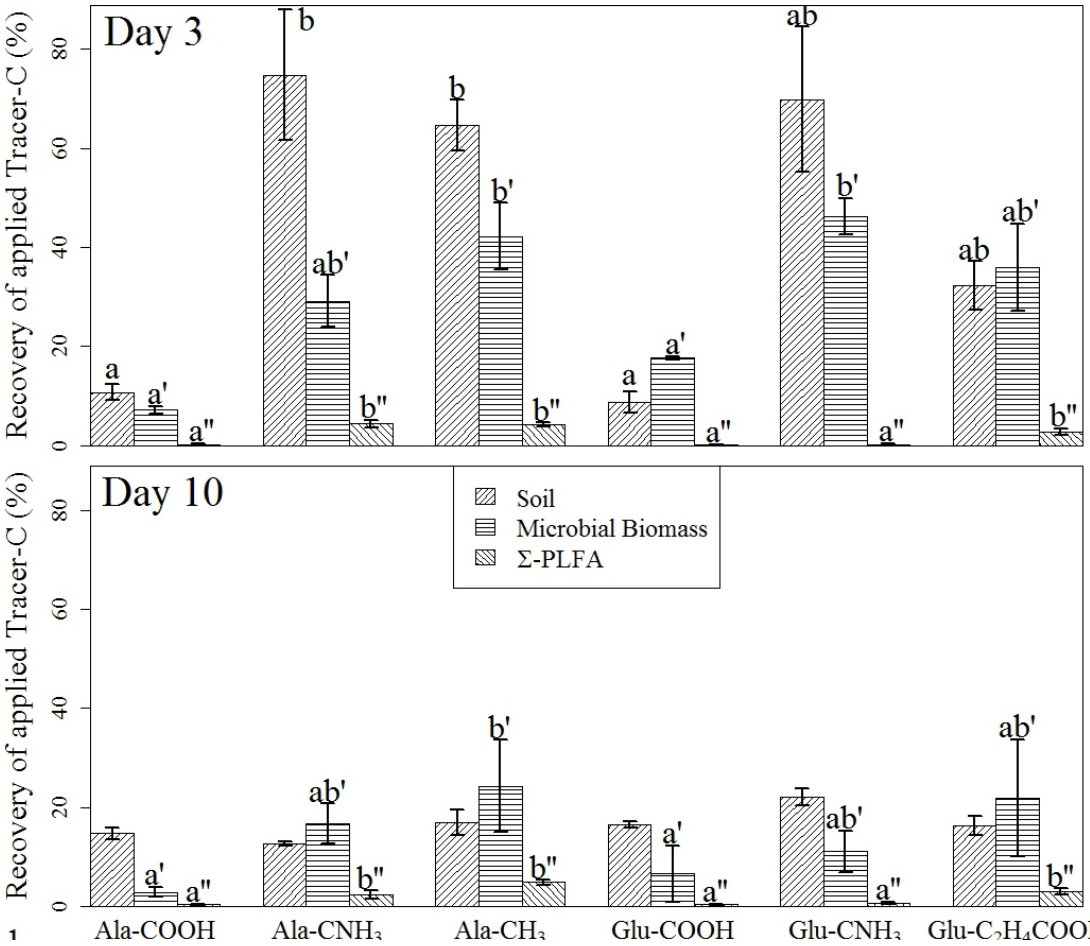

Fig. 1 Recovery of position-specifically ^{13}C labeled Ala and Glu in soil, microbial biomass and Σ-PLFA, 3 (top) and 10 days (bottom) after application. Letters indicate significant differences ($p < 0.05$) between recovery bulk soil (a), microbial biomass (a') and Σ-PLFA (a")

To identify microbial groups, a PCA was performed on the PLFAs C-content from both sampling times. By comparing classification in the literature (ZELLES 1999; ZELLES et al. 1995), the fatty acid groups were matched to microbial groups and through factor loadings they were further subdivided (Supplementary Table 3). Recovery of applied position-specifically labeled C from the two amino acids in most microbial groups (Fig. 2) shows the same pattern as recovery of applied C in Σ-PLFA: The ^{13}C recovery from the carboxyl groups is less than 0.1% of ^{13}C input of both amino acids on both days. On day 3, the recoveries of the amino-bound and the methyl C from alanine were similar. This pattern was different on day 10, when the recovery of alanines amino-bound C was lower than that of its methyl group. In the first days after being taken up by microorganisms and utilized in the cell membrane, only the C_1 position was split from the alanine molecule, while the C_2 and C_3 positions were utilized together.

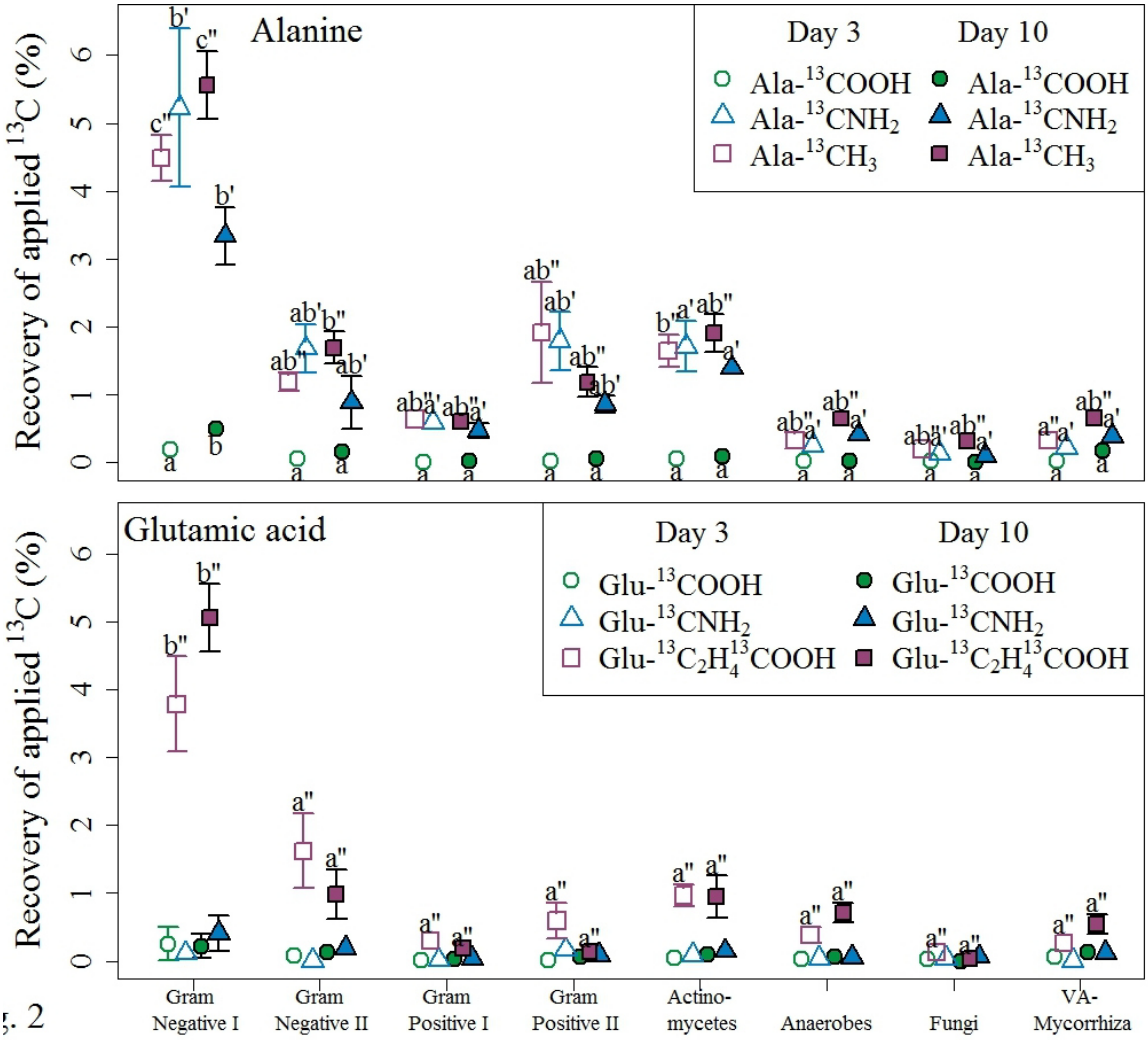

Fig. 2 Recovery of applied ^{13}C from positions of alanine (top) and glutamic acid (bottom) in microbial groups after 3 and 10 days. Letters indicate significant differences ($p < 0.05$) between carboxyl C (a), amino-bound C (a') and methyl C of alanine or the residual molecule of glutamic acid (a'').

The ^{13}C recovery of glutamic acid's positions reveals that it is transformed differently than alanine. From both the amino-bound and the carboxyl C, less than 0.4% were recovered in Σ-PLFA on both days. In contrast, nearly 4% of the residual amino acid C were recovered in Σ-PLFA. In the microbial pathways both C_1 and the C_2 from glutamic acid were split from the residual molecule, which was then incorporated into PLFAs.

The maximum incorporation of C into PLFAs from all positions of both amino acids was achieved by the group of gram negative I (18:1ω7c, 18:1ω9c) (Fig. 2). This group of gram negative prokaryotes took up 4.5 - 5.5% of the methyl C from alanine and also of the residual molecules C from glutamic acid. No other microbial group took up more than 2% from any position. Most prokaryotic groups incorporated more C from C_2 and C_3 positions of alanine than the anaerobic bacteria (cy17:0) and the two eukaryotic groups (Fungi (20:1ω9c, 18:2ω6,9) and VA-Mycorrhiza (16:1ω5c)).

2.3.3.3 Divergence Index

The divergence index (DI) was used to compare the extent of incorporational discrimination of C from different positions between the pools (Fig. 3) and microbial groups (Fig. 4).The DI relativizes differences in absolute uptake. Regarding the DI in soil, microbial biomass and Σ-PLFA (Fig. 3), differences between days 3 and 10 after tracer application can be observed (Fig. 3). The relative incorporation in soil on day 3 shows a clear discrimination against the carboxyl positions; on day 10, there is no significant difference in DI between any position of alanine or glutamic acid. Although only the declined discrimination against alanines carboxyl C between day 3 and 10 is significant ($p < 0.05$), the reduced discrimination between all positions in soil between day 3 and day 10 shows that during the initial reactions, the pathways of C from different positions of the two amino acids differ greatly. However, ten days after application, the source position in the molecules is not determining for fixation in soil. In microbial biomass, a reduction of discrimination between positions may be taking place – the discrimination between positions is not significant anymore – but high standard errors prevent any certain conclusions. In Σ-PLFA, alanine's amino-bound position had a DI equal to that of its methyl position on day 3 but a lower DI on day 10. The incorporation pattern of glutamic acids positions into Σ-PLFA did not change between day 3 and day 10.

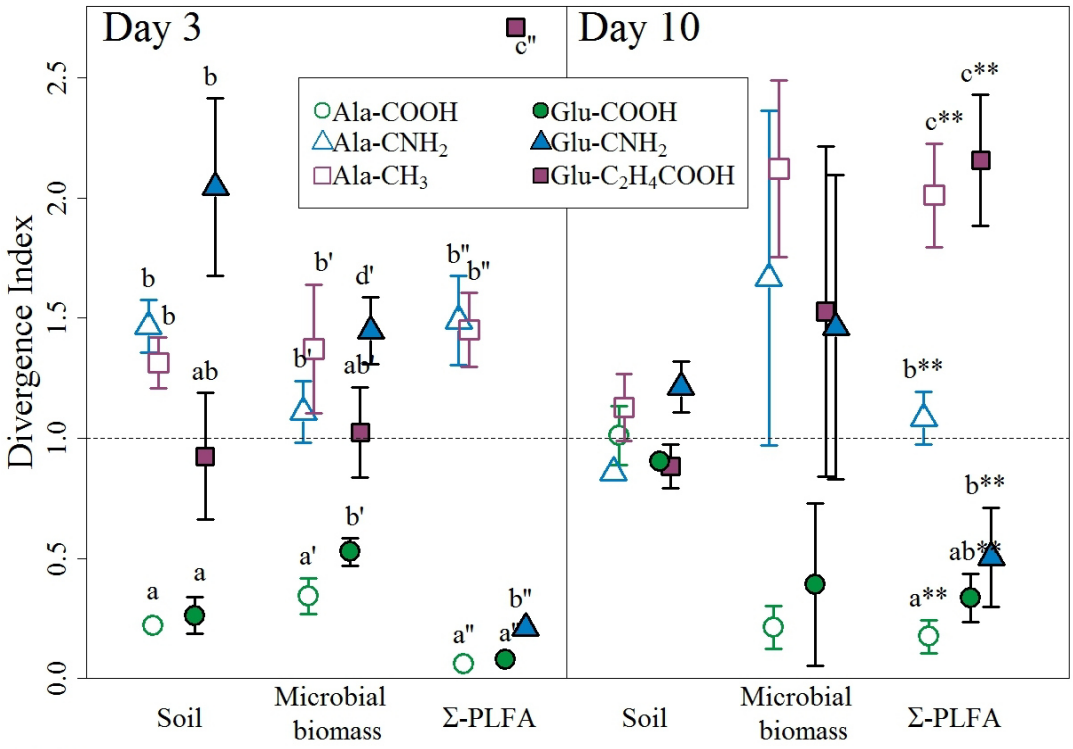

*Fig. 3 Divergence index (DI) reflecting incorporational discrimination between C positions into soil, microbial biomass and Σ-PLFA, 3 (left) and 10 (right) days after applying ^{13}C-labeled alanine (Ala) and glutamic acid (Glu). Letters indicate significant differences ($p < 0.05$) in the relative incorporation of the C positions into soil (a), microbial biomass (a'), Σ-PLFA (a") on day 3, and into Σ-PLFA (a**) on day 10 after tracer application.*

Despite the differences in absolute ^{13}C recovery in PLFAs between the microbial groups (Fig. 2), the DI (relative ^{13}C recovery) of most microbial groups PLFAs (Fig. 4) shows a similar pattern, which also generally reflects the pattern described by Σ-PLFA (Fig. 3). In most PLFAs, there was an average to above-average relative incorporation of the amino-bound group of alanine on day 3, which is prominent on day 10 (Fig. 4). The DI for the methyl position of alanine and the residual molecule of glutamic acid was above-average in all microbial groups and on both days. The amino-bound C of glutamic acid was incorporated less than average in all microbial groups and on both days. Exceptions to this pattern were the groups of gram positive II (i15:0, i17:0) and fungi (20:1ω9c, 18:2ω6,9), which both showed no significant discrimination against any position on either days.

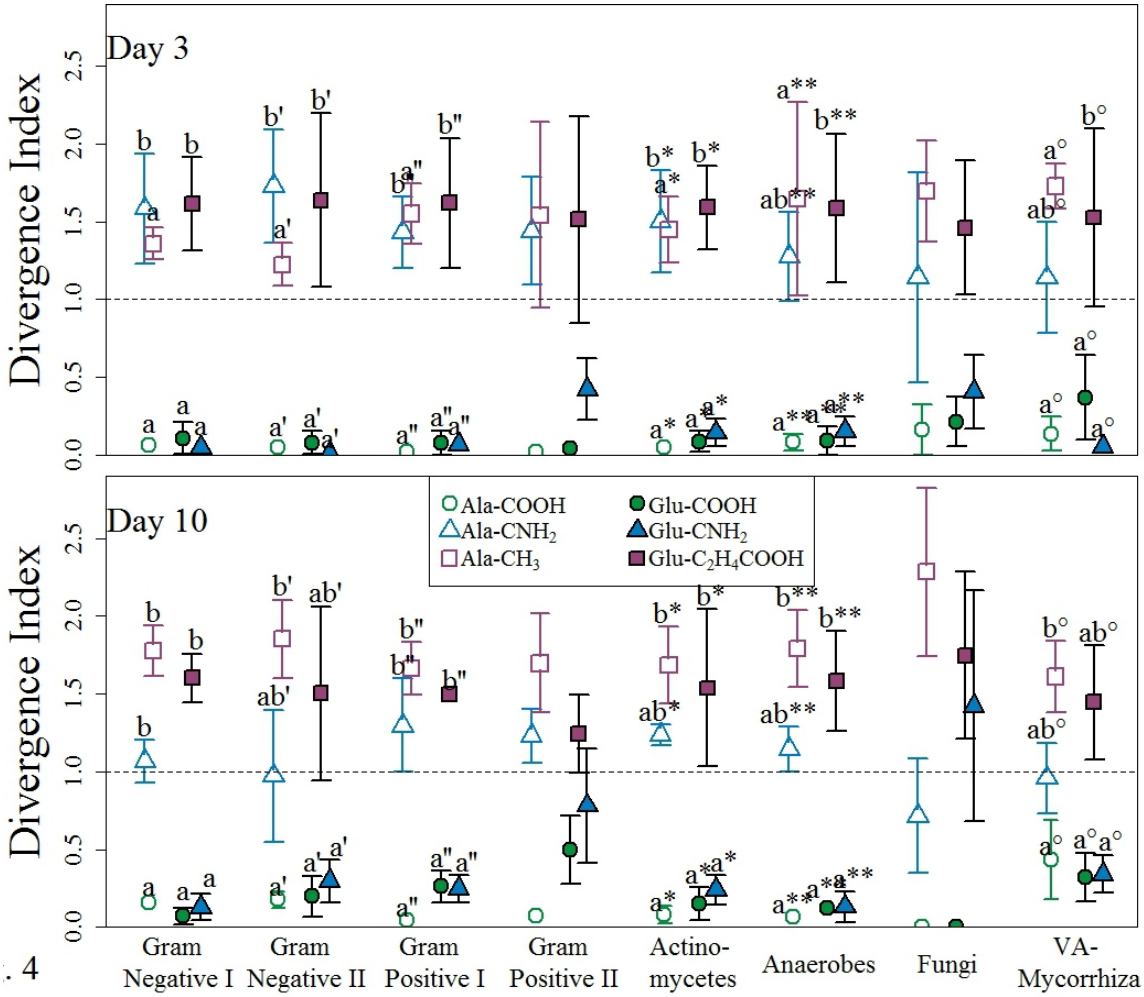

Fig. 4 Divergence Index (DI), reflecting discrimination between C positions of alanine (Ala) and glutamic acid (Glu), 3 (top) and 10 (bottom) days after application. Letters indicate significant differences ($p < 0.05$) between the relative incorporation of the C positions into the microbial group a: gram negative I, a': gram negative II, a'': gram positive I, a: actinomycetes, a°: VA-mycorrhiza.*

2.3.4 Discussion

2.3.4.1 Incorporation of carbon from amino acids in soil and microbial biomass

On day 3, the ^{13}C recovery from alanine and glutamic acid in soil, microbial biomass and Σ-PLFA shows the same pattern (Fig. 3). C from the carboxyl group is recovered less than that from the amino-bound and methyl positions and the residual molecule of glutamic acid in all pools. This was expected as the carboxyl C has the highest oxidation state and is therefore most prone to being removed from the molecules. This is achieved by decarboxylation. Enzymes necessary for decarboxylation of amino acids have been found in soil (Braun et al., 1992; Tena et al., 1986) as well as in prokaryotic

and eukaryotic microorganisms (Caspi et al., 2008). On day 10, the amount of carboxyl C from both amino acids in soil remained stable, while the recovery of all other positions decreased. Although it seems contradictory this can also be explained by the high reactivity of the carboxyl C: not only can it be oxidized to CO_2 easily, but it can also react with other soil components and be thus stabilized. This possible stabilization mechanism is supported by results of Kuzyakov (1997), who found position-specifically labeled ^{14}C from the carboxyl position of alanine in humic and fulvic acids. As complex macromolecules, humic and fulvic acids contain a variety of functional groups such as hydroxyl groups, methylenes, ethers and esters in aliphatic chains (Simpson et al., 2002). It is possible for carboxyl C from microbial sources to react with humic macromolecules, e.g. by forming ester-linkages with hydroxy groups. Esters are highly inert, therefore the former carboxyl C will be stabilized from further degradation.

In contrast to carboxyl C, the ^{13}C recovery from the amino-bound and methyl group of alanine in soil decreased by up to 60% between days 3 and 10. Compared to the decrease in recovery of these positions in soil, the amount incorporated into microbial biomass is still high on day 10. In microorganisms, alanine can be used catabolically, in the citric acid cycle and anabolically, e.g. to produce sugars or fatty acids (Fig. 5) (Caspi et al., 2008). The first reactions for both pathways are the same. Alanine is first deaminated and then decarboxylated, thereafter the resulting acetyl reacts with coenzyme A to form acetyl-CoA. The acetyl-CoA, which consists of the former amino-bound and methyl C from alanine, is then fed into the citric acid cycle or used for biosynthesis. This explains why C from those two positions is recovered in PLFAs, but C from the carboxyl group is not. After incorporation into PLFAs, C from the former amino-bound position is on the terminal position and thus most prone to being oxidized and decarboxylated (Caspi et al., 2008).This process is hinted at by the slight decrease in relative incorporation of alanines amino-bound position between day 3 and 10. The incorporation of the C from the methyl position of alanine and from the residual molecule of glutamic acid in Σ-PLFAs is still high on day 10.

2.3.4.2 Incorporation of tracer into the microbial groups

The ^{13}C incorporation into PLFAs of microbial groups differed by more than one order of magnitude (Fig. 2). As hypothesized, the highest incorporation, with more than 5% ^{13}C uptake, was recorded for a group of gram negative prokaryotes (gram negative I). This fits well with the observations by Griffiths et al. (1999) that gram negatives react fastest to addition of LMWOS, which gives them a competitive advantage.

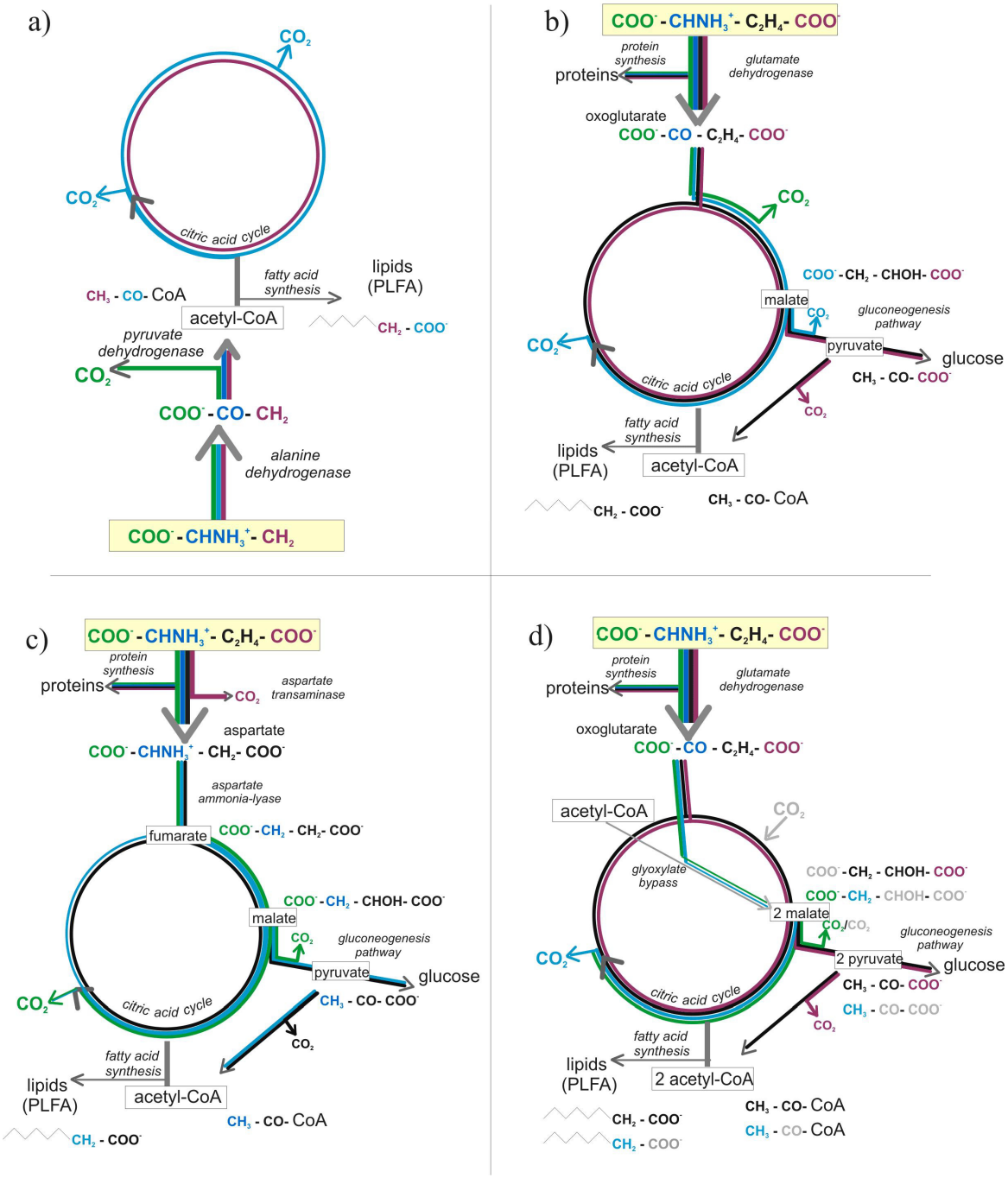

Fig. 5 Microbial transformation pathways of alanine (a) and glutamic acid (b, c, d). As there are different transformation pathways for glutamic acid, it is presented in 3 subfigures. The entrance of alanine (a) occurs from the bottom (in contrast to glutamic acid, b, c, d,) of the citric acid cycle because of its initial transformation to acetyl-CoA.

Three other prokaryotic groups (gram negatives II, actinomycetes and gram positive II), also achieved moderate ^{13}C incorporation. The two eukaryotic groups – fungi and VA-mycorrhiza – were unable to take up as much of the applied amino acid C as the prokaryotic group. This is unsurprising because the turnover of the larger, more complex eukaryotes' biomass is slower than that of prokaryotes' (Bååth 1998, Rousk & Bååth 2007). Accordingly, enrichment of eukaryotic cell components takes longer (Moore et al.,

2005). Apart from a slower turnover, the larger size of eukaryotic cells results in a smaller ratio of surface to volume. As PLFAs are utilized as cell membranes on the surface of the organism and the difference in the ratios of ^{13}C in PLFA to ^{13}C in microbial biomass for various microbial groups is unknown, there is no full comparability between cells of different size. It is also well known that fungi are specialized on more complex substrate than LMWOS.

As in the eukaryotes, the anaerobic bacteria also incorporate only a maximum of 0.7% of the applied C. As the roof we installed prevented excess wetting, the soil was well aerated, so the anaerobic microorganisms could only persist inside anaerobic microhabitats such as microaggregates. Thus, only ^{13}C that permeated into those anaerobic microhabitats could be taken up by anaerobic microorganisms.

2.3.4.3 Discrimination of individual carbon positions by microbial utilization differs depending on oxidation state, amino acid and time

As in soil and microbial biomass, discrimination of the individual C positions of both amino acids also took place in the microbial PLFA. As the percent of ^{13}C recovery (Fig. 2) between the microbial groups' PLFA differs greatly, the discrimination between the positions of alanine and glutamic acid is best evaluated with the DI (Fig. 4).

On day 3, there was no difference in relative incorporation of ^{13}C from the methyl and amino-bound C of alanine for most microbial groups. Nearly no ^{13}C from alanine's carboxyl group was recovered in the PLFAs and the incorporation of alanine's ^{13}C into microbial biomass is much lower than that of its amino-bound and methyl position. Accordingly, we can conclude that during the three days after applying the amino acid, the C_1 atom in alanine is split from the molecule quickly, whereas C_2 and C_3 remain bonded. Presumably, the alanine molecule is taken up and then metabolized in the main alanine utilization pathway: deamination to pyruvate and after decarboxylation by pyruvate dehydrogenase, transformation to acetyl-CoA (Fig. 5a). This molecule then either enters the citric acid cycle (de Kok et al., 1998) or fatty acid synthesis (Caspi et al., 2008). On day 10, the DI of the amino-bound C is slightly lower than that of alanine's methyl C in most microbial groups, which can be explained by the further reactions in microorganisms: If the molecule is used catabolically in the citric acid cycle, then the acetyl-CoA condensates with oxalate to citric acid. After this reaction, the former amino-bound C of alanine is one of citric acid's carboxyl groups. Thus, the chance for the amino-bound position to be degraded into CO_2 during the next step – the formation of 2-oxoglutarate (Camacho et al., 1995) – is about 1:3, whereas the methyl position is still incorporated in the non-reactive chain. After every circuit of the citric acid cycle, C from the alanine molecule can

either be transferred to a biosynthesis pathway or continue partaking in this cycle (Caspi et al., 2008). We detected ^{13}C in the extracted PLFAs. Thus, the alanine molecules were fed either into the fatty acid biosynthesis pathway or the fatty acid elongation pathway. The initial substance for both these pathways is also acetyl-CoA. As in the citric acid cycle, the former amino-bound C will be the terminal C on the fatty acid molecule and is thus more prone to being degraded than the former methyl C (Caspi et al., 2008). In summary, regardless of whether alanine is used anabolically or catabolically, the former amino-bound C will be degraded before the former methyl C (Fig. 5a).

The DI of glutamic acid shows that it is transformed in different pathways than alanine (Fig. 5 b, c and d). We find a discrimination against C from both the carboxyl and the amino-bound position, which means that either C_1 and C_2 were split from the residual molecule together or in short succession. Glutamic acid most commonly enters the citric acid cycle after being transformed into oxoglutarate (Caspi et al., 2008). Oxoglutarate has the original five atoms in its chain and loses the carboxyl group immediately after entering the citric acid cycle. This transformation does not yet explain why the amino-bound C has a DI as low as the carboxyl group. One possible explanation is that the metabolization of the former glutamic acid molecule is so fast that, after three days, the citric acid cycle has already removed most of the formerly amino-bound C. However, the DI of microbial biomass (Fig. 3) does not shows a discrimination against glutamic acid's amino-bound C. If glutamic acids amino-bound positions would be solely lost by repetitive oxidation in the citric acid cycle, we would not only see a discrimination in PLFA but also in overall microbial biomass. Therefore, the explanation for the less than average incorporation of glutamic acids amino-bound position might be found by tracing the anabolic pathway that leads to fatty acid formation in microbial cells. As opposed to alanine, glutamic acid is not transformed into acetyl-CoA (the starting substance for fatty acid synthesis) before it is fed into the citric acid cycle. But in that cycle, glutamate is transformed into malate, which can be fed into the gluconeogenesis pathway, producing sugars and other anabolic products from non-sugar substrates (Caspi et al., 2008; Katz and Tayek, 1999). One of the intermediaries in this pathway is pyruvate, which can be transformed into acetyl-CoA. Following these transformations, the acetyl-CoA molecule will consist of two of glutamic acid residual Cs (Fig 5b).

In contrast to alanine, the DI for glutamic acid's amino-bound C is not convergent for all microorganisms: the groups gram positives II and fungi show specific incorporation patterns. The difference between glutamic acid's amino-bound position and its methyl position is not significant. Following from the aforementioned transformation pathways of glutamic acid, it is impossible for the amino-bound C to be incorporated into microbial PLFAs, so there should be a significant difference between the amino-bound and methyl

C. Detection of this position in the PLFAs can be only be explained by the use of alternative pathways. Two pathways for glutamic acid utilization exist: aspartate production from glutamic acid prior to the citric acid cycle (Fig. 5c) and the glyoxylate bypass (Fig. 5d). Glutamic acid is transformed into aspartate by removing the C_5 position; thereafter, the aspartate is deaminated and fed into the citric acid cycle. The glyoxylate bypass avoids the exhaustion of CO_2. This yields two instead of one malate molecules, but will produce no energy. The glyoxylate bypass in the citric acid cycle is used by bacteria, and its enzymes have also been found in fungi (Maxwell et al., 1977; Munir et al., 2001). Again, for fatty acid production, pyruvate has to be produced by the gluconeogensis pathway. In contrast to the "common" pathway mentioned above, glutamic acid's former amino-bound position will remain in the molecule. Therefore, both the production of aspartate and the utilization of the glyoxylate bypass can explain why we find no significant difference between the amino-bound and methyl C in the PLFAs of gram positives II and fungi (Fig. 5c and d) (Caspi et al., 2008).

As mentioned above, the bypass produces no energy and is thus only relevant at C deficiency conditions. This indicates that the gram positives II and fungi might be suffering from C deficiency and need to utilize specific pathways to meet their anabolic demands. Such groups might be of special interest when environmental conditions change, especially an altered C input.

2.3.5 Conclusions

This study has shown that position-specific ^{13}C labeling and compound-specific ^{13}C-PLFA analysis are a valuable combination to gain new insights into microbiological transformations of amino acids in soil. As hypothesized, the carboxyl C of both amino acids is oxidized rapidly by microorganisms. Methyl C from alanine and glutamic acid residual molecules showed high recoveries in all microbial groups 10 days after the application. While functional group and oxidation state help to predict the incorporational behavior for carboxyl, methyl and residual positions, the amino-bound C from two amino acids is transformed differently. C_2 from alanine is incorporated like its methyl C on day 3, but its recovery decreased slightly on day 10. The DI revealed that, although C_2 from glutamic acid is lost from most microbial groups, gram positives II and fungi incorporate it into their PLFA. This was explained by special microbiological pathways - the glyoxolate bypass and the transformation of glutamic acid into aspartate prior to being fed into the citric acid cycle - used under C deficiency. As glutamic acid has proven to be a sensitive tracer for environmental conditions, it could be applied to observe metabolic changes under environmental gradients.

None of these findings could have been achieved without using position-specifically labeled substances. The method of coupled position-specific ^{13}C labeling and compound-specific isotope analysis can in the future be further expanded to investigate pathways of other microbial or soil constituents, including other amino acids and amino sugars, carboxyl acids, sugars, humic and fulvic acids. This would help to identify further transformation and stabilization processes and improve our knowledge about soil C fluxes.

Acknowledgements

We thank the DFG for the funding for the funding of this work (KU1184 19/1) and Stefanie Boesel from the GC-EA-IRMS Laboratory of the Department of Soil Biogeochemistry (Halle) for the reliable measurement of all bulk isotope samples.

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Supplementary Data

Supplementary Table A1: Fatty acids in the external standard

FA-type	Name	Common name	Abbreviation	Retention time (s)*
Saturated	Tetradecanoic acid	Myristic acid	14:0	150
	Pentadecanoic acid	-	15:0	355
	Hexadecanoic acid	Palmitic acid	16:0	630
	Heptadecanoic acid	Margaric acid	17:0	1010
	Octadecanioc acid	Stearic acid	18:0	1520
	Eicosanoic acid	Arachidic acid	20:0	2930
Branched chain	11-Methyltridecanoic acid	Anteisomyristic acid	a14:0	100
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	90
	12-Methyltetradecanoic acid	12-Methylmyristic acid	a15:0	290
	13-Methyltetradecanoic acid	13-Methylmyristic acid	i15:0	270
	13-Methylpentadecanoic acid	Anteisopalmitic acid	a16:0	545
	14-Methylpentadecanoic acid	Isopalmitic acid	i16:0	525
	14-Methylhexadecanoic acid	14-Methylpalmitic acid	a17:0	890
	15-Methylhexadecan acid	15-Methylpalmitic acid	i17:0	860
Cyclo-propane	cis-9,10-Methylenhexadecanioc acid	cis-9,10-Methylpalmitic acid	cy17:0	940
	cis-9,10-Methylenoctadecanoic acid	Dihydrosterculic acid	cy19:0	2025
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic acid	10Me16:0	780
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	1745
Mono-unsaturated	9-Tetradecaeoic acid	Myristoleic acid	14:1w5c	130
	cis-11-Hexadecenoic acid	-	16:1w5c	595
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1w7c	570
	cis-Octadecenoic acid	Cis-Vaccenic acid	18:1w7c	1400
	cis-9-Octadecenoic acid	Oleic acid	18:1w9c	1375
	11-Eicosenoic acid	Eicosenoic acid	20:1w9c	2700
Poly-unsaturated	cis,cis-9,12-Octadecadienoic acid	Linoleic acid	18:2w6,9	1320
	6,9,12-Octadecatrienoic acid	g-linolenic acid	18:3w6,9,12	1355
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4w6	2320

*for 45 m ± 0.5 m column lengths, deviations of ± 15 s possible

Supplementary Table A2: Results of factor analysis

Fatty acid	Factor 1	Factor 2	Factor 3	Factor 4	Microbial group
i16:0	**0.76**	-0.12	-0.48	0.19	Actinomycetes
10Me16:0	**0.83**	-0.26	0.26	0.17	
a17:0	**0.79**	0.19	-0.41	0.06	
10Me18:0	**0.59**	0.62	-0.21	0.28	
18:1w9c	**-0.76**	0.28	0.22	0.15	Gram negatives I
18:1w7c	**-0.73**	-0.61	0.13	0.07	
20:4w6	**0.83**	-0.01	-0.06	0.18	Protozoa
20:1w9	**0.80**	-0.02	0.18	0.11	Fungi
18:2w6,9	**0.79**	-0.05	0.45	0.03	
a15:0	0.50	**-0.57**	0.13	0.07	Gram positives I
16:1w5c	-0.10	**0.93**	0.04	0.15	VA-mycorrhyza
cy17:0	0.55	-0.23	**0.57**	0.06	Anaerobes
i15:0	-0.31	-0.48	**-0.67**	-0.02	Gram positives II
i17:0	0.36	-0.37	**-0.69**	-0.30	
a16:0	0.13	0.35	0.12	**-0.71**	Gram positives III
16:1w7c	-0.50	-0.19	0.24	**0.51**	Gram negatives II
i14:0	-0.38	-0.15	-0.47	0.48	N/A
cy19:0	0.11	-0.46	0.07	-0.20	
18:3w6,9,12	-0.02	0.95	-0.19	0.00	

2.4 Study 4: Biogeochemical transformations of amino acids in soil assessed by position-specific labeling

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Abstract

BACKGROUND AND AIMS: Amino acid turnover in soil is an important element of terrestrial carbon and nitrogen cycles. This study accounts for their driver - the microbial metabolism - by tracing them via the unique isotopic approach of position-specific labeling.

METHODS: Three ^{14}C isotopomers of alanine at five concentration levels combined with selective sterilization were used to distinguish sorption mechanisms, exoenzymatic and microbial utilization of amino acids in soil.

RESULTS: Sorption and microbial uptake occurred immediately. Unspecific microbial uptake followed a linear kinetic, whereas energy-dependent uptake followed Michaelis-Menten. Less than 6% of the initially added alanine was sorbed to soil, but after microbial transformation products were bound to the soil matrix at higher proportions (5-25%). The carboxyl group (C-1) was rapidly oxidized by microorganisms, whereas C-2 and C-3 positions were preferentially incorporated into microbial biomass. Dependency of C metabolization on amino acid concentration reflected individual alanine transformation pathways for starvation, maintenance and growth conditions.

CONCLUSIONS: This study demonstrates that position-specific labeling determines the mechanisms and rates of C cycling from individual functional groups. This approach reflected underlying metabolic pathways and revealed the formation of new organic matter. We therefore conclude that position-specific labeling is a unique tool for detailed insights into submolecular transformation pathways and their regulation factors.

Keywords: Position-specific tracers, Amino acids stabilization, Sorption, Exoenzyme and uptake kinetics, Metabolic tracing, Soil organic matter formation, Sterilization and inhibition methods, Biochemical pathways

2.4.1 Introduction

Studies on transformation of organic substances in soils are important for understanding of C and N cycles in terrestrial ecosystems. Plant residues and rhizodeposits are the main sources of organic matter in soils (Rasse et al., 2005). Therefore, many studies have focused on decomposition, microbial utilization and stabilization of C from these sources (von Luetzow et al., 2006).

During decomposition of litter, macromolecular compounds are depolymerized by enzymes into low molecular weight organic substances (LMWOS) (Cadisch and Giller, 1996). LMWOS are the lightest (<250 Da) components of DOC (Boddy et al., 2007) from substance classes such as organic acids, amino acids, mono- and disaccarides, amino sugars, phenols and many more (van Hees et al., 2005a). In addition to litter, rhizodeposition is a source of LMWOS in soil. Microorganisms determine the fate of LMWOS in soil because they either produce them, decompose them to CO_2 and NH_4^+ (catabolism) or incorporate them in cellular compounds (anabolism). The importance of LMWOS is not connected with their pool size (Fischer et al., 2007), but with the huge fluxes that pass through this pool. Therefore, the transformation pathways of LMWOS represent a crucial step of soil C and N fluxes, and a molecular-level knowledge of these processes is needed (van Hees et al., 2005a).

Within the LMWOS, amino acids play an important role because they are the quantitatively most important compounds coupling the C and N cycle. In topsoil, amino bound N constitutes 7-50% of the total organic N (Gardenas et al., 2011; Stevenson, 1982a). Thus, many recent studies focused on the fate of N-containing LMWOS (Hobbie and Hobbie, 2010; Jones et al., 2004b; Knowles et al., 2010; Kuzyakov, 1996; Lipson et al., 2001; Vinolas et al., 2001a) and investigated the three major pathways of amino acid utilization in soil: 1) sorption (Jones, 1999), 2) extracellular transformation, and 3) intracellular metabolization (Vinolas et al., 2001a; Vinolas et al., 2001b) which can be separated by selective inhibition of biotic processes.

Sorption strongly depends on the functional group of the amino acid (Jones and Hodge, 1999): it can occur by ion exchange of positively charged amino groups, by ligand exchange of carboxyl groups and by hydrophobic interactions with alkyl groups. To date, nearly all studies assumed sorption of the entire molecule by soil sorbents. Only a few studies on glycine sorption indicated abiotic degradation of the sorbed amino acid (Wang and Huang, 2003, , 2005).

Amino acids can be transformed extracellularly, mainly by exoenzymes attached to cell surfaces (Geisseler et al., 2010). Deamininating (Killham, 1986) and oxidizing (Bohmer et al., 1989; Braun et al., 1992) extracellular systems are described in the litera-

ture, but neither their relevance nor the differences between extra- and intracellular pathways have been investigated (Burns, 1982).

Intracellular amino acid metabolization follows the uptake by transport systems (Anraku, 1980; Hediger, 1994; Hosie and Poole, 2001). Uptake kinetics of some amino acids has already been elucidated (Vinolas et al., 2001a; Vinolas et al., 2001b). Barraclough (1997) showed that the majority of N mineralization of amino acids occurred inside the cells. Knowles et al. (2010) described for the first time the decoupling of N and C metabolization in soil, discovering a preferential retention of amino acid N with respect to C. Nonetheless, as they used uniformly labeled tracers, they could not determine the fate of the C skeleton. We hypothesize that the fate of amino acid C and N in soil is mainly determined by the dominating intracellular metabolization pathways. Therefore, identification of microbial metabolization is a crucial step for understanding and predicting C and N fluxes.

In addition to abiotic factors such as temperature (Dijkstra et al., 2011c; Vinolas et al., 2001b) or soil properties (Gonod et al., 2006; Kemmitt et al., 2008), the concentration of a substrate is a key driver of the intracellular metabolization (Dijkstra et al., 2011a; Fischer and Kuzyakov, 2010b; Schneckenberger et al., 2008). Soil amino acid concentrations range from 0.5 µM in root-free bulk soil to 5 mM directly next to bursting cells (Fischer et al., 2007; Jones and Hodge, 1999). We expect cellular uptake and metabolism always dominate the amino acid removal from soil solution and that sorption only plays a relevant role at low substrate concentrations. For our study, we chose alanine as a representative amino acid for the neutral amino acids as it is one of the most dominant amino acids in soil solution (Fischer et al., 2007). In addition, alanine was chosen because it is very close to the basic C metabolism of the cell: by oxidative deamination alanine can be transferred to pyruvate, which is a suitable substrate for metabolic tracing experiments in plants and soils (Dijkstra et al., 2011a; Tcherkez et al., 2005).

To elucidate intra- and extracellular alanine transformation pathways, we used the approach of position-specific labeling. This tool is commonly used in biochemistry to investigate metabolization pathways, but has rarely been applied in soil science (Dijkstra et al., 2011a; Dijkstra et al., 2011b; Dijkstra et al., 2011c; Fischer and Kuzyakov, 2010b; Fokin et al., 1993, , 1994; Haider and Martin, 1975; Kuzyakov, 1997; Nasholm et al., 2001). It overcomes the limitations of uniform labeling because it allows differentiating between incorporation of fragments vs. incorporation of entire molecules.

Coupling of position-specific labeling with soil sterilization enables us to separate abiotic splitting of alanine from extracellular and from cellular metabolism. We assume that extra– and intracellular transformation differ from each other as they are based on different enzymes. By comparison of the kinetics of alanine removal from soil solution in

the non-inhibited and respiration-inhibited treatments, the relevance of extra- versus intracellular transformations of alanine was compared. We hypothesize that under soil conditions microbial uptake systems and intracellular metabolization dominate the fate of alanine in soil. Comparing our results with known microbial metabolization pathways enables the identification of metabolic changes depending on substrate concentration.

2.4.2 Material and Methods

2.4.2.1 Soil

Topsoil (0-10 cm) from the Ap horizon of a silt loam haplic Luvisol (WRB, 2006) was collected from a field in Bavaria with a crop sequencing of barley, wheat and triticale (49.907 N, 11.152 E, 501 m asl, mean annual temperature 6-7 °C, mean annual precipitation 874 mm). The soil had a pH_{KCl} of 4.88 and pH_{H2O} of 6.49, total organic C and total N content were 1.77% and 0.19%, respectively, and potential CEC was 13.6 $cmol_c$ kg^{-1}. Soil was sieved to 2 mm, and all roots were removed with tweezers. Soil was stored at field moisture at 5 °C not longer than one week until the experiment started.

2.4.2.2 Chemicals and radiochemicals

Stock solutions with 1, 10, 100, 1000, and 10000 µM alanine and an equal activity of 10^4 DPM ml^{-1} (Disintegrations Per Minute and ml) were prepared from U-^{14}C-labeled alanine and the position-specifically labeled isotopomers 1-^{14}C-, 2-^{14}C- and 3-^{14}C-labeled alanine (American Radiolabeled Chemicals Inc, St. Louis, USA) as well as non-labeled alanine (Sigma-Aldrich, Taufkirchen, Germany).

Sterilization solutions were produced with 1 mM NaN_3 to inactivate aerobic microbial respiration or with 1 mM NaN_3 and 1 mM $HgCl_2$ to denaturate all proteins and reach full inhibition of biotic processes. Effectiveness of the chosen azide inhibitor was evaluated by a qualitative 2,3,5-triphenyltetrazoliumchloride incubation (TCC, Sigma-Aldrich, Taufkirchen, Germany). Therefore 0.63 µg of the yellow dye TCC were added to the 1 ml of soil suspension in this preexperiment.

2.4.2.3 Experimental setup

The effects of two factors on alanine transformations in soil were investigated: 1) the concentration of alanine, and 2) the extra- and intracellular as well as abiotic processes of alanine removal from soil solution, separated by sterilization. Therefore, three

sterilization treatments were used (Fig. 1): 1) treatments without any inhibition, where three groups of processes occured: intracellular metabolism, extracellular transformation and physicochemical sorption, 2) treatments with inhibition of aerobic respiratory chains by azides (Burns, 1982), where only extracellular processes are active and sorption could occur, and 3) treatments with full inhibition, where microbial metabolism as well as exoenzymes were inhibited by $HgCl_2$ (Stevenson and Verburg, 2006; Wolf et al., 1989) and only sorption could remove alanine from the soil solution (Fig. 1). We define here as extracellular transformations all processes (decomposition, decarboxylation, condensation, etc.) localized in the soil solution or periplasm (Glenn, 1976) which don't depend on intracellular energy metabolism (i.e. proton gradient or ATP) and can not be inhibited by NaN_3. Biotic transformations sums up extra- as well as intracellular processes.

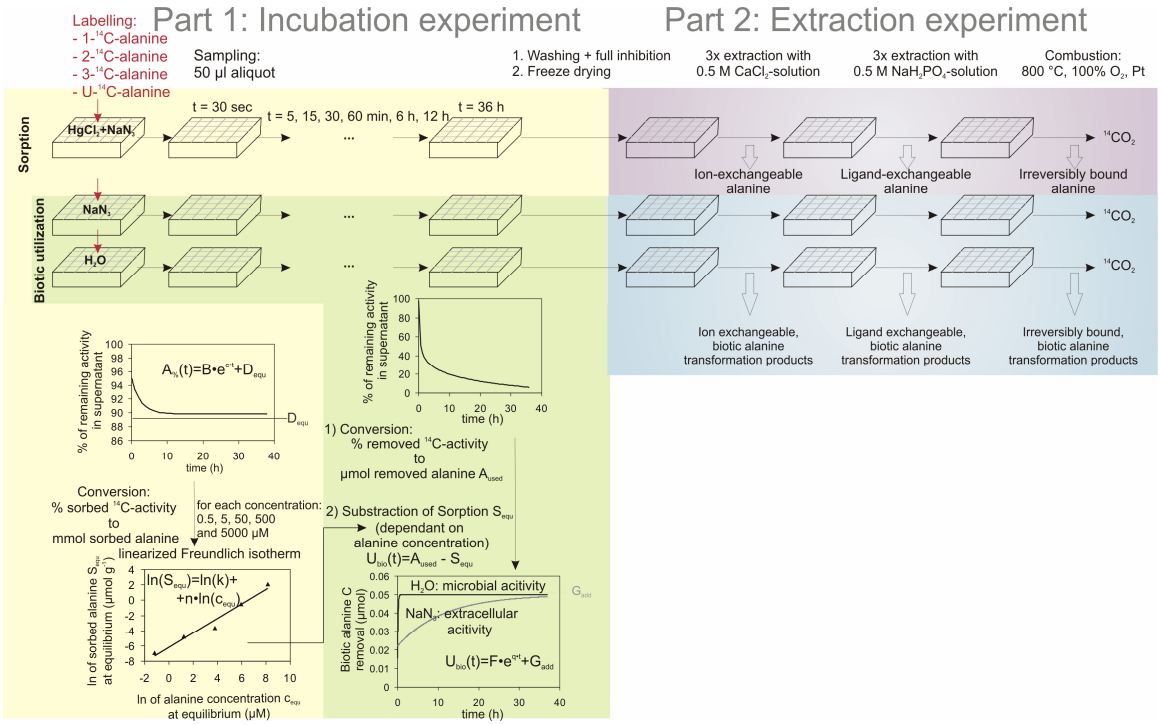

Fig. 1 Scheme of the experimental design for one of the five concentrations: in part 1 on the left side (incubation experiment) yellow-shaded plates shows fully-inhibited treatment to investigate sorption whereas green-shaded plates reflect biotic utilization (upper line with only extracellular activity and lower line with extra- and intracellular activity). Yellow-shaded graphs demonstrate the calculation of the sorbed proportion of alanine by the sorption isotherm, which is derived from the fully inhibited treatment. Green-shaded graphs reveal the calculation of the biotic utilization by substracting the sorption from the percentage of alanine removal from supernatant. In part 2 on the right side (extraction experiment) purple-shaded plates reflect the fully-inhibited treatment and thus extraction of untransformed alanine by the sequential procedure. Blue-shaded plates show desorption of biotic alanine transformation products (upper plate with only extracellular activity and lower plate with extra- and intracellular activity).

The experiment consisted of two parts (Fig. 1): In the first part – the incubation experiment- the processes removing alanine from the supernatant were investigated. The incubation was performed in 24-deep-well plates (6 ml volume per well) on a rotational shaker at 200 rpm with 200 mg field fresh soil per replication. Before adding the alanine, the soil was pre-incubated for 1 h with 0.5 ml of 1 mM sterilization solutions or distilled water, respectively. Pre-incubation was performed under intensive shaking to enable a homogenous sterilization of the entire soil volume under high oxygen supply. Thus, during pre-incubation anaerobic processes were prevented, the stored energy could be consumed and no new energy reserves were produced.

In the treatment with extracellular processes, the intracellular respiration was inhibited with 0.5 ml 1 mM NaN_3. Although chosen NaN_3-concentrations are far above those described for respiratory chain inhibition (Kita et al., 1984) some activity may remain in the soil suspension. This was evaluated by a triphenyl-tetrazolium chloride assay. This dye is intracellularly reduced by various dehydrogenases (Kvasnikov et al., 1974; Mohammadzadeh et al., 2006). An active intracellular metabolism leads to the formation of insoluble red formazan crystals within living cells. In the treatment with full inhibition, denaturation of proteins was achieved by adding 0.5 ml of 1 mM $HgCl_2$ and 1 mM NaN_3.

After pre-incubation, 0.5 ml of the alanine-solution was added. All experiments were performed with uniformly labeled alanine and the three isotopomers. The soil suspension was shaken for 30 seconds, centrifuged at 2000 rpm and an aliquot of 50 µl was removed for ^{14}C measurement. After remixing, incubation was continued, and further 50 µl were sampled 5, 15, 30 and 60 min and 6 h, 12 h and 36 h after addition of ^{14}C labeled alanine. After incubation, the remaining supernatant was removed and soil was washed three times – first with distilled water, then with full inhibition solution and finally with distilled water.

In the second part of the study - the desorption experiment - we evaluated the binding mechanisms of alanine C in soil (Fig. 1). In treatments with full inhibition, the extracted C reflects alanine C itself, as no biotic transformation occurred. In treatments with biotic activity, the microbial or extracellular transformation products were extracted. The washing step with $HgCl_2$ led to denaturation of membrane proteins and thus a loss of membrane integrity. This allowed the joint extraction of water soluble cytoplasm compounds and extracellular transformation products. Macromolecular compounds like proteins, polysaccharides or peptidoglycan as well as hydrophobic compounds like the membrane lipids could not be extracted by a salt solution.

For the desorption experiment, 0.5 ml of 0.5 M $CaCl_2$ solution was added to the soil and shaken for 2 h. The solution was centrifuged, and supernatant was removed and stored for ^{14}C analysis. Desorption was repeated three times, and the supernatants were

combined to one solution, in which ^{14}C was analyzed. This desorption treatment with $CaCl_2$ enabled evaluating the amount of alanine being weakly bound, mainly by ion exchange. After extraction with $CaCl_2$ the same procedure was done three times with 0.5 M NaH_2PO_4 solution to extract the alanine bound by ligand-exchange. To estimate irreversibly bound alanine C, the soil was freeze-dried and combusted at 600 °C for 10 min under a constant O_2 stream with a HT 1300 solid combustion module of the multi N/C 2100 analyzer (Analytik Jena, Jena Germany). $^{14}CO_2$ released by combustion was trapped in 10 ml of 1 M NaOH. The irreversibly bound pool contains untransformed, irreversibly bound alanine C as well as macromolecular, hydrophobic or irreversibly bound microbial transformation products.

2.4.2.4 Radiochemical analyses

^{14}C activity of the supernatants was determined using a scintillation counter (Wallac 1450, MicroBeta® TriLux, PerkinElmer, Walham MA; USA) by adding 50 µl of the supernatant directly to 0.6 ml scintillation cocktail (EcoPlus, Roth Company, Germany) in transparent 24-well plates. Remaining supernatant, washing solution and desorption solution were measured in glass scintillation vials with the LS 6500 scintillation counter (LS 6500, Beckman-Coulter, Krefeld, Germany) with a 1:2 ratio of solution to scintillation cocktail and a 1:8 ratio for the $CaCl_2$ and NaH_2PO_4 solutions. ^{14}C activity in the NaOH solution was measured with a 1:2 ratio of sample to scintillation cocktail after 24 h of dark storage after disappearance of chemoluminescence. All measurements with the LS 6500 were also performed with blanks of the respective solutions ($CaCl_2$, NaH_2PO_4 or NaOH) and background corrected by subtracting this value from each measurement result.

2.4.2.5 Calculation of the kinetics of alanine utilization

To calculate the biotic utilization the amount of sorbed alanine C has to be subtracted from the total removal from soil suspension. Therefore, the decrease in ^{14}C activity in the supernatant of the fully inhibited treatment ($A_{\%}(t)$ in percent of added activity) was fitted to an exponential equation (Fig. 1) where B (% of added activity) and c (1/h) are the fitted parameters and D_{equ} is the remaining percentage of activity in the supernatant at equilibrium. The remaining activity D_{equ} was converted into the amounts of sorbed alanine per g soil (S_{equ} in µmol g^{-1}) and the dissolved alanine concentration c_{equ} (µM) were calculated and all five concentration treatments were fitted by a linearized Freundlich sorption isotherm with the sorption affinity constants k and n (Fig. 1). Based on Fischer and Kuzyakov (2010b), the fitted sorption isotherm was used to calculate the

sorbed amount of alanine (S_{equ} in µmol g^{-1}) at different concentration (c_{equ}) in the supernatant .

The biotic alanine C utilization $U_{bio}(t)$ (µmol) per well was calculated by subtracting the sorbed amount of alanine S_{equ} (µmol) from the amount of alanine $A_{used}(t)$ (µmol) removed from the supernatant (Fig. 1). For those concentration treatments in which nearly all of the added alanine was biologically used after 36 h (0.5 µM to 500 µM), biotic utilization $U_{bio}(t)$ was fitted by an exponential model (Fig. 1) which is approaching the total amount of added alanine G_{add} (µmol) after 36 h. The fitted parameters F (µmol) and q (h^{-1}) are the amount of initial utilization (µmol) and the rate of biotic utilization (h^{-1}), respectively. This equation was used to linearize the measured data for statistical tests.

For the whole concentration range, curves for the reaction kinetics v at a distinct substrate concentration (S_0 + S_{add}: alanine concentration derived from soil solution + alanine concentration from the addition of labeling solution) were fitted to the initial rate of biotic utilization q (e.g. the slope of the function $U_{bio}(t)$ at an initial time point). The extracellular processes showed saturation kinetics according to Michaelis-Menten. Thus, the Michaelis-Menten constant K_m, the maxima rate V_{max} and the alanine concentration S_0 (µM) of the soil was calculated from the results of curve fitting (equation 1).

$$v = \frac{V_{max} \cdot (S_{add} + S_0)}{K_m + (S_{add} + S_0)} \qquad (1)$$

The curves for cellular uptake showed no saturation. Thus, their equation (2) contains an additional linear component expressed by the linear utilization rate constant L, as observed by Jones and Hodge (1999). Equations were linearized as proposed by Hobbie and Hobbie (2010).

$$v = \frac{V_{max} \cdot (S_{add} + S_0)}{K_m + (S_{add} + S_0)} + L \cdot (S_{add} + S_0) \qquad (2)$$

2.4.2.6 Calculation of the distribution of alanine-C in transformation products

For the second part of the experiment, the desorption experiment, the distribution of the remaining alanine C in the soil suspension after 36 h was determined. Alanine C in the various bound fractions ($CaCl_2$-extractable, NaH_2PO_4-extractable, irreversibly bound) and dissolved as well as decomposed alanine C was calculated as relative percentage of the added ^{14}C activity.

For a presentation of transformation specifics of C from individual molecule positions, the Divergence Index DI_i was defined:

$$DI_i = \frac{n \cdot [C_i]}{\sum_{i=1}^{i=n} [C_i]} = \frac{3 \cdot [Ala_i]}{\sum_{1}^{3} [Ala_i]} \quad (3)$$

This index shows the fate of individual C atoms from the position i within a transformation process relative to the mean transformation of the n total number of C atoms in the substance. Thus, a DI_i of 1 means that the transformation of this C atom, e.g. Ala_1 position, in the investigated pool corresponds to that of uniformly labeled substance (average of all C atoms e.g. Ala_1+Ala_2+Ala_3). The DI_i ranges from 0 to n, and values between 0 and 1 reflect reduced incorporation of the C into the investigated pool, whereas values between 1 and n show increased incorporation of the C atom into this pool as compared to the average. This index is not dependent on absolute amounts or proportions of the substance used in individual processes. Therefore, it enables comparing the distribution of individual alanine C atoms over the whole range of investigated concentrations.

2.4.2.7 Statistics

All experiments were done with six replications, and the values on figures and in tables present a mean ± standard error of mean (± SEM). SEM of divergence index was calculated by Gaussian error propagation (Gottwald, 2000). Measured variables were screened for outliers using the Nalimov test (Gottwald, 2000) and tested for normal distribution using the Kolmogorov Smirnoff test. Less than 1% of the values were excluded as outliers. Nested ANOVA, with the factor C position nested within the factor inhibition treatment, and non-linear curve fitting were done using Statistica (version 7.0, Statsoft GmbH, Hamburg, Germany). ANOVA of the divergence index was calculated by a procedure proposed by Cohen (2002) from means and standard deviations. Nonlinear curve fitting of the Michaelis Menten equations was done minimizing least-squares with the nonlinear estimation tool of Statistica based on a Levenberg-Marquardt algorithm. Tests of regressions for linearity and significant differences of linear regression parameters were performed by GraphPad Prism (version 4.01, GraphPad Software Inc, San Diego CA, USA). Linear regressions were tested for deviation from linearity by Steven's Runs Test and significant differences between regression lines were identified by covariance analyses (ANCOVA) according to Zar (1984).

2.4.3 Results

2.4.3.1 Evaluation of results quality

We evaluated four quality aspects of the experimental data. The first approach tested for ^{14}C losses by calculating the tracer budget of the full inhibition treatments. For the five concentrations the sum of irreversibly bound, extractable and dissolved ^{14}C activity was between 90 and 97% of the added ^{14}C activity. This high tracer recovery enables the calculation of respired alanine C in the treatments with biotic activity based on the difference between the added and recovered ^{14}C activity.

The second approach checked for sterilization efficiency of the $HgCl_2$ + NaN_3 – solution based on the sorption kinetics in the soil with full inhibition: Sorption occurred completely within the first hour. Within the further 35 h the amount of alanine C in the soil suspension remained constant without any position-specific differences (Supplementary, Fig. A1 shows the example of 50 µM treatment). Thus, no respiration of alanine occurred in the fully inhibited soil.

In a third approach sterilization efficiency of the respiration-inhibited treatment with NaN_3 was tested by the dehydrogenase substrate tetrazoliumchlorid (Kvasnikov et al., 1974; Mohammadzadeh et al., 2006): after 36 h under identical incubation conditions only the non-inhibited treatment showed a clear red precipitation at the bottom of the well. Much longer incubation time was needed (> 3-5 days) until first precipitates could be observed in the respiration-inhibited soil.

The fourth approach evaluated the quality of position-specific data and was based on comparing the mean of the ^{14}C activity in three positions with the ^{14}C activity in uniformly labeled alanine. For the fitted curves of sorption and of biotic utilization (Fig. 2), no significant differences between uniformly labeled alanine and the mean of the three isotopomers could be detected.

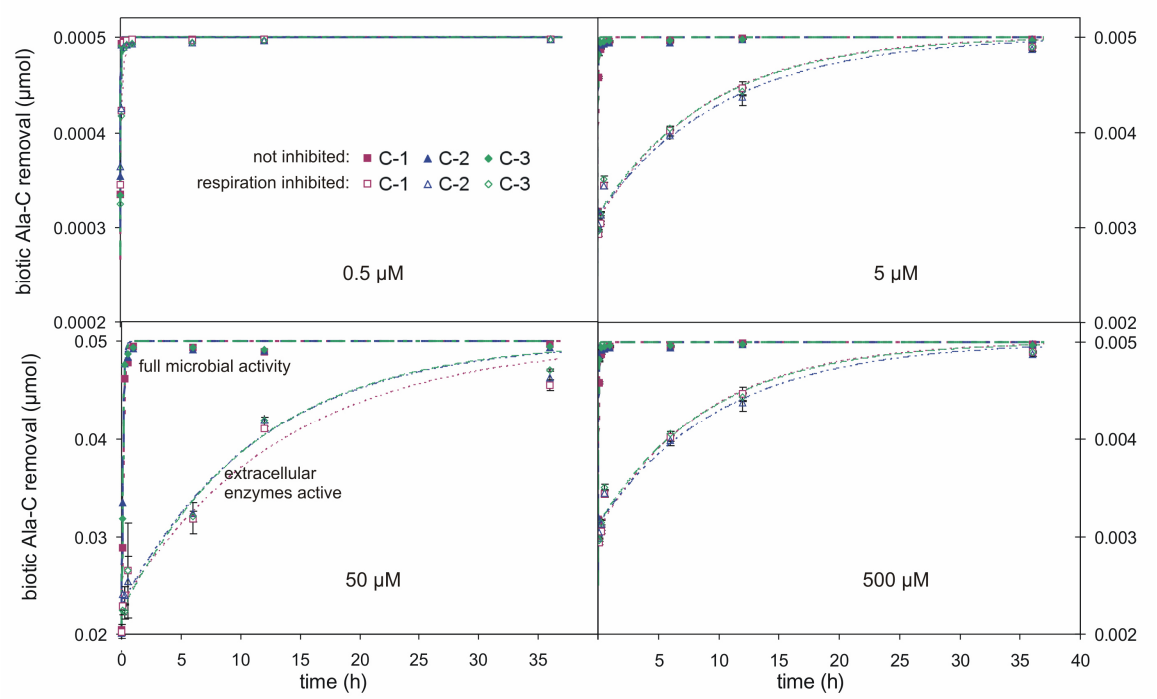

Fig. 2 Removal of alanine from soil solution by extra- and intracellular processes without inhibition (filled symbols) and by extracellular transformation in respiration-inhibited treatments (open symbols) depending on alanine concentration. Experimental points (means ± SEM, N=6) and fitted curves based on the exponential utilization model (see Fig. 1) are presented. Removal of alanine from soil solution is identical for treatments with low concentrations (lines for C-1, C-2 and C-3 overlap) and starts to differentiate for concentrations higher 50 µM.

2.4.3.2 Sorption of alanine to the soil matrix

For the treatment with full inhibition the amount of bound alanine at each concentration was calculated and an exponential curve was fitted to these data. Equilibrium of sorption (95% of fitted line of sorption at equilibrium) was reached within 1 h. The linearized Freundlich Isotherms (Supplementary, Fig A2) showed no significant deviation from linearity based on Stevens Runs Test. ANCOVA of the fitted regression parameters of the linearized Freundlich data showed no differences between the individual C positions and uniformly labeled alanine for the slopes ($p=0.9991$) and the intercepts ($p=0.9997$). Thus, data of isotopomers and U-alanine were pooled and the affinity constants were determined by curve fitting on the entire dataset: $k=0.002$ and $n=0.965$.

The second part of the experiment revealed the extractability of the bound alanine. The sorbed portion ranged from 3 to 6% of the added alanine (Fig. 3). Less than 7.3% of the totally sorbed alanine was adsorbed irreversibly to the soil matrix (<0.4% of the added alanine), and only a portion of <11.70% could be extracted with NaH_2PO_4 (<0.7% of the

totally sorbed alanine). 83-90% of the totally sorbed alanine were extracted with $CaCl_2$ (2.5 to 5.4% of the added alanine) (Fig. 3).

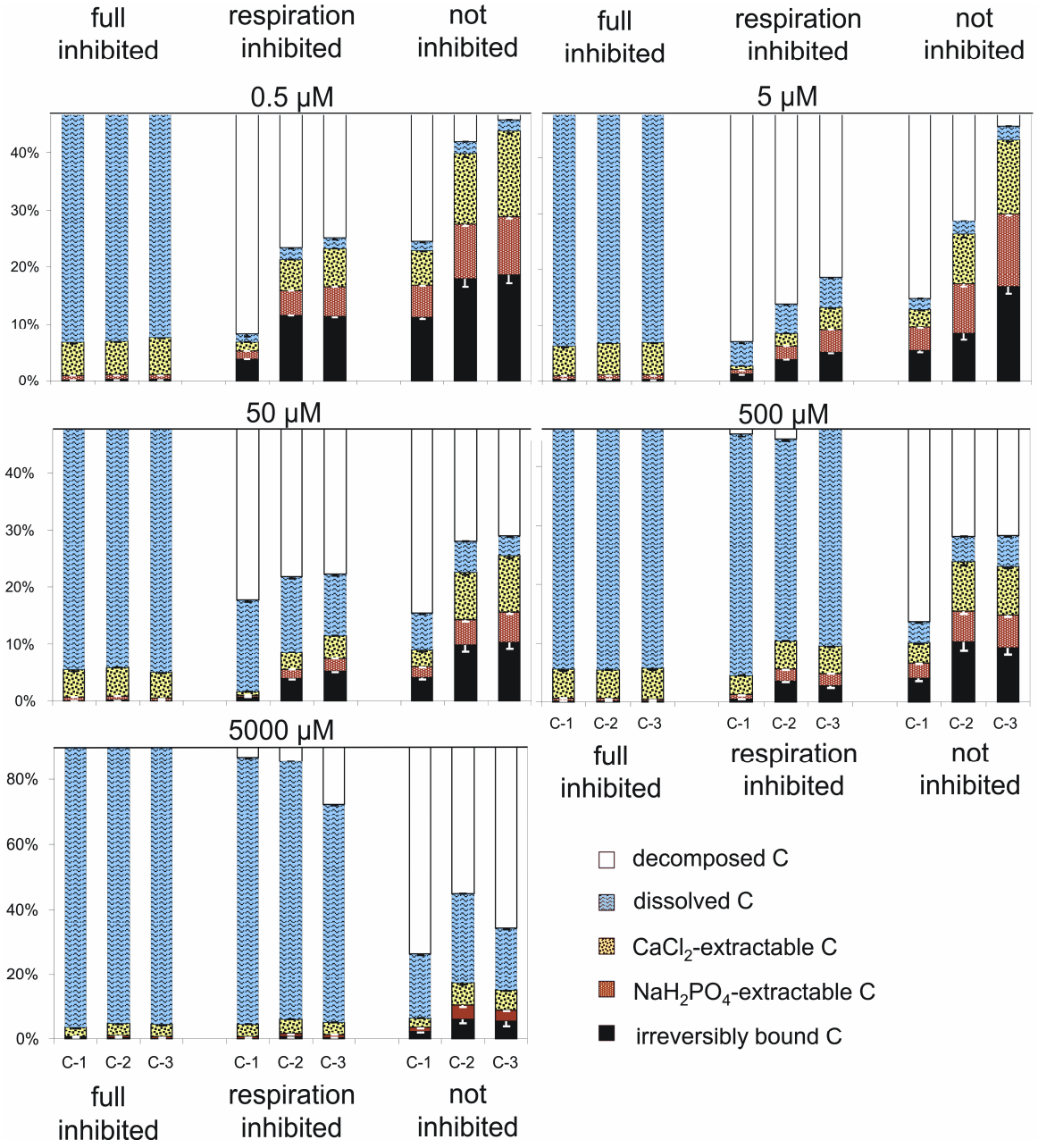

Fig. 3 Amounts of alanine C in differently bound, dissolved or degraded forms in treatments with full inhibition (left), treatments with respiration inhibition (middle) and treatments without inhibition (right). Values show means ± SEM (N=6) depending on alanine concentration.

2.4.3.3 Kinetics of biotic alanine utilization

An exponential curve was fitted to the data of biotic alanine utilization (Fig. 2) for the concentration range from 0.5 to 500 µM. Curve fitting was impossible for the 5 mM treatment because the equilibrium was not reached after 36 h. The rate of biotic utiliza-

tion p (equation see Fig. 1) ranged from low to high concentrations from -11.3 to -0.05 s^{-1} for the exoenzymatic and from -41.7 to -0.13 s^{-1} for the non-inhibited treatment. For lowest alanine concentration extracellular and biotic removal of alanine from the soil suspension were similiarly fast: more than 95% of the added alanine was used within the first 5 min. In contrast, biotic alanine utilization is significantly faster than extracellular removal ($p<0.001$) at medium and high alanine concentrations (5-500 µM). In the 5 and 50 µM treatments, microbial uptake removed 95% of the added alanine in less than 15 and 30 min, respectively, whereas extracellular systems needed about 1 day.

The effect of C position on alanine removal from the soil solution was tested using the linearized function of $U_{bio}(t)$. No significant difference in the removal of alanine C from the three positions by extracellular processes or total biotic utilization was detected over the 36 h of experiment duration.

The extracellular alanine transformation rate (Fig. 4) followed Michaelis-Menten kinetics for all alanine C positions ($p<0.001$, $R^2>0.95$) (Table 1). Significant differences in the kinetics of extracellular transformations of C from individual positions were identified ($F=44.4$, $p<0.0001$). The initial alanine concentration S_0 in the soil was 0.39 µM and thus in the range of the lowest amino acid concentration added.

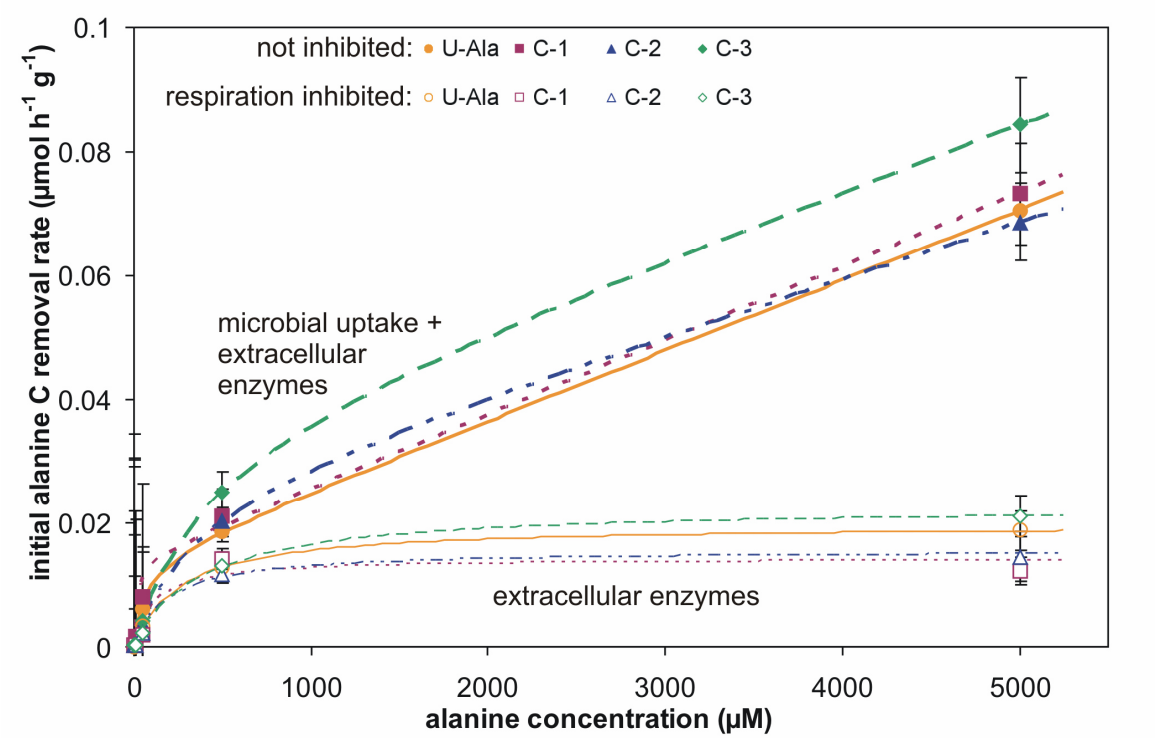

Fig. 4 Initial rate of alanine removal from soil suspension in treatment without inhibition (filled symbols) and treatments with respiration-inhibition (open symbols); Experimental points (means ± SEM calculated by Gaussian error propagation, N=6) and fitted kinetic curves (parameters see Tab. 2) are presented.

The rate of alanine removal from soil suspension in the non-inhibited treatment followed a mixed linear and Michaelis-Menten model ($p<0.001$, $R^2>0.99$) i.e. had no saturation level within the range of amino acids concentration investigated (Fig. 4). The linear uptake rate constant L did not differ significantly between the positions (slope: $F=2.45$, $p>0.05$; intercept: $F=2.76$, $p>0.05$). In contrast at low alanine concentrations (<50 µM) the K_m values were always lowest for the carboxyl group (K_m = 16.4 µM) and highest for methyl groups (384 µM), and V_{max} behaved opposite ($p<0.001$) (Table 1). ANCOVA revealed significantly different uptake behaviour of the individual alanine C positions ($F = 314$; $p < 0.0001$).

Table 1 Parameters of the Michaelis-Menten kinetics for treatments with inhibition of respiration (eq. 2) and treatments without inhibition (eq. 6). R^2 is the coefficient of determination and stars show significance of the respective non-linear fitting result (respectives curves are plotted in Figure 4).

Position	Extracellular transformation			Microbial uptake and intracellular metabolization			
	K_M (µM)±SE	V_{max} (nmol h^{-1} g^{-1})±SE	R^2	K_M (µM)±SE	V_{max} (nmol h^{-1} g^{-1})±SE	L (nmol h^{-1} g^{-1})±SE	R^2
U-Ala	266.4±16.0**	19.70±0.26 **	0.9998	62.1±49.2***	14.29±3.45***	0.011±0.000***	0.9976
C-1	124.0±122.4**	14.21±2.75 *	0.9179	16.4±18.1***	13.89±3.53***	0.012±0.000***	0.9948
C-2	199.1±56.9**	15.51±0.91 **	0.9930	323.6±407.1***	25.87±16.05***	0.009±0.000***	0.9978
C-3	385.6±24.7**	22.69±0.35***	0.9996	383.9±512.3***	34.88±24.98***	0.010±0.000***	0.9981

* $p<0,05$; ** $p<0,01$; *** $p<0,001$

2.4.3.4 Biotic transformation products of alanine

Sequential desorption by $CaCl_2$ and NaH_2PO_4 gives first information about physicochemical properties of alanine transformation products (Fig. 1). The extractability of alanine C significantly changed due to biotic transformation. Comparing full-inhibited treatments with those with extracellular activity revealed that - with the exception of the highest alanine concentration - extracellular transformation always caused an increase in the irreversibly bound and a decrease in the $CaCl_2$-extractable alanine C. In treatments with intracellular metabolization of alanine the increase of NaH_2PO_4-extractable alanine C is even higher. In summary, the portion of C associated with the soil matrix (in the irreversibly bound or NaH_2PO_4-extractable fraction) strongly increased after biotic transformations (Fig. 3). Note, however, that the irreversibly bound pool would contain many cellular components being pelleted out during centrifugation.

At very low concentrations (0.5-5 µM), nearly no alanine C remained in the soil solution; at medium and high concentrations, however, exoenzymes were saturated and were unable to use the whole added alanine C within 36 h (Fig. 2). Thus, significantly more alanine C remained in the supernatant if only extracellular processes used the

alanine. Incomplete removal of the added alanine (>50 µM) always caused significant position-specific differences in the dissolved alanine C (Supplementary Table A1), irrespective of the utilization mechanism. If only trace amounts of alanine remained in the soil solution (<50 µM), no significant position-specific differences were observed (Supplementary Table A1).

2.4.3.5 Position-specific differences of the alanine transformation pathways

Biotic transformations discriminated between the three C positions (Fig. 3). At all concentrations in treatments with biotic activity, the carboxyl group was preferentially decomposed to CO_2 ($p < 0.001$), whereas the C-2 and C-3 positions of alanine were incorporated into various transformation products after 36 h (Fig. 3). Only in the absence of microbial uptake and metabolization the NaH_2PO_4-extractable products at 5 µM and the irreversibly bound forms at 50 µM exhibited a significantly higher portion of the C-3 versus C-2 alanine C. The methyl group was preferentially incorporated in transformation products at the 5 µM concentration in the $CaCl_2$-extractable products at 0.5 µM and in the NaH_2PO_4-extractable pool at medium alanine concentrations (50-500 µM) (Supplementary Table A1). Comparing absolute values of alanine C incorporation depending on concentration is hardly possible, because the very broad concentration range leads to a different utilization of alanine C (e.g. alanine as a growth substrate is incorporated in different absolute and relative amounts than under maintenance conditions). Thus, completely different portions of C are incorporated in the various pools (see Fig. 3: from 0.5 µM to 5 mM the dissolved alanine C pool changed from 1.2 to 8.0%) and direct comparison of C positions is complicated. Less pronounced differences in the position-specific behavior will be lost, if percentages of alanine C allocation are compared. Instead a relative index ignoring these absolute differences is needed to enable comparison of transformation over the investigated concentration range. The Divergence Index DI_i was calculated for degraded alanine C, $CaCl_2$-extractable, NaH_2PO_4-extractable and irreversibly bound transformation products (Fig. 5). The DI_i shows that C-1 was preferentially degraded by intracellular metabolism at all concentrations, whereas the C-2 and C-3 positions were preferentially incorporated into cellular compounds. A significant effect of concentration was observed for intracellular alanine C transformation to NaH_2PO_4-extractable and irreversibly bound products (Supplementary, Table A2): with increasing alanine concentration less splitting between the C-2 and C-3 position occurred, and with decreasing concentration the preferential C-1 decarboxylation by intracellular metabolism tended to be reduced.

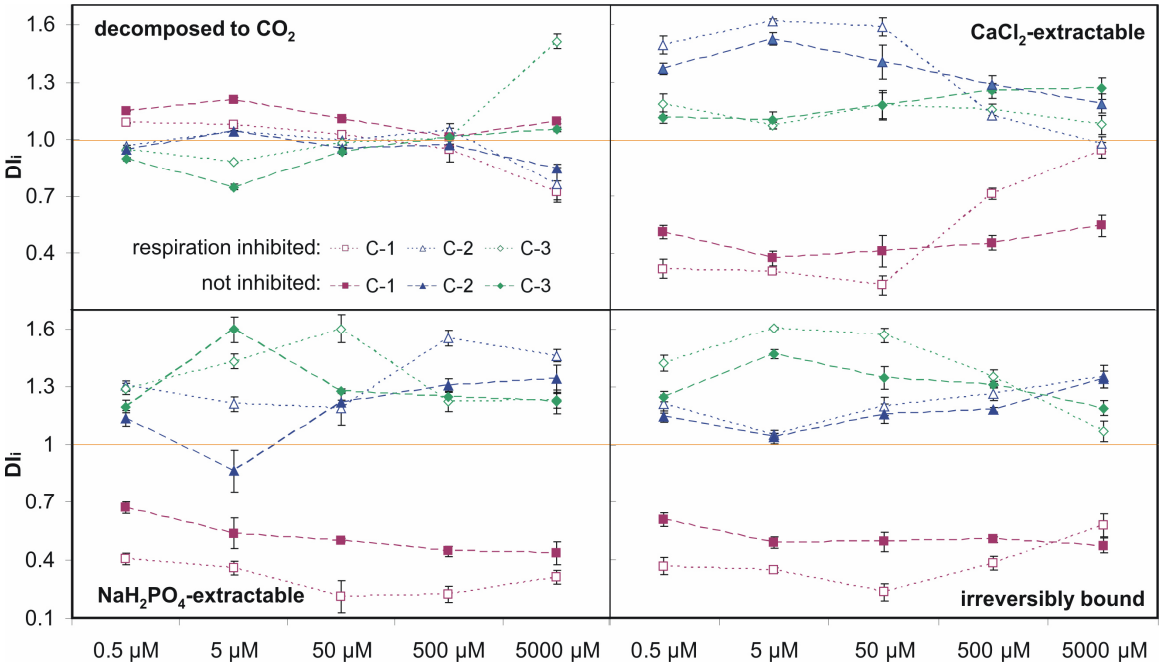

Fig. 5 Concentration-dependent position-specific transformation index DI_i (N=6, ± SEM) of alanine C positions for treatments without inhibition (filled symbols) and treatments with respiration inhibition (open symbols) for four pools. The SEMs were calculated by Gaussian error propagation.

In contrast, extracellular degradation showed a lower preferential decarboxylation. At the highest concentration the C-3 oxidation even exceeded the C-1 oxidation (Fig. 5). With increasing concentration a highly significant decrease in the discrimination between alanine positions for the extractable transformation products was observed. In addition a change in the preferential incorporation from C-2 to C-3 occurred with increasing concentrations (Fig. 5).

2.4.4 Discussion

2.4.4.1 Sorption of alanine occurs as a whole molecule

The goodness of fit of the Freundlich isotherms shows that the sorption capacity of a loamy soil for alanine has no limit within naturally occurring concentrations. Sorption of the dipolar ion alanine can occur by ion exchange via the amino group, ligand exchange via the carboxyl group and hydrophobic interactions via the methyl group and may explain the observed non-saturable Freundlich isotherm. Sequential desorption is an attempt to differentiate these mechanisms. As $CaCl_2$ is a very potent cation exchange reagent, it is likely that the majority of the alanine molecules were bound by ion exchange (83-90%), i.e. via the positively charged amino group.

Wang and Huang (2003) observed abiotic degradation of amino acids by the interaction with mineral phases. In their experiment, the abiotic oxidations of glycine showed highly significant position-specific differences with a preferential decarboxylation of the carboxyl group (Wang and Huang, 2003, , 2005). We did not observe this in our treatments with full inhibition, neither for the sorption isotherms nor for the desorbed transformation products. Thus, we conclude that sorption of the intact alanine C skeleton occurred. Theoretically, the used ^{14}C labeling did not enable to observe deamination of amino acids. However, deamination is very unlikely as the majority of the sorbed alanine is $CaCl_2$-extractable and thus, is mainly bound by cation exchange via the amino group. Thus, we conclude that abiotic cleavage of amino acids is of minor relevance. We explain this lack of abiotic molecule splitting by the short incubation time of our experiment and the physico-chemical differences of the investigated soils compared to the subsoils used by Wang and Huang (2003).

2.4.4.2 Kinetics of extracellular transformation and microbial uptake

In general, differentiation between extra- and intracellular metabolization in soil can either be performed by using selective inhibition of the exoenzymes (Martens and Frankenberger, 1993) or by using selective sterilization that inhibits intracellular metabolism (Gibson and Burns, 1977; Hope and Burns, 1987). As a broad set of enzymes is available to transform alanine (a very common substrate), we used the inhibition of the active microbial cells. We tried to reduce artifacts of the inhibition (e.g. remaining activity in dead cells or continuing fermentation processes) by a pre-incubation with double concentrated NaN_3 and high oxygen supply (Burns, 1982). The lack of visible red crystals after 36 h confirms that the inhibition of intracellular processes was successful. Thus, we conclude that extracellular enzymes which were stabilized for more than the 1 h of preincubation in the soil dominated the alanine removal from soil suspension. Extracellular kinetics as well as transformation products differed significantly from those formed by intracellular metabolism (Fig. 3). This confirms that remaining intracellular activity is of minor importance after NaN_3 inhibition. The observed Michaelis-Menten constants are in a similar range than values observed for other exoenzymes (Braun et al., 1992).

Under medium and high concentrations, microbial uptake systems were much faster than extracellular enzymes (Fig. 2 and 4). Rate of alanine utilization resembled removal of an amino acid mixture observed by Rousk and Jones (2010): they observed a loss of 90% of ^{14}C activity within the first hour of incubation. At low concentrations this microbial alanine uptake also follows a Michaelis-Menten kinetics (Vinolas et al., 2001a). The linear kinetic term of microbial uptake at higher concentrations is similar to the kinet-

ics observed by Vinolas (2001a). These combined kinetics reflect different types of microbial uptake and enzyme systems involved in alanine utilization (Anraku, 1980; Piperno and Oxender, 1968): 1) high-affinity, energy-dependent active uptake systems at low concentrations and 2) additional uptake mechanisms with linear non-saturable kinetics at high substrate concentrations. The non-saturable kinetics showed identical behavior of all C positions. This experimentally confirmed the assumption of Jones and Hodge (1999) that the non-saturable kinetics is based on uptake by permeases or ion channels which do not split the molecule. In contrast, the processes that follow Michaelis-Menten kinetics revealed individual position-specific kinetics (Fig. 4). For extracellular enzymes this is a result of alanine splitting processes like decarboxylation. However, alanine uptake into cells also revealed molecule splitting. This can either be uptake of fragments or the uptaken alanine was split and fragments were secreted. Both possible processes can not be distinguished by our approach.

To summarize, C-1 decarboxylation by extracellular enzymes as well as C-1 removal by cellular uptake are the fastest biotic processes under low alanine concentrations. At medium and high alanine concentration microbial cells take up alanine without splitting by unsaturated enzyme systems. Thus, the position-specific transformations revealed that multiphase kinetics of LMWOS uptake by microbial cells reflect a change of the underlying biochemical processes and thus the fate of LMWOS depending on their concentration in soil.

2.4.4.3 Exoenzymatic transformation products

We know little about the exoenzymatic transformation of amino acids because the tacit assumption is that LMWOS are completely taken up into microbial cells. It was shown that some organisms such as *Cellulomonas cellulans*, *Corynebacterium* or *Proteus rettgeri* produce extracellular amino acid oxidase with broad substrate specificity (Braun et al., 1992). These enzymes catalyze the oxidative deamination of alanine, forming pyruvate, which could be decarboxylated in further steps. This might e.g. be done by unspecific decarboxylation by manganese peroxidase (Hofrichter et al., 1998). Exact mechanisms cannot be identified without analysis of the enzymatic products, but it can be shown that the discrimination between C-2 and C-3 of the exchangeable products is higher compared to microbial metabolites (Fig. 5). This might indicate a stepwise oxidation reaction from C-1 to C-3, at least at low alanine concentrations.

At the two highest concentrations, alanine was not completely removed from the solution (Fig. 2) and thus, discrimination between C positions decreased due to an increasing portion of untransformed alanine (Fig. 5). This saturation of exoenzymes at high

concentrations supports the hypothesis that mainly unspecific oxidizing enzymes are involved. The desorption experiment showed that a significant part of the not-extractable products are formed after exoenzymatic transformation. This is probably a first step of extracellular amino acid C stabilization in soil. Further metabolite tracing of the transformation products is needed to understand LMWOS stabilization mechanisms in soil.

Extracellular pathways were less important because active microorganisms take up LMWOS much faster (Fig. 4 and Table 1). Nonetheless, these pathways may explain transformation in aggregate cores or micropores, where microbial cells are size-excluded (von Luetzow et al., 2006). Thus, these transformation pathways are expected to take place parallel to microbial uptake and metabolization. However, if a potential overestimation of extracellular activity due to insufficient microbial inhibition by NaN_3 is considered, the extracellular transformation of alanine is of minor importance for microbially active soil.

2.4.4.4 Metabolic pathways and their intracellular transformation products

Intracellular metabolism products showed a strong increase of irreversibly bound and ligand exchangeable substances (Fig. 3). Non-extractable products were expected to be either macromolecular or hydrophobic e.g. proteins, peptidoglycan or lipids: they are not extractable in polar reagents with low salt molarity. Ligand-exchangeable transformation products are characterized by either hydroxyl or more probably carboxyl groups (Gu et al., 1994) like alcohols and mono- or poly-carboxylic acids.

Direct decarboxylation of alanine, producing amine, has been described only for cucumber and tea plants (Takeo, 1978), and no evidence for this reaction within microorganisms is available. In contrast, the oxidation of the C-1 group was most likely caused by the fundamental alanine degradation pathway of prokaryotes under aerobic conditions: the oxidative deamination to pyruvate by alanine dehydrogenase (Caspi et al., 2008; Gottschalk, 1986; Keseler et al., 2009). This reaction, shown in Fig. 6 (first arrow down from alanine), decouples the C and N fate in the microbial metabolism (Knowles et al., 2010). Additional pathways of alanine utilization like transamination or oxidoreduction also lead to the formation of pyruvate (Caspi et al., 2008; Gottschalk, 1986; Keseler et al., 2009). Pyruvate as the main alanine transformation product allows a qualitative metabolic tracing approach (Dijkstra et al., 2011a). Citric acid cycle leading to the oxidation of the C-1 position by pyruvate dehydrogenase can be distinguished from alternative C utilization pathways like protein biosynthesis or gluconeogenesis (Fig. 6, arrows branching left from the main pathway), which commonly use the entire C skeleton of alanine (Caspi et al., 2008; Keseler et al., 2009).

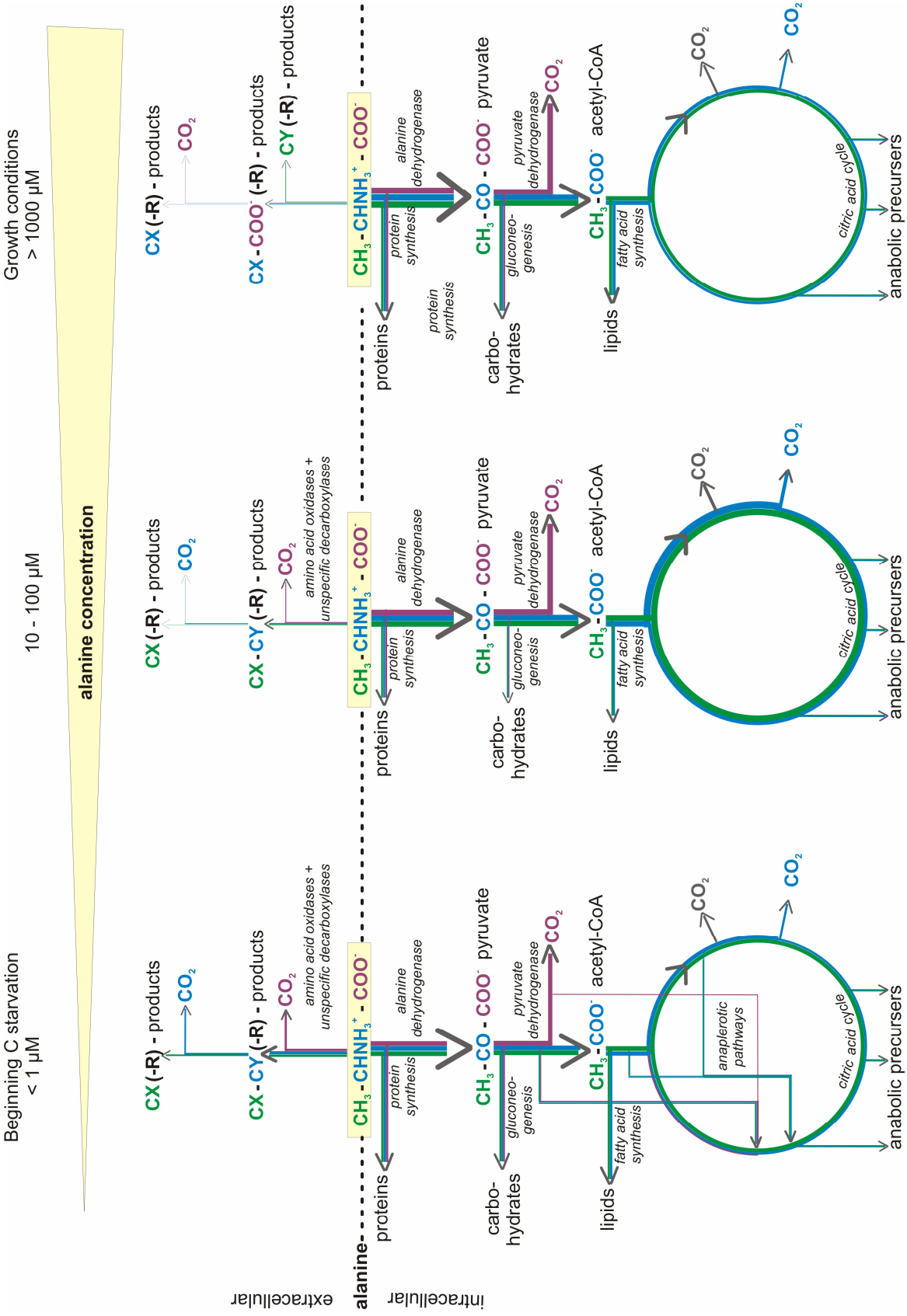

Fig. 6 *General biochemical pathways of extra- and intracellular amino acids transformation in soil in dependence on alanine availability. Line width represents the qualitatively estimated relative shifts of alanine C between certain pathways dependent on the alanine concentration.*

In general for all concentrations, only a minor part of the C-1 group is incorporated into newly formed cellular compounds (Fig. 3 and Fig. 5). This reveals citric acid cycle as the major pathway of alanine i.e. pyruvate C metabolization (Fig. 6). Further cycling of the C skeleton through the citric acid cycle led to a partial oxidation of the C-2 position, which is maximized under medium alanine concentrations (Fig. 5).

At lowest alanine concentration the incorporation of C-1 slightly increased in the extractable transformation products (Fig. 5) and significantly increased in irreversibly bound microbial products (Supplementary, Table A2). This might reflect the slight increase of anapleurotic carboxylation pathways, protein biosynthesis or gluconeogenesis with increasing C deficiency (Dijkstra et al., 2011a). For these pathways the C skeleton of alanine needn't be split (Fig. 6, left). Which of these pathways is driven by microbial anabolism can be clarified only if transformation products (e.g. amino sugars or acids) are investigated compound-specifically.

At high alanine concentrations we experimentally simulated growth conditions like representative for soil hot-spots: the microbial community was not energy deficient because oxidative deamination of alanine along with decarboxylation of pyruvate to Acetyl-CoA are energy-supplying reactions producing ATP (Caspi et al., 2008; Keseler et al., 2009). Thus, C and energy were available in excess and allowed microbial growth, at least for a part of the microbial community, presumably bacterial groups (Rousk and Baath, 2011). A high portion of C-2 and C-3 positions was found in irreversibly bound, cellular products under these conditions (Fig. 5). Biosyntheses pathways common for microbial growth like fatty acid synthesis start with Acetyl-CoA (Fig. 6, arrow to left) and do not split this C-2-C-3-fragment (Caspi et al., 2008; Keseler et al., 2009). Thus, such growth pathways produce not-extractable, macromolecular or hydrophobic compounds containing the C-2 and C-3 positions (Fig. 6, right). Hence, an increased incorporation by biosynthesis would explain the changes of alanine transformation under high C supply (Fig. 6).

To summarize, position-specific labeling enables to trace changes in microbial substrate metabolization due to changes in C availability: from starvation over maintenance and growth concentrations a shift in the microbial pathways (Fig. 6) leading to formation of different cellular compounds from alanine C could be observed in this study.

2.4.5 Conclusions and Outlook

Next to multiple isotope labeling (Knowles et al., 2010) the position-specific labeling is a preeminent tool to identify and trace the major pathways of LMWOS transformation in soil (Dijkstra et al., 2011a; Dijkstra et al., 2011b). This study demonstrates that position-

specific labeling provides a new insight into amino acid transformation on a submolecular level. Cellular uptake always outcompetes sorption and extracellular transformations, which are quantitatively relevant only at very low alanine concentrations or in specific microhabitats and occurs as a stepwise extracellular oxidation. In general, however, cellular uptake and metabolization dominate the fate of alanine C in soil. Two mechanisms underlying the microbial uptake kinetic were identified: an unsaturable unspecific uptake of intact alanine at hot-spot concentrations and specific uptake mechanisms at low alanine concentrations. In addition, this tool also enabled us to detect minor changes of the intracellular alanine metabolization, which were a result of the switch from anabolic pathways characteristic for C deficiency to those common for growing cells. However, without a quantitative detection of the metabolic products this assay remains qualitative. Coupling this sensitive, submolecularly operating technique with compound-specific isotope analysis of the transformation products is the next step to shed light on the black box of C transformations in soil. As opposed to closer examination of transformation pathways, generalization (by further compounds, compound classes and environmental conditions) and upscaling are the future demands: Once, general principles controlling LMWOS metabolization and the effects of environmental conditions are identified, the fate of C entering the soil can be determined based on its chemical structure. A detailed understanding of the general principles of LMWOS transformation, the used pathways and the regulating factors is crucial to understand and predict the SOC dynamics under changing environmental conditions.

Acknowledgement

We thank the Deutsche Forschungsgemeinschaft (DFG) for funding.

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Supplementary Data

Supplementary Table A1: Nested ANOVA between single alanine positions nested in sterilization treatment of decomposed, dissolved, $CaCl_2$- and NaH_2PO_4-extractable and irreversibly bound alanine C. Degrees of freedom (df), F-values and significance level (p) are shown for the five concentration treatments.

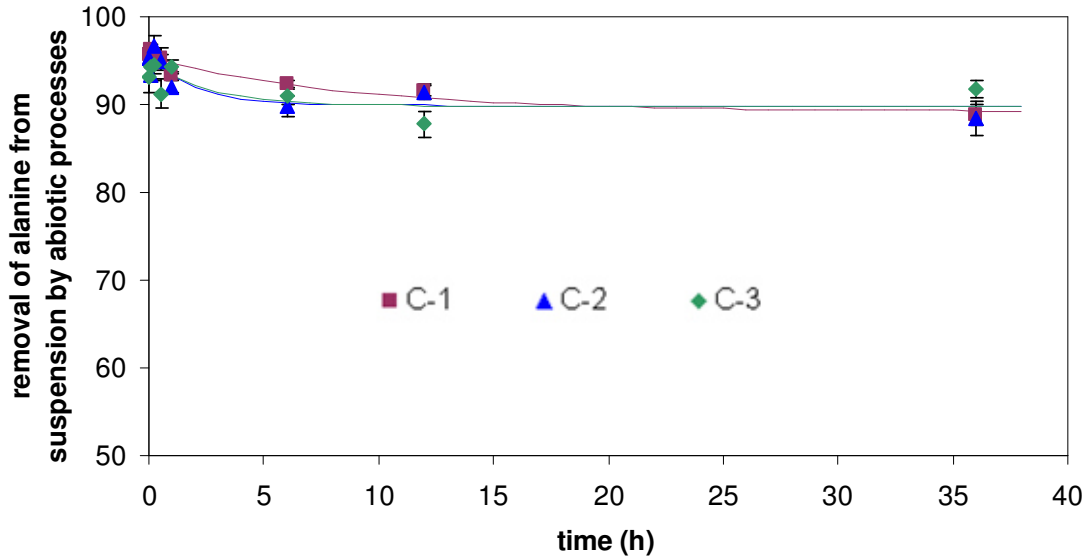
Ala concentration	factor	degraded Ala-C			dissolved Ala-C			$CaCl_2$-extractable Ala-C			NaH_2PO_4-extractable Ala-C			irreversibly bound Ala-C		
		df	F	p	df	F	p	df	F	p	df	F	p	df	F	p
0.5 µM	inhibition	2	10794.7	***	2	10794.7	***	2	199	***	2	1037	***	2	1792	***
	Ala-C position	6	0.5	***	6	0.5	n.s.	6	62	***	6	97	***	6	135	***
5 µM	inhibition	2	1222.3	***	2	1222.3	***	2	393	***	2	1291	***	2	106	***
	Ala-C position	6	1.0	***	6	1.0	n.s.	6	101	***	6	134	***	6	14	***
50 µM	inhibition	2	7787.0	***	2	7787.0	***	2	64	***	2	346	***	2	1504	***
	Ala-C position	6	3.4	***	6	3.4	*	6	25	***	6	55	***	6	178	***
500 µM	inhibition	2	1404.5	***	2	1404.5	***	2	66	***	2	2658	***	2	2015	***
	Ala-C position	6	3.8	***	6	3.8	**	6	74	***	6	436	***	6	287	***
5000 µM	inhibition	2	574.5	***	2	574.5	***	2	4	***	2	658	***	2	380	***
	Ala-C position	6	3.7	***	6	3.7	**	6	24	***	6	78	***	6	35	***

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

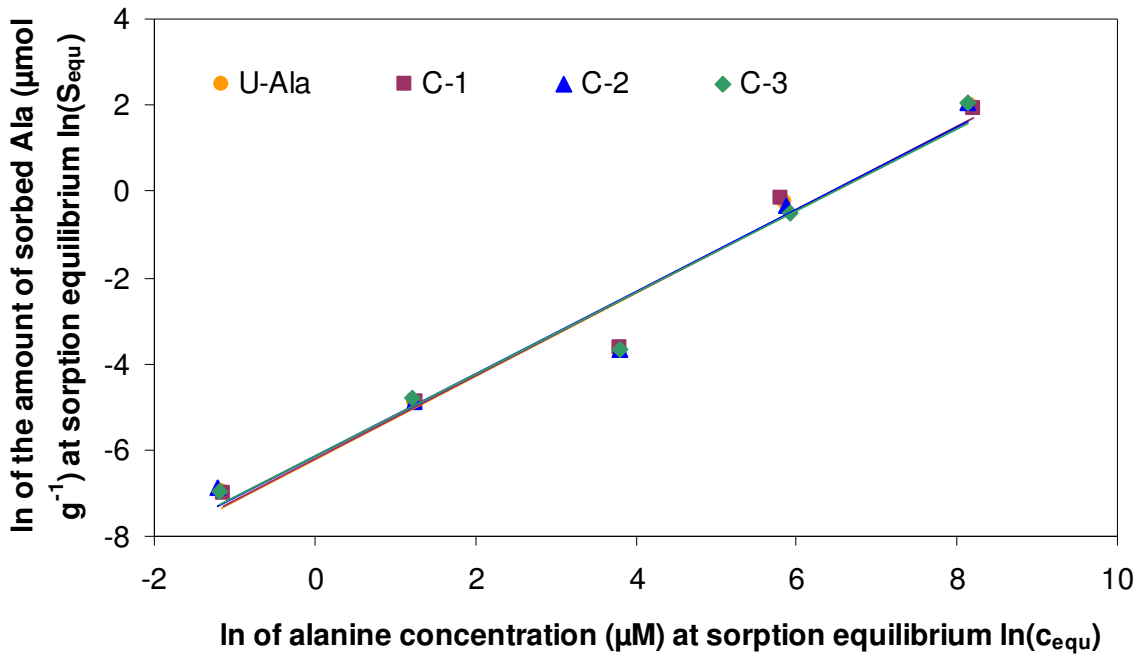
Supplemetary Table A2: ANOVA, calculated according to Cohen (2002), for the divergence index DI between the five concentration treatments. Degrees of freedom (df), F-values and significance level (p) are shown for the three alanine positions and the two inhibition treatments with biotic activity.

Divergence Index DI_i	inhibiton treatment	degraded Ala-C			$CaCl_2$-extractable-Ala-C			NaH_2PO_4-extractable Ala-C			irreversibly bound Ala-C		
		df	F	p	df	F	p	df	F	p	df	F	p
C-1	respiration-inhibited	4	9.338	***	4	52.843	***	4	7.512	***	4	2.890	*
	no-inhibition	4	9.338	***	4	1.175	n.s.	4	1.766	n.s.	4	3.7	*
C-2	respiration-inhibited	4	0.523	n.s.	4	0.642	n.s.	4	2.904	*	4	7.416	***
	no-inhibition	4	0.115	n.s.	4	1.135	n.s.	4	4.532	**	4	5.68	**
C-3	respiration-inhibited	4	12.15	***	4	42.77	***	4	20.098	***	4	8.616	***
	no-inhibition	4	8.772	***	4	2.596	n.s.	4	3.014	*	4	5.014	**

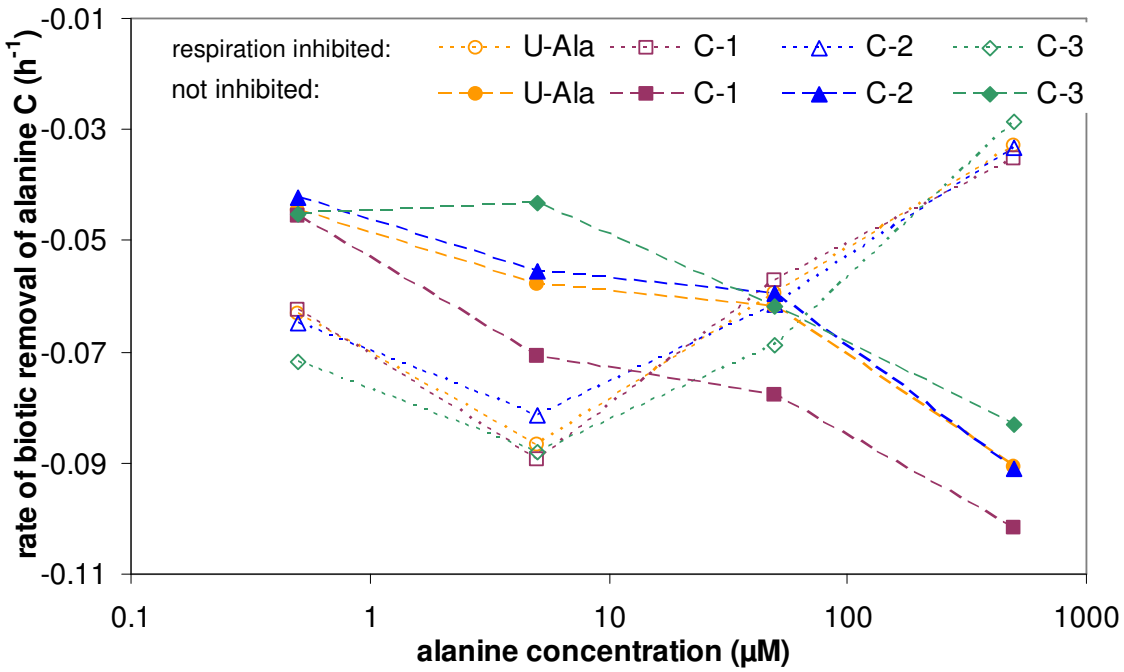
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$



Supplemetary Figure A1: Alanine ^{14}C-removal from soil suspension in % of the added ^{14}C activity for the alanine C positions C-1, C-2 and C-3 in the full inhibited treatment; Added concentration was 50 µM alanine.



Supplemetary Figure A2: Linearized Freundlich Isotherm as schematized in Figure 1: ln of the amount of sorbed alanine at equilibrium is plotted against ln of the alanine concentration (µM); No significant differences between the fitted linear regressions could be detected.



Supplementary Figure A3: Rate h of biotic removal of alanine C (h^{-1}) fitted according to Figure 1 to the data of alanine removal from soil suspension (Figure 2) for concentration treatments from 0.5 to 500 µM

2.5 Study 5: Sorption affects amino acid pathways in soil: Implication from position-specific labeling of alanine

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Abstract

Organo-mineral interactions are the most important mechanisms of long-term C stabilization in soils. Nevertheless, a part of the sorbed low molecular weight organic substances (LMWOS) remains bioavailable. Uniformly labeling of substances by ^{14}C or ^{13}C reflects only the average fate of C atoms of an LMWOS molecule. The submolecular tool of position-specific labeling allows to analyze metabolic pathways of individual functional groups and thus reveals deeper insight into mechanisms of sorption and microbial utilization.

Alanine labeled with ^{14}C in the 1st, 2nd or 3rd position was adsorbed to five sorbents: two iron oxides with different crystalline structure: goethite and haematite; two clay minerals with 2:1 layers – smectite, and 1:1 layers – kaolinite; and activated charcoal. After subsequent addition of these sorbents to a loamy haplic Luvisol, we analyzed ^{14}C release into the soil solution, its microbial utilization and $^{14}CO_2$ efflux from individual C positions of alanine.

All sorbents bound alanine as an intact molecule (identical sorption of 1st, 2nd or 3rd positions). The bioavailability of sorbed alanine and its microbial transformation pathways depended strongly on the sorbent. Goethite and activated charcoal sorbed the highest amount of alanine (~45% of the input), and the lowest portion of the sorbed alanine C was microbially utilized (26 and 22%, respectively). Mineralization of the desorbed alanine peaked within the first 5 h and was most pronounced for alanine bound to clay minerals. The initial mineralization to CO_2 of bound alanine was always highest for the C-1 position (-COOH group). Mineralization rates of C-2 and C-3 exceeded the C-1 oxidation after 10-50 h, reflecting the classical biochemical pathways: 1) deamination, 2) decarboxylation of C-1 within glycolysis, and further 3) oxidation of C-2 and C-3 in the citric acid cycle. The ratio between two metabolic pathways – glycolysis (C-1 oxidation) versus citric-acid cycle (oxidation of C-2 and C-3) – was dependent on the microbial availability of sorbed alanine. High availability causes a peak in glycolysis C-1 oxidation followed by an abrupt shift to oxidation via the citric acid cycle. Low microbial availability of sorbed alanine, in turn, leads to a less pronounced, parallel oxidation of all three positions and to a higher relative incorporation of alanine C into microbial compounds. Modeling of C fluxes revealed that a significant portion of the sorbed alanine was incorporated in microbial biomass after 78 h and was further stabilized at the sorbents' surfaces.

Position-specific labeling enabled determination of pathways and rates of C utilization from individual molecule positions and its dependence on various sorption mechanisms. We conclude that position-specific labeling is a unique tool for detailed insights

into the submolecular transformation processes, mechanisms and rates of C stabilization in soil.

Keywords: position-specific tracers, sorption mechanisms, metabolic tracing, C mineralization and stabilization, iron oxides, clay minerals, active coal, soil organic matter formation, biochar, organo-mineral interactions

2.5.1 Introduction

Soil organic carbon (SOC) represents a major terrestrial carbon (C) sink. This makes studies on the transformation of organic substances in soils important for understanding the C cycle in terrestrial ecosystems. Plant residues, rhizodeposits and pyrogenic organic matter are the main sources of organic matter in soils (Knicker, 2011a; Kuzyakov and Domanski, 2000; Rasse et al., 2005). Accordingly, many studies have focused on decomposition, microbial utilization and stabilization of C from these sources in soils (Dungait et al., 2012; Rasse et al., 2005; von Luetzow et al., 2006).

During litter decomposition, all macromolecular compounds are split by enzymes into low molecular weight organic substances (Cadisch and Giller, 1996). Thus, transformation of LMWOS is a key step in biogeochemical processes in soils: all high molecular substances pass this stage during their degradation. Microorganisms determine the fate of LMWOS in soil because they either decompose them to CO_2 (catabolism) or incorporate them into cellular compounds (anabolism). Both main branches of metabolism occur in parallel and C partitioning between them depends on environmental conditions.

Within the LMWOS, amino acids play an important role because they are the quantitatively most important compound class coupling the C and N cycles. In topsoils, amino acid N - mainly bound in proteins - constitutes 7-50% of the total organic N (Knicker, 2011b; Stevenson, 1982), and concentrations of free amino acids range from 0.5 µM in root-free bulk soil up to 5 mM directly next to bursting cells (Fischer et al., 2007; Jones and Hodge, 1999). Thus, many recent studies focused on the fate of N-containing LMWOS (Kuzyakov, 1996, 1997; Lipson et al., 2001) and investigated the three major pathways of amino acid in soil: 1) sorption (Jones, 1999), 2) microbial utilization (Vinolas et al., 2001a; Vinolas et al., 2001b), and 3) plant uptake (Lipson and Nasholm, 2001; Nasholm et al., 1998). Whereas the importance of plant uptake strongly depends on the type of ecosystem (especially its N limitation) (Jones et al., 2005; Lipson and Nasholm, 2001; Sauheitl et al., 2009), sorption and microbial utilization are significant in all ecosystems and are competing processes. They lead either to a stabilization of amino acid C and N in soils or to their decomposition.

Many studies on the microbial utilization of free amino acids showed that their metabolization occurs mainly intracellularly after uptake by transport systems (Anraku, 1980; Dippold and Kuzyakov, 2013; Hediger, 1994; Hosie and Poole, 2001). Based on the uptake kinetics of some single amino acids, microbial uptake outcompetes sorption in soils (Fischer et al., 2010; Jones and Hodge, 1999; Vinolas et al., 2001a). Nevertheless, sorption is thought to be the most relevant long-term stabilization mechanism for amino acids in soils. This is even more relevant for their amino acid polymers – the proteins – which

accumulate at mineral surfaces (Duemig et al., 2012; Spence and Kelleher, 2012). However, neither the exact processes nor the relevance of amino acid sorption compared to stabilization by inaccessibility in micropores or aggregates has been analyzed (Sollins et al., 1996; von Luetzow et al., 2006).

The lack of accumulation of strongly sorbed amino acids in ecosystems (Stevenson, 1982) supports the idea that sorption does not completely protect amino acids from biodegradation. They do, however, remain at least partially bioavailable (Gonod et al., 2006). Jones and Hodge (1999) demonstrated that sorption strength of three amino acids (lysine>glycine>glutamic acid) behaves contrary to microbial utilization (glutamic acid>glycine>lysine). These results support the idea that the presence of substrate in solution and consequently sorption is key driver regulating the fate of amino acids. Furthermore, both the quantity of microbial uptake and the metabolization pathways of LMWOS are affected by sorption (Dijkstra et al., 2011a; Fischer and Kuzyakov, 2010; Schneckenberger et al., 2008).

Broadly, amino acids can be adsorbed by three types of functional groups (Jones and Hodge, 1999): 1) ion exchange by the positively charged amino groups, 2) ligand exchange by the carboxyl groups and 3) hydrophobic interactions by the alkyl groups. In addition, weak electrostatic interactions (H-bondings, dipole-dipole-interactions, van-der-Waals bondings) are possible by the C skeleton (Brigatti et al., 1999) and intercalation into the clay mineral interlayer has also been discussed (Wattel-Koekkoek et al., 2003). There is strong evidence that soil properties (Kemmitt et al., 2008) such as soil mineralogy (Strahm and Harrison, 2008) influence the microbial utilization of LMWOS, but detailed studies comparing sorption on various mineral phases and their effect on microbial utilization of amino acids are rare (Dashman and Stotzky, 1982).

Here, we use the approach of position-specific labeling to elucidate the shifts in microbial amino acid transformation pathways caused by sorption. This tool, commonly used in biochemistry to reconstruct metabolic pathways, has increasingly been applied in soil science in recent years (Dijkstra et al., 2011a; Dijkstra et al., 2011b; Dijkstra et al., 2011c; Fischer and Kuzyakov, 2010). It overcomes the limitations of uniform labeling because it helps differentiate between cleavage of a molecule vs. utilization of the entire molecules.

Our model amino acid – alanine (one of the most abundant amino acids in soil) – occurs under soil conditions as a dipolar ion: it has a positive charge, a negative charge and a hydrophobic methyl group – enabling different sorption mechanisms. As a representative subset of sorbents common in soils and representative for three basic sorption mechanisms, we chose a three- and a two-layer clay mineral (smectite and kaolinite), two iron oxides (goethite and haematite) and activated charcoal. Thus, we present here a

submolecular approach to elucidate LMWOS stabilization on sorbents and effects of sorption on amino acid transformations in soil. We assume that alanine interacts differently with various mineral phases and that interaction strength and mechanism strongly affects its subsequent microbial utilization, e.g. we assume a preferred allocation of LMWOS-C into the anabolic pathways of maintenance metabolism, the lower the availability of a substrate is. We hypothesize that various sorption mechanisms affect the stabilization of amino acid C on mineral surfaces not only by direct interaction of LMWOS-C with the mineral but also by changing C allocation: The stronger a substrate is sorbed, the more its C is transformed to products, which are prone to be stabilized on the sorbents. In the case of polymeric microbial products (proteins, cell walls,…), this process could be identified by an equal incorporation of all three alanine C positions.

2.5.2 Material and Methods

2.5.2.1 Soil

Soil samples were taken from an Ap horizon of a loamy silt, haplic Luvisol (WRB 2006) from a long-term cultivated field in Bavaria near Hohenpölz (49.907 N, 11.152 E, 501 m asl, mean annual temperature 6.7 °C, mean annual precipitation 874 mm), where a continuous rotation of corn, barley, wheat and triticale was established. The soil had the following characteristics: pH_{KCl} 4.9 and pH_{H2O} 6.5, TOC and TN content were 1.77% and 0.19%, respectively, and potential CEC was 174 $mmol_c$ kg^{-1}. The soil was stored field-moist at 5 °C for less than 1 month, sieved to 2 mm, and all roots were removed manually before adding to the minerals. 800 mg of field-moist soil were used per replicate.

2.5.2.2 Sorbents

Minerals were ordered from Kremer pigments (Aichstetten/Allgäu, Germany): smectite-dominated Bentonite (58900), kaolinite-dominated Kaolin (58250), heamatite-dominated “Eisenoxid rot” (48600) and goethite-dominated “Eisenoxidocker” (40301). Activated charcoal was ordered from Sigma-Aldrich (Taufkirchen, Germany) and ball-milled until the texture was comparable to the mineral phases. Specific surface area (SSA) was determined with a Quantachrome Nova 4000 surface analyzer (Quantachrome GmbH, Odelzhausen, Germany) by N_2 adsorption using the BET method (Mikhail and Brunauer, 1975). Effective cation exchange capacity (CEC_{eff}) was determined for all sorbents and the soil by exchange of the cations with Ba^{2+} according to NF ISO 11260 1994 (Rhoades, 1982) (Table 1). SSA and CEC_{eff} were determined with sterilized, heated minerals (300 °C over night) to have identical conditions than with the minerals used for

the main experiment. Whereas loss of smectite crystal water is a reversible process, 300 °C heating might have caused some transformations in goethite structure lowering its adsorption capacity. However, values of SSA and CEC_{eff} shown in Table 1 characterize the sorbent properties of the main experiment.

Table 1 Effective cation exchange capacity and specific surface area of the five sorbents and the soil used for this experiment

	Soil	Goethite	Haematite	Smectite	Kaolinite	Activated charcoal
Effective CEC ($mmol_c\ kg^{-1}$)	119.5	133.0	84.5	208.5	54.0	50.0
Internal surface ($m^2\ g^{-1}$)	16.8	36.2	26.3	58.0	18.3	980

2.5.2.3 Chemicals and radiochemicals

Sterile stock solutions with 50 µM alanine and an equal ^{14}C activity of 830 kBq ml^{-1} were prepared from U-^{14}C-labelled alanine and the position-specifically labeled isotopomeres 1-^{14}C-, 2-^{14}C- and 3-^{14}C-labeled alanine (American Radiolabeled Chemicals Inc, St. Louis, USA) as well as non-labeled alanine (Sigma-Aldrich, Taufkirchen, Germany).

The incubation of the sorbed alanine with soil was conducted in 24 well microtiter plates. The CO_2 efflux from the wells was trapped in 2.0 M NaOH-solution placed on a filter mat on top of the microtiter plates. The filter mat segments were separated by a thin line of silicone oil added on the well boarders which diffused into the mat. In addition 1 M NaOH solution was prepared to trap the CO_2 after combustion (Sigma-Aldrich, Taufkirchen, Germany).

2.5.2.4 Pre-experiments

Two preliminary experiments were performed to evaluate parameters and optimize the experiment design: First, the time needed for the sorption experiment was determined by adding U-^{14}C-labeled alanine to the five sterile sorbents (five replications). 200 mg of each sorbent were added per well of a 24 deep-well plate, and 1.0 ml of the U-^{14}C-labeled alanine solution was added to each well. The plate was closed and shaken on a horizontal shaker with 120 rpm. An aliquot of 50 µl was taken after 0.5, 1, 1.5, 4, 10 and 24 h and the ^{14}C activity in the supernatant was determined.

Second, the efficiency of the CO_2-trap was tested. A glassfiber-filtermat was installed on top of the 24 well plate. The preprinted well borders on the filtermat were redrawn with silicon-oil to avoid diffusion of the NaOH-drops between the filter segments

above each well. 70 µl of 2 M NaOH were added onto the segments above each well to capture the evolved CO_2. To test the capacity and efficiency of this trap, 500 µl of a 0.08 M ^{14}C-Na_2CO_3 solution with an activity of 0.083 kBq were transferred into the wells. The amount of CO_2 derived from the carbonate reflects the maximum of soil respiration expected during the 78 h of the experiment (approx. 3% of TC). The CO_2-trap was installed above these wells and the CO_2 volatilized completely by adding several drops of 2 M HCl by a syringe directly into the well. The ^{14}C activity trapped on the filter mat during incubation was compared with the added ^{14}C activity. Efficiency of the trap was around 90% and CO_2 efflux from the wells was corrected for this efficiency.

2.5.2.5 Experimental Setup

The availability of absorbed alanine was analyzed in two steps: 1) Alanine was absorbed on sterilized sorbents (heated for 300 °C for 12 h) and the not absorbed alanine was removed by washing with distilled water. This step reflected the affinity of the sorbent to alanine. 2) Thereafter, the sorbed alanine was mixed with the soil and incubated for 3 days. This step showed the effect of sorption on microbial utilization and decomposition.

For the main experiment the sorbents were sterilized by heating overnight at 300 °C and afterward cooled down and stored in a dry air desiccator. 200 mg of sorbent were added in each of 20 wells of the 24 well plate. Four wells (one per line: A5, B5, C5 and D5) without mineral and ^{14}C addition were used as controls e.g. to check for diffusion from one filter segment to another through the silicon oil and to check for contamination by suspension drops into the wells. Solution in the well and on the filter segment was never contaminated by neighboring wells and was used to correct for the background ^{14}C activity. To the five remaining wells per row, 1 ml of a 50 µM alanine solution with a ^{14}C activity of 0.83 kBq was added. The four rows represented one treatment of U-^{14}C-labeled alanine (row A) and the three position-specifically labeled isotopomers 1-^{14}C- (row B), 2-^{14}C- (row C) and 3-^{14}C-labeled alanine (row D). After adding the sorbent the plate was shaken for 24 h on the horizontal shaker (120 rpm). The plate was then centrifuged at 4000 rpm for 10 min and all the supernatant was removed. The sorbent was washed 3 additional times with millipore water to remove all unbound alanine. Until the experiment start the prepared plates were stored frozen (-20 °C) to prevent microbial degradation of the sorbed alanine.

After defrosting the plate, the sorbent was resuspended in 3 ml millipore water and 800 mg of fresh soil were added. The plate was covered by the CO_2-trap (with filter segments separated by silicon oil as described above), which was fixed by a complete cover with parafilm. This was fixed under slight pressure on a metal construction and shaken at

120 rpm (shaking intensity was optimized that no cross-contamination between the wells occurred). Sampling took place after 1, 3, 6, 12, 24, 36, 52 and 78 h. The filtermat was removed and cut into segments. Simultaneously the plate was centrifuged for 5 min at 4000 rpm and a 70 µl aliquot of the supernatant was removed to measure the ^{14}C activity. Afterward, soil and sorbent were resuspended, a new CO_2-trap was installed and incubation continued as before. After the last sampling, the supernatant was removed, the plates were frozen, freeze-dried and the soil was combusted at 600 °C for 10 min under a constant O_2 stream with an HT 1300 solid combustion module of the multi N/C 2100 analyzer (Analytik Jena, Jena, Germany). $^{14}CO_2$ released by combustion was trapped in 10 ml of 1 M NaOH in a vigreux column.

2.5.2.6 Chemical and radiochemical analyses

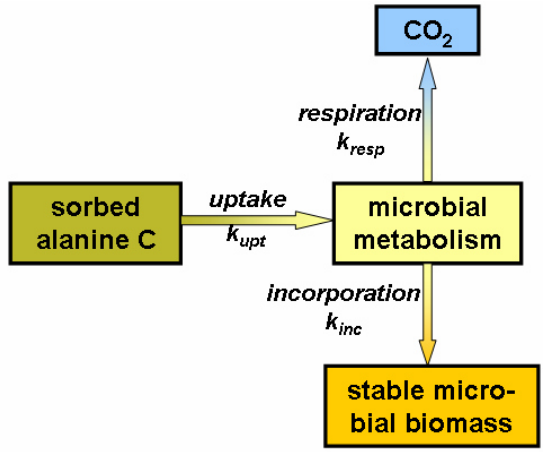
^{14}C activity of the supernatants was determined on a scintillation counter (Wallac 1450, MicroBeta® TriLux, PerkinElmer, Walham MA; USA) by adding the 70 µl aliquot to 0.6 ml scintillation cocktail in 24 well plates. Filter segments and NaOH-solution after combustion were measured in glass scintillation vials on the LS 6500 scintillation counter (LS 6500, Beckman-Coulter, Krefeld, Germany). 3 ml of the NaOH-solution was mixed with 6 ml of scintillation cocktail (EcoPlus, Roth Company, Germany). Filter segements were also added to 6 ml of scintillation cocktail, preconditioned with 0.5 ml of 2 M NaOH.. Each scintillation vial was stored for 24 h in dark until disappearance of chemoluminescence.

2.5.2.7 Calculations and modeling

The initially sorbed ^{14}C activity per well was calculated by the sum of 1) the captured CO_2 over 72 h, 2) the dissolved activity in the supernatant and 3) the activity of the combusted soil-sorbent mixture. All graphs and calculations were done in percent of the initially sorbed ^{14}C activity.

Microbial decomposition of alanine was expressed in % of sorbed alanine ^{14}C per h and as cumulative respiration of all sampling times. A four pool model, based on first-order kinetics according to Kuzyakov and Demin (1998) – considering sorbed alanine C, microbially uptaken C, respired CO_2 and alanine C incorporated in microbial biomass (Fig. 1) – was adapted to these data. Curve fitting by simplex algorithm was done by Model Maker (Model Maker, Version 3.1 MMAN 1, CHEM Research GmbH, Hamburg) and delivered the following parameters: microbially available alanine C, the uptake constant k_{upt}, the incorporation constant k_{inc} and the respiration constant k_{resp}. The three con-

stants reflect the kinetic constant of alanine transfer between the pools (see Figure 1). The decrease in sorbed alanine $\frac{d(sorbAlaC)}{dt}$ by time occurs by microbial uptake (-Uptake) (first equation). The incorporation rate into stable microbial biomass (Inc = $\frac{d(stableMB)}{dt}$) as well as the respiration rate (Resp = CO_2 ($\frac{d(CO2)}{dt}$)) originates from C incorporated the pool of microbial metabolites (micMetab) (second and third equation). Consequently uptake is a source for microbial metabolites whereas respiration to CO_2 and stabilization in stable microbial biomass are C sinks for microbial metabolites (fourth equation).



with

$$\frac{d(sorbAla)}{dt} = -Uptake = -k_{upt} \cdot sorbAla$$

$$\frac{d(stableMB)}{dt} = Inc = k_{inc} \cdot micMetab$$

$$\frac{d(CO_2)}{dt} = Resp = k_{resp} \cdot micMetab$$

$$\frac{d(micMetab)}{dt} = Uptake - Resp - Stab = k_{upt} \cdot sorbAlaC - (k_{stab} + k_{resp}) \cdot micMetab$$

Fig. 1 Scheme of the adapted four-pool model to the measured $^{14}CO_2$ efflux.

In this model we disregard the other fluxes (from stableMB to micMetabol and stableMB to sorbAlaC) because of their minor importance within the few days of the experiment.

2.5.2.8 Calculation of the C-1/C-2,3-ratio and the Divergence Index DI_i

According to Djikstra et al. (2011a; 2011c) the C-1/C-2,3-ratio was calculated for 78 h of incubation from the fitted CO_2-release (equation 1). Ala-C-j represents the percent of Ala-C incorporation from the position j of the molecule into that pool. The ratio reflects the relative activity of glycolysis (leading to C-1 respiration) compared to the citric acid cycle (causing C-2 and C-3 respiration):

$$C\text{-}1 \Big/ C\text{-}2,3 = \frac{[Ala - C\text{-}1]}{\sum_{j=2}^{3}[Ala - C\text{-}j]/2} \quad (1)$$

After 78 hours of incubation the alanine C remaining in the soil and that decomposed to CO_2 was determined as a relative percentage of the sum of total ^{14}C activity. According to Dippold and Kuzyakov (2013) the transformation of C from individual molecule positions was presented by the Divergence Index DI_i, which was calculated for each of the three labeled C positions of alanine, whereas Ala-C-i is accordingly the percentage of incorporation of the position i into the respective pool:

$$DI_i = \frac{n \cdot [C\text{-}i]}{\sum_{j=1}^{n}[C\text{-}j]} = \frac{3 \cdot [Ala - C\text{-}i]}{\sum_{j=1}^{3}[Ala - C\text{-}j]} \quad (2)$$

This index reflects the fate of individual C atoms from the position i relative to the mean transformation of the total number of C atoms n within a transformation process. Thus, a DI_i of 1 means that the transformation of this position in the investigated pool corresponds to the transformation of uniformly labeled substance (average of all C atoms). DI_i ranges from 0 to n, and values between 0 and 1 reflect reduced incorporation of the C into the investigated pool, whereas values between 1 and n show increased incorporation of the C atom into this pool as compared to the average. As this index is independent of absolute amounts of the substance, it enables comparing the alanine C distribution in various pools.

2.5.2.9 Statistics

All experiments were done with five replications, and the values on figures present mean ± standard error of mean (± SEM). SEM of the divergence index was gained by Gaussian error propagation. Measured variables were screened for outliners using the Nalimov test (Gottwald, 2000), tested for normal distribution using the Kolmogorov Smirnoff test and for homogeneous variances using Levene's test. Nested ANOVA, with the factor C position being nested in the factor sorbent treatment, were done using Statistica (version 7.0, Statsoft GmbH, Hamburg, Germany). If assumptions such as normal distribution or homogeneous variances were not met, the result of the nested ANOVA was confirmed by non-parametric Kruskal-Wallis ANOVA before performing a Tukey HSD post-hoc test for unequal sample size.

2.5.3 Results

2.5.3.1 Sorption and microbial utilization of uniformly labeled alanine

In the first part of the experiment, sorption of alanine to various sorbents was compared. There were no differences in the sorption of the three alanine positions and uniformly labeled alanine onto the sterile sorbents. Thus, sorption affinity is represented by the results of the uniformly labeled treatment (Table 2): activated charcoal and goethite showed highest sorption of the added alanine (45%), with lower values in kaolinite, haematite and smectite (26, 19 and 12%, respectively). This sorbed amount of alanine was set to 100% for all further calculations of microbial utilization in soil.

In the second part of the experiment the minerals with sorbed alanine were added to soil to prove the effects of an active microbial community. This experiment demonstrated that the sorption strength varied strongly between the minerals: Within the iron minerals, goethite sorbed most alanine, and the lowest portion – only 26% – of this U-^{14}C alanine was available to microorganisms. In contrast, haematite sorbed only 19% of the added alanine and 44% of this U-^{14}C alanine was microbially available. Within the clay minerals a quite similar percentage of 30 to 35% of the sorbed U-^{14}C alanine was usable by microorganisms (Table 2). Activated charcoal was the most efficient sorbent for alanine: it did not only adsorb the most of the added alanine (45%), but also the smallest portion (22%) was accessible.

Table 2 Initially sorbed alanine C and fitted parameters to the four-pool model for microbial utilization of sorbed alanine (Fig. 1) fitted to the data of uniform alanine labeling

	Sorbed Ala C (% of added)	Available Ala C (% of sorbed)	Uptake rate k_{upt} (% h^{-1})	Incorporation rate k_{inc} (% h^{-1})	Mineralization rate k_{resp} (% h^{-1})	R^2 of model
Goethite	44.37	26.23 ± 0.39	0.1627 ± 0.0068	2.098 ± 0.105	4.852 ± 0.237	0.993
Haematite	18.50	43.61 ± 0.88	0.0841 ± 0.0041	1.923 ± 0.146	5.331 ± 0.401	0.983
Smectite	11.84	33.42 ± 0.61	0.6390 ± 0.0405	5.067 ± 0.411	17.486 ± 1.392	0.980
Kaolinite	26.08	32.69 ± 0.69	0.6268 ± 0.0497	6.584 ± 0.666	24.875 ± 2.497	0.990
Activated charcoal	45.41	22.31 ± 0.38	0.1585 ± 0.0066	8.330 ± 0.319	10.200 ± 0.390	0.991

The decomposition to CO_2 of the U-^{14}C labeled alanine bound to clay minerals peaked within the first 5 h, with subsequent fast decrease (Fig 2). In contrast, the peak of decomposed alanine bound to iron minerals and to activated charcoal was ca. 5 times lower, with a subsequent slow decrease. It has to be considered that the continued low mineralization of clay mineral bound alanine is not represented well by the model, which approaches an equilibrium state for the last hours of the experiment. However, the faster initial decomposition of clay mineral bound alanine is also reflected by the fitted microbial uptake rate for bound alanine k_{upt}, which was highest for the clay minerals (~0.6% h^{-1}) and at least 5 times lower for the iron minerals and activated charcoal (0.08 to 0.16% h^{-1}).

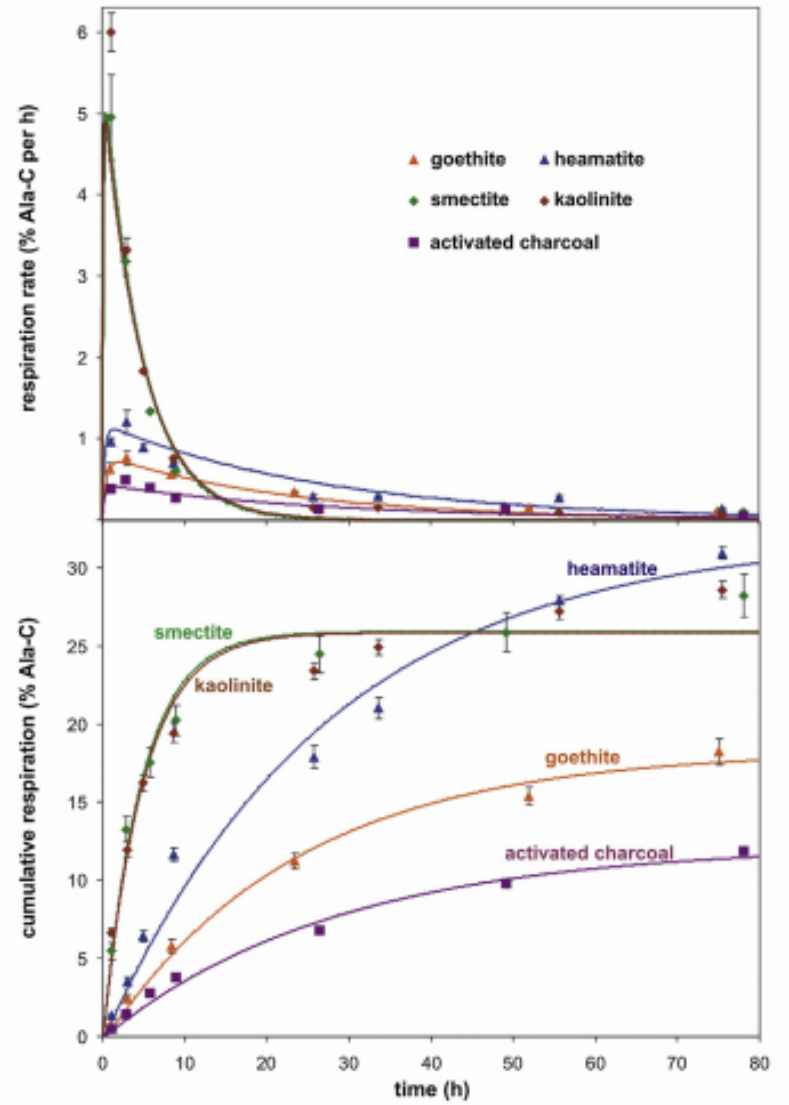


Fig. 2 Respiration rate (in % of sorbed alanine C per h) and cumulative $^{14}CO_2$ efflux from alanine adsorbed to various sorbents; Experimental points (means ± SEM, N=6) and fitted curves based on the microbial utilization model (Fig. 1) are presented.

This microbial availability and the respective uptake rate of sorbed alanine affected the metabolic utilization: the faster the desorption and uptake took place, the higher the portion of microbial mineralization versus incorporation. The mineralization rate of alanine sorbed on clay minerals was 3-4 higher than the incorporation rate, whereas for iron minerals this ratio lies between 2 and 3, with activated charcoal having the lowest value (1.2). Thus, the availability of alanine C and desorption kinetics of individual minerals had a distinct effect on C allocation between cata- and anabolism in microorganisms.

2.5.3.2 Kinetics of position-specific utilization of sorbed alanine C

In the first part of the experiment the sorption of individual alanine molecule positions was identical. However, microbial transformation of sorbed alanine in the second part of the experiment was strongly different for individual molecule positions. Therefore, a more detailed insight into the mechanisms of catabolic to anabolic alanine utilization can be gained by comparing the kinetics of the utilization of individual positions of alanine C: For each of the sorbents, the mineralization to CO_2 followed the order C-1 > C-2 > C-3 (Fig. 3 and Table 3). This is also reflected in the fitted amount of available C and uptake constants k_{upt} (Table 3). Thus, irrespective of the sorbent, the carboxyl group was less stabilized and the highest amount was respired. Consequently, the alanine was either 1) bound to all sorbents not by the carboxyl group, but by the methyl or alkyl-amino group and extracellularly cleaved or 2) the desorption of alanine occurs as the whole molecule, and microbial metabolism accounts for the preferential decarboxylation of C-1.

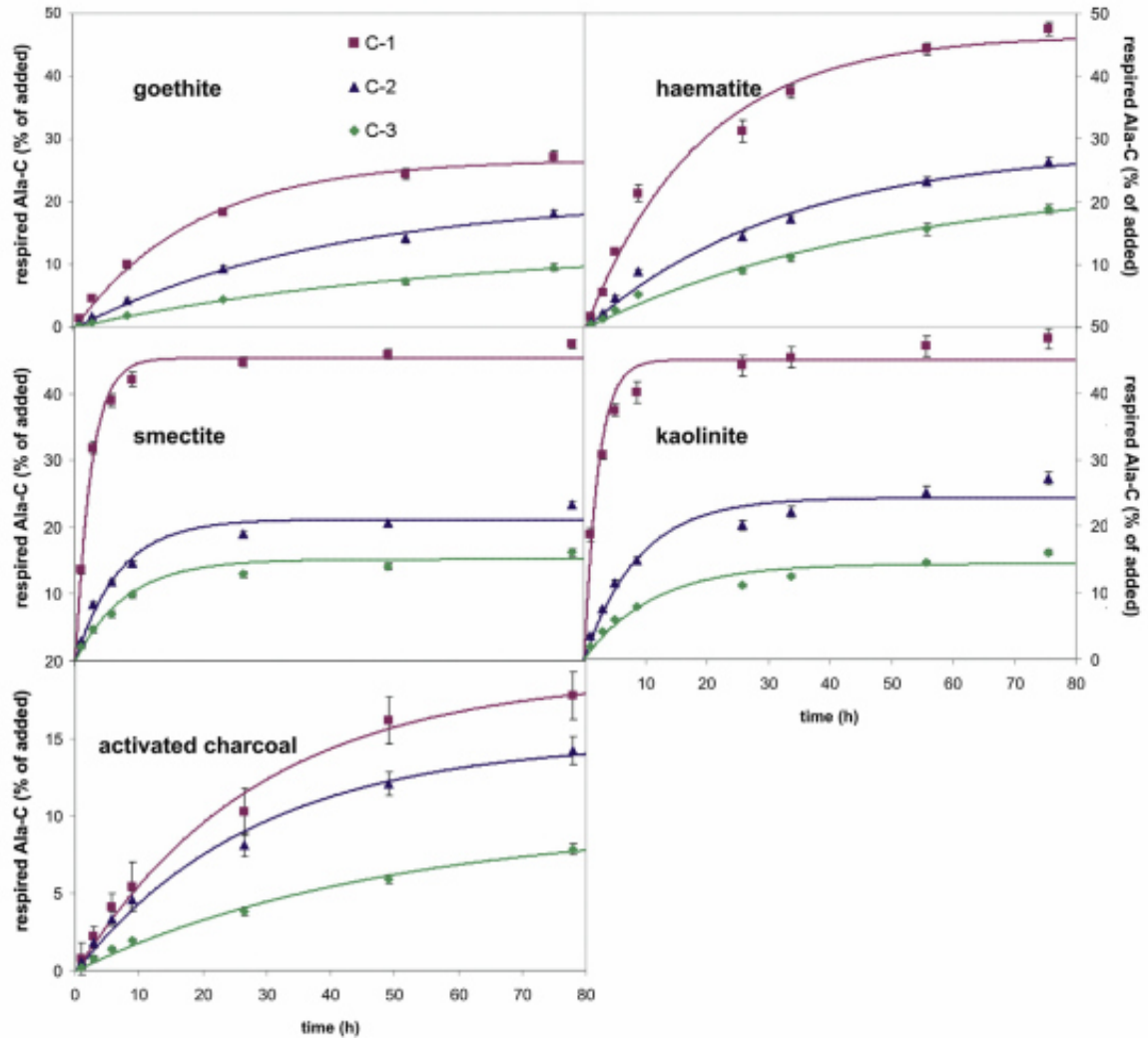


Fig. 3 Cumulative $^{14}CO_2$ efflux (in % of initially sorbed alanine C) from individual molecule positions of alanine; Experimental points (means ± SEM, N=6) and fitted curves on the microbial utilization model (Fig. 1) are presented.

The initial mineralization peak was most pronounced for the alanine sorbed on clay minerals. During this peak, the mineralization rate was highest for C-1 and differences between C-1 and both other positions (C-2 + C-3) were most pronounced within the first hours (Fig. 4). Later, the C-1 mineralization rate was even lower than that of C-2- and C-3 mineralization (Fig. 4). This C-1 oxidation peak was less pronounced for alanine sorbed to iron minerals and did not occur for activated charcoal sorbed alanine (Fig. 4). For this most efficient sorbent the general behavior of the positions changed. C-1 and C-2 behaved more similarly, whereas C-3 was best stabilized by activated charcoal (Fig. 3 and Fig. 4). This trend was also visible in the fitted microbially available C and the uptake constant k_{upt}: for all minerals, k_{upt} of C-1 was higher than the respective values for C-2 and C-3, whereas in the case of activated charcoal the C-3 and C-2 exceeded C-1 (Table 3). In addition, the C-1/C-2,3 ratio shown in Figure 4 reflects a constant ratio of positon-specific respiration for the whole experiment. This is in contrast to the other four treatments with mineral-associated alanine, where always an initial peak of C-1 respiration was followed by a peak in C-2,3 oxidation for the late period of the experiments. These divergences in the curve shape (Figure 4) as well as position-specific individualities (Figure 4) suggest of special transformation pathways observed for alanine bound to activated charcoal.

Table 3 Fitted parameters of the four-pool model for microbial utilization of sorbed alanine (Fig. 1) for the individual alanine C positions

		Available Ala C (% of sorbed)	Uptake const. k_{upt} (% h^{-1})	Incorporation const. k_{inc} (% h^{-1})	Mineralization const. k_{resp} (% h^{-1})	R^2 of model
Goethite	1-Ala	31.54 ± 0.40	0.168 ± 0.007	1.055 ± 0.089	5.869 ± 0.468	0.995
	2-Ala	28.76 ± 0.41	0.089 ± 0.003	1.427 ± 0.075	3.648 ± 0.183	0.994
	3-Ala	19.53 ± 0.25	0.091 ± 0.002	2.771 ± 0.098	5.186 ± 0.191	0.996
Haematite	1-Ala	56.56 ± 1.02	0.096 ± 0.045	0.949 ± 0.097	4.393 ± 0.441	0.985
	2-Ala	43.07 ± 0.75	0.071 ± 0.034	2.165 ± 0.112	4.257 ± 0.218	0.988
	3-Ala	36.53 ± 0.62	0.056 ± 0.023	2.243 ± 0.108	4.027 ± 0.191	0.989
Smectite	1-Ala	59.79 ± 0.62	0.685 ± 0.031	2.065 ± 0.090	6.449 ± 0.271	0.992
	2-Ala	28.28 ± 0.58	0.515 ± 0.035	5.738 ± 0.476	17.120 ± 1.353	0.977
	3-Ala	18.98 ± 0.38	0.636 ± 0.042	9.134 ± 0.810	31.090 ± 2.759	0.980
Kaolinite	1-Ala	55.83 ± 1.00	0.798 ± 0.067	2.537 ± 0.236	10.559 ± 0.965	0.969
	2-Ala	31.65 ± 0.72	0.359 ± 0.029	7.216 ± 0.707	23.860 ± 2.327	0.969
	3-Ala	18.48 ± 0.48	0.484 ± 0.044	14.855 ± 1.732	51.958 ± 5.977	0.962
Activated charcoal	1-Ala	36.20 ± 0.58	0.097 ± 0.004	3.740 ± 0.026	4.155 ± 0.141	0.992
	2-Ala	25.62 ± 0.45	0.138 ± 0.006	5.909 ± 0.032	8.184 ± 0.341	0.990
	3-Ala	16.35 ± 0.25	0.128 ± 0.004	12.832 ± 0.034	18.113 ± 0.664	0.993

The sorbent affected not only microbial uptake but also the intracellular metabolization of C from individual amino acid positions. Whereas mineralization and the incorporation constants of alanine bound on clay minerals and activated charcoal followed the order C-3 > C-2 > C-1, the alanine bound on iron oxides showed also the lowest incorporation rate for C-1, but similar mineralization rates for C-1, C-2 and C-3. Intracellular metabolization is also reflected by the C-1/C-2,3-ratio, which provides information about the relative intensity of glycolysis to citric acid cycle (Fig. 4). A clear peak in glycolysis intensity was observed for the mineralization of clay-mineral-bound alanine, but after an initial phase of C-1 oxidation the alanine metabolization changed to an increasing proportion of

citric acid cycle activity. In contrast, this initial glycolysis activity was much less expressed for iron-oxide-bound alanine, and no change in the metabolization pathway over time was observed for the microbial utilization of active-coal-bound alanine.

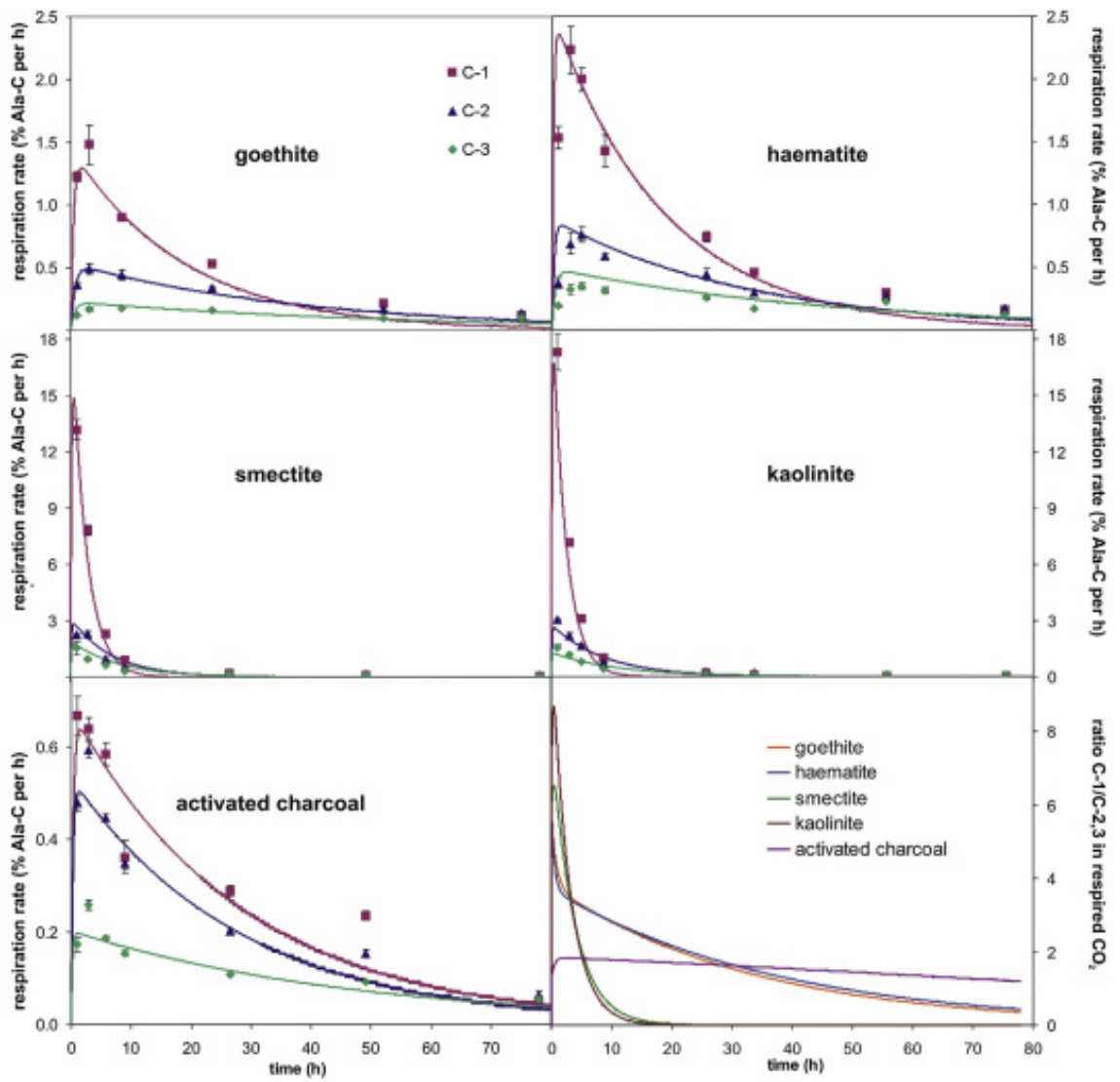


Fig. 4 Respiration rate (in % of initially sorbed alanine C per h) of individual molecule positions of alanine and ratio of C-1 to (C-2+C-3)/2 respiration of alanine C for the used sorbents calculated from the fitted position-specific oxidation rate; Experimental points (means ± SEM, N=6) and fitted curves on the microbial utilization model (Fig. 1) are presented.

Thus, the sorbed amount as well as sorption strength of the sorbents affected not only the availability and uptake rate of alanine, but apparently influenced the uptake processes. Subsequent splitting of the molecule, as well as intracellular utilization by cata- and anabolism of the microorganisms, are controlled by the way alanine is sorbed in soils.

2.5.3.3 Incorporation of C from alanine positions in stabilized pools and decomposition to CO_2

During the incubation of sorbed alanine with soil, the portion of dissolved and respired alanine C was measured. Whereas cumulative CO_2 efflux for each position and each sorbent showed a saturation curve (Fig. 4), the amount of dissolved alanine C was always very low, never exceeding 1% of the total alanine C in the system (Fig. 5). Thus, desorbed alanine or alanine fragments persisted only extremely briefly in the dissolved state or were even directly exchanged from the sorbed form. As there was no significant difference in the alanine C positions in solution (Suppl. Table 1), the measured ^{14}C in the supernatant is intact alanine or the deaminated product pyruvate.

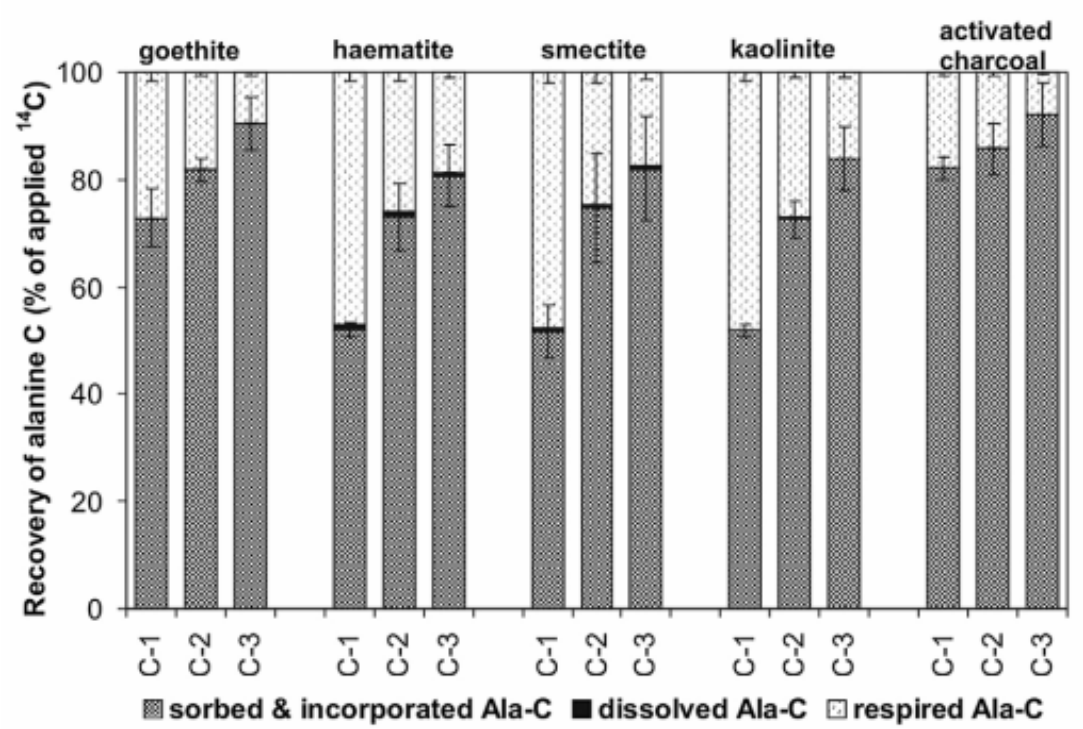


Fig. 5 Percentage of alanine C in bound fraction, dissolved fraction or respired to CO2 after 78 h. Values show means ± SEM (N=5) depending on the sorbents for the individual alanine C positions.

After 72 h, between 8 and 48% of alanine C were decomposed to CO_2 and 52–92% were still bound to the sorbents or incorporated into microbial biomass (Fig. 5). The absolute portion of ^{14}C incorporation per pool (Fig. 5, Suppl. Table 1) as well as the divergence index (Fig. 6, Suppl. Table 2) revealed that the general trend is similar irrespective of the sorbents: C-1 is preferentially mineralized to CO_2, whereas C-3 either remains bound or is incorporated into microbial biomass.

Considering the relative C allocation by the DI, released CO_2 – the product of microbial catabolism – had much higher discrimination between the positions than bound alanine C (Fig. 6). Hence, the DI of respired alanine C has to reflect a value inverse to

the incorporation into microbial biomass – the anabolic branch. Fig. 6, however, shows that DI of respired CO_2 and sorbed C are not inverse and highest DI was observed for smectite, kaolinite and haematite (Fig. 6). These were the minerals with the highest percentage of microbially available alanine. In contrast, activated charcoal and goethite showed the lowest range in DI for the sorbed alanine C (Fig. 6).

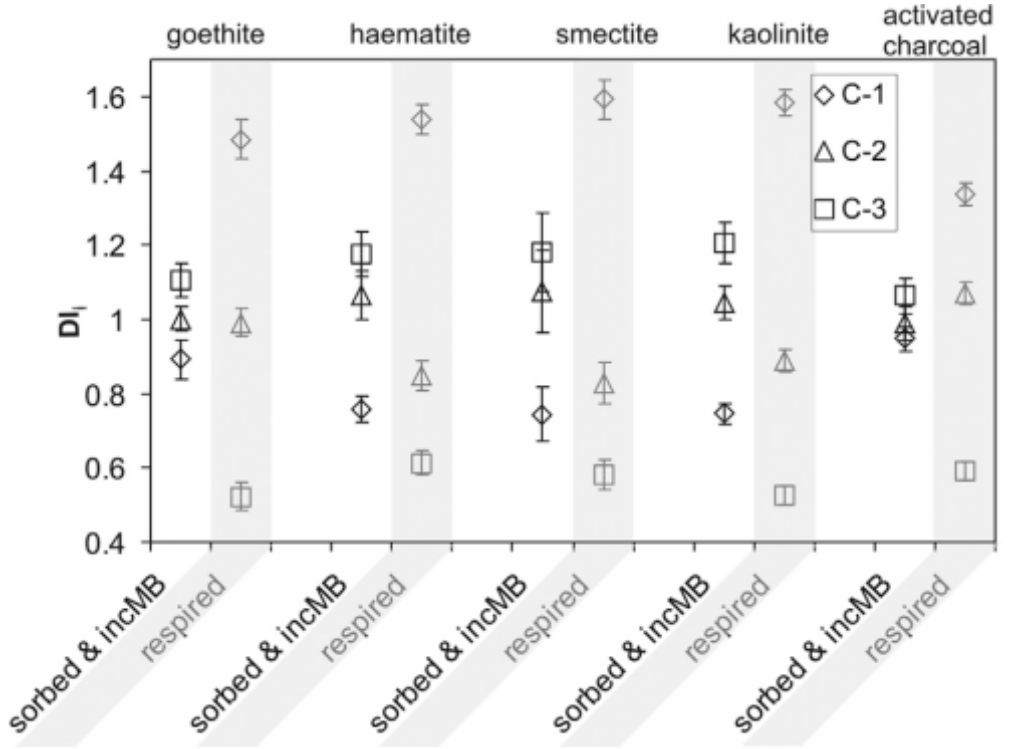


Fig. 6 Divergence Index (DI) of sorbed alanine and alanine incorporated into microbial biomass (sorbed & incMB) and respired alanine C for the five sorbents after 78 h; Values show means ± SEM (N=5) calculated by Gaussian error propagation.

The DI of CO_2, which represents a pool passed to 100% through microbial catabolism, reflected differences in metabolic utilization of C from the three positions of alanine. Alanine sorbed to clay minerals and haematite showed a clear preferential degradation of C-1 compared to C-2 and C-3. In goethite-sorbed alanine, the C-2 was not preferentially degraded or incorporated, but showed a DI of 1. In contrast, alanine bound on charcoal reflected a preferential degradation of C-1 and C-2, and only C-3 was preferentially protected from decomposition. Thus, sorption mechanisms as well as microbial availability affected alanine transformation (Suppl. Table 2), at least over the first 78 h.

2.5.4 Discussion

2.5.4.1 Sorption mechanisms of amino acids

Preliminary studies to this experiment with mineral phases (results not shown) as well as sorption studies with soil (Dippold and Kuzyakov, 2013) confirmed the hypothesis that sorption of alanine occurs as a whole molecule. Abiotic splitting as described for glycine by Wang and Huang (2003) seemed to be of minor importance for alanine sorption.

The high range of microbial uptake rates of alanine bound to various sorbents – differing by a factor of 6 – suggests various sorption strengths and presumably also sorption mechanisms. For a eutric Cambisol, Jones and Hodge (1999) found the highest sorption for positively charged lysine compared to the dipolar glycine and lowest sorption for the negatively charged glutamic acid. Similarly, the mineralization of cationic lysine was higher compared to the dipolar ion leucine in an eutric Cambisol (Gonod et al., 2006). These results clearly support the concept of cation exchange by positively charged amino groups as the main sorption mechanism for amino acids in soil especially for amino acids with a net positive charge.

Investigations of negatively charged LMWOS focused mainly on organic acids. Many studies have demonstrated the ability of LMWOS to sorb by their carboxyl group (Jones, 1998; Jones and Brassington, 1998; Strahm and Harrison, 2008) either by direct ligand-exchange or via cation bridges. Iron oxides are known to effectively bind and stabilize them (Jones and Edwards, 1998; Kaiser and Zech, 2000a). Nagarajah (1970), however, showed that the ability of alanine as a dipolar ion for ligand-exchange mechanism is much lower than for most of the investigated organic acids, which were confirmed by Rothstein (2010). Accordingly, neutral amino acids such as alanine have no clear preference for a charge-dependant sorption mechanism and are either able to interact 1) by ligand exchange via their carboxyl group (Strahm and Harrison, 2008), 2) by cation- or anion exchange with the charged group (Rothstein, 2010) or 3) show non-specific weak interactions such as dipole-dipole and H-bondings, van-der-Waals or entropy-driven hydrophobic interactions (Brigatti et al., 1999).

Our results support diverse but specific sorption mechanisms for alanine: Amount of sorbed alanine is clearly related to the specific surface area of the sorbents, especially in the case of activated charcoal. Specific sorption mechanisms of the investigated sorbents can be concluded from differences in the sorbed amount as well as from differences in the percentage of microbially available alanine. Fast desorption and microbial uptake of alanine sorbed by clay minerals shows its weak bonding, which can either be cation exchange or non-specific weak interactions (Fig. 7). Both mechanisms show exchange reactions with competing molecules. Thus, when sorbent with alanine was added

to the soil, cations as well as DOC compounds caused immediate exchange reactions, releasing bound alanine (Fig. 7). This mechanism explains the immediate and pronounced peak in respiration (Fig. 2). This initial $^{14}CO_2$ peak, however, was only a small portion of the totally sorbed alanine (33%). This indicates an additional binding mechanism causing the portion of non-bioavailable alanine in this study. Intercalation into clays is unlikely because this mechanism preferentially stabilizes hydrophobic, aromatic compounds and typically occurs with smectites rather than kaolinites (Wattel-Koekkoek et al., 2001). As the total amount of sorbed alanine is higher for kaolinite than for smectite, another mechanism has to dominate. Boudot (1992) observed that mainly surface $Al(OH)_3$-groups stabilize organic C, probably by interactions with the carboxyl groups of LMWOS. Only kaolinite (not smectite) possesses free $Al(OH)_3$ groups, explaining the higher sorption capacity of kaolinite in this experiment (Fig. 7).

A comparable initial $^{14}CO_2$ peak cannot be observed with alanine sorbed to iron oxides (Fig. 2) indicating other, stronger binding mechanisms. Many studies showed that iron oxides are very strong and efficient sorbents in soil exceeding the capacity of clay minerals (Jones and Edwards, 1998; Kaiser and Zech, 2000a, 2000b). These studies suggested a sorption mechanism based on the carboxyl group of the LMWOS – presumably ligand exchange (Jones and Edwards, 1998; Kaiser and Zech, 2000b; Strahm and Harrison, 2008). This helps explain the strong sorption and slow desorption and microbial utilization of iron-oxide-bound alanine (Fig. 7). Our result that goethite sorbed more alanine, along with the lower bioavailability of the sorbed alanine, supports the mechanistic view of ligand exchange: Goethite is known to have a higher portion of OH-groups than haematite, which is the relevant functional group for ligand bonding. Nevertheless, a combination of binding mechanisms as well as multi-side coordinative bonds should be considered for interpretation (Kaiser and Zech, 1999).

Activated charcoal is a relevant sorbent in hydrophobic interactions (Choi et al., 2008), and sorption studies with organic molecules of various properties show that aromatic rings are not necessary for interaction with coal structures (Cornelissen et al., 2005b). Hydrophobic interactions by the methyl group of alanine as well as polar-π-interactions with COO^-, $-NH_2$ or NH_3^+ groups are possible (Keiluweit and Kleber, 2009) – all reflecting very strong binding mechanisms. Thus, theoretically each functional group of amino acids can interact with the activated charcoal (Fig. 7). In combination with the large surface of the activated charcoal, this explains its high amount of sorbed alanine. The strong binding is shown by the missing initial mineralization peak and activated charcoal showed the slowest respiration kinetics as well as the lowest proportion of bioavailable alanine (Fig. 2 and Table 2). This confirms the view of Keiluweit and Kleber (2009) and

Cornellissen et al. (2005a) on coal's high sorption capacity for a broad range of LMWOS, which is presumably even higher for activated charcoal.

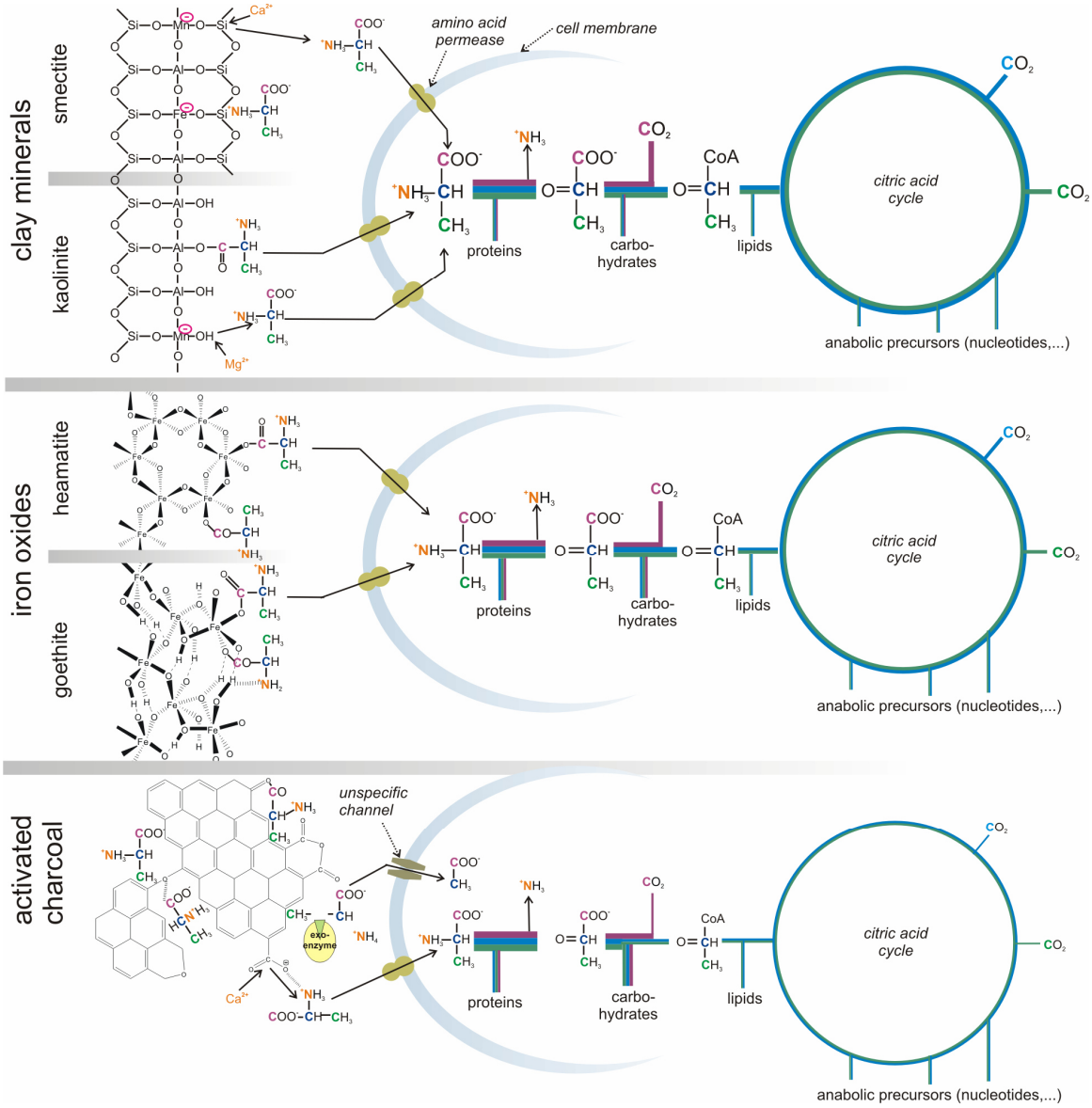


Fig. 7 *Metabolic pathways of alanine sorbed on clay minerals (smectite and kaolinite), iron oxides (haematite and goethite) and active coal. Detailed explanations in text. Various colors show the pathways of C from individual positions of alanine. Line width represents the qualitatively estimated relative shifts in the fate of alanine C positions between certain pathways dependent on the sorbent class.*

Caution should be exercised in transferring these results and supposed mechanisms directly to natural soil conditions, because we used systems showing no initial covering of the sorbents' surface at the experiment start. Under soil conditions, however, a high proportion of sorbent positions are likely to be occupied by a diverse spectrum of ions and organic compounds. In addition, the adsorption properties of sorbents may strongly change if multilayer sorption (as described by the zonal model of Sollins et al. (2007)) takes place in soils. Assuming this sorption model, the mechanisms investigated

here reflect only contact zone sorption, and the situation under soil conditions becomes more complex. At the same time, only a small percentage of the sorbent's surface may be covered by organic films according to the multilayer model (Heister et al., 2012). Therefore, the relevance of complex multilayer sorption versus direct interaction with the mineral surfaces is not yet quantified.

2.5.4.2 Bioavailability of sorbed alanine

The available C in the soil as well as the bioavailable portion of the sorbed alanine are potential microbial C sources. The results showed that for each of the sorbents a distinct portion of the sorbed alanine is bioavailable and can be mineralized by microorganisms (Table 2). Dashman and Stotzky (1982) showed, for clay minerals, that permeases have a higher affinity for amino acids than their sorbents. Hence, from a kinetic and energetic point of view, microorganisms located close to a sorbent can take up at least parts of the sorbed amino acids. Thus, two mechanisms can explain the partial bioavailabilty of sorbed amino acids: 1) passive desorbing due to exchange with DOC and ions from the soil and 2) active desorbing by microorganisms that is followed by active uptake through their membranes (Fig. 7).

Protein expression of the respective transport proteins is a fast process (Jones et al., 1996). The respective amino acid transporters as well as degradation pathways are ubiquitous, enzymes and transporters constitutively expressed (Anraku, 1980; Gonod et al., 2006) and consequently it can be expected that they are already present in the microbial community living based on the available soil C sources when the experiment started. The establishment of microbial communities that live near or on the sorbents' surface is a more time consuming process (Chenu and Stotzky, 2002) and probably explains the observed delay in microbial utilization of sorbed amino acids (Gonod et al., 2006; Jones and Hodge, 1999). An additional explanation might be that alanine is slowly desorbed from inaccessible domains of the sorbents and time for desorption as well as diffusion delays the uptake of alanine by microorganisms. Nonetheless, after establishment of such a sorbent-associated microbial community, e.g. in biofilms, a positive feedback reaction can be expected: The sorbent might provide a co-location of various growth resources (sorbed DOC + nutrients from the soil), and biofilms themselves are able to establish synthrophies (Schink, 1997; Schink and Friedrich, 1994). This is consistent with our results, especially for the activated charcoal: The decomposition of alanine to CO_2 is still ongoing at 78 h. Accordingly, even after 78 h, microorganisms may still not have reached all parts of the high specific surface area of activated charcoal (Table 1). Continued mineralization of alanine in each of the treatments (but most pronounced for acti-

vated charcoal) can be explained by microorganisms gaining access and colonizing new surfaces. This process was also observed but less expressed for each of the mineral phases.

Conversely, inaccessibility of parts of the sorbent surfaces for microorganisms explains the portion of irreversible sorption and thus stabilization of LMWOS observed in this and other studies (Table 2 and Fig. 7). This inaccessibility is likely to occur in activated charcoal, where large DOC molecules can fill pores and block accessibility and exchange of LMWOS from these pores (Nguyen et al., 2007). Together with the strong sorption mechanism of activated charcoal, this explains the high portion of non-bioavailable amino acids (Table 2). In addition to inaccessibility of the LMWOS, microbial cells or exoenzymes can be immobilized by sorbents and reduce mineralization (Jin, 2010; Novick and Rozell, 2005; von Luetzow et al., 2006). The immobilization of microbial cells and exoenzymes by charcoal can strongly affect the subsequent transformation pathways of alanine.

Sorption occurs as a whole molecule, i.e. all three positions are sorbed together. Accordingly, an increase in inaccessible, not bioavailable alanine at the sorbent would cause an approximation of the DI of all three positions to 1.0. On contrast alanine incorporated into microbial biomass should have a DI complimentary to respired CO_2. This study showed that sorbents with a low portion of accessible alanine and thus a high portion of untransformed alanine bound to the sorbent (such as activated charcoal or goethite) have DIs closest to one (Fig. 6). On contrast, haematite and the clay minerals reveal significant differences between the DI of the individual positions. Hence, a DI close to one reflects a high proportion of untransformed alanine C bound to the sorbent whereas a DI close to the complimentary value of respired CO_2 reflects a dominance of microbial biomass C associated to the mineral surface (Fig. 6). The DI of alanine C, however, was never exactly 1.0 for any of the sorbents after 78 h (Fig. 6). This shows that at least some of the surface-associated alanine C was transformed to metabolites. This part shows the classical pattern of alanine-C utilization of microbial cells (Dippold and Kuzyakov, 2013): preferential incorporation of C-2 and C-3 in microbial biomass due to a preferential mineralization of the carboxyl group (C-1). This result supports the idea that cells that approach the sorbents or even establish as biofilms on their surfaces are mainly responsible for the utilization of sorbed LMWOS.

2.5.4.3 Pathways of microbial metabolization of sorbed alanine

The DI enables a better comparison between the sorbents. This is because DI is not overprinted by absolute differences in the uptake and utilization rates and amounts.

The DIs of incorporation of single C positions into the decomposed and sorbed pool were calculated and revealed significant differences (Suppl. Table 1): DI of the respired CO_2 showed, for each sorbent, a preferential respiration of the C-1 group and a preferential stabilization of the C-3 group (Fig. 3). Thus, the metabolization of bound alanine generally follows the same pathways as demonstrated for free alanine (Dippold and Kuzyakov, 2013): 1) deamination to pyruvate, 2) entering glycolysis and decarboxylation to acetyl-CoA and 3) successive oxidation in the citric acid cycle. This enables applying the C-1/C-2,3-ratio – used by Djikstra et al. (2011a) for pyruvate metabolization in soil – to alanine metabolization to determine the ratio of glycolysis to citric-acid cycle oxidation. Both this ratio (Fig. 4) and the fitted kinetic constants for C metabolization via ana- and catabolism (k_{inc} and k_{resp} in Tab. 2) showed a clear effect of the sorbent, i.e. the sorption mechanism, on the metabolization of alanine. The uptake rate k_{upt} showed that, in contrast to sorption, desorption and microbial uptake do not necessarily occur as an intact molecule. This means that the alanine molecule will be split just before, during or immediately after microbial uptake. The exoenzymes using alanine exist in soil but play a minor role for the utilization of free alanine (Dippold and Kuzyakov, 2013). For strongly sorbed molecules the uptake of intact alanine by microbial cells may no longer be possible. Indeed, some functional groups of alanine (e.g. COOH groups or amino-bound C) might still be accessible for exoenzymes and consequently will be split before transformation products enter microbial cells (Fig. 7). This was already described for charcoal surfaces by the co-location model of Lehmann et al. (2011), which explains the accumulation of microbial cells as well as their enzymes on the sorbent surface. Thus, parts of the molecules might be taken up as split fragments after exoenzymatic cleavage. However, the kinetic constants k_{inc} and k_{resp} differed more strongly between the positions than the uptake constant k_{upt}. This indicates that the main splitting of the C skeleton of alanine occurred within the microbial cells (Fig. 7) and that further specific pathways of cells and enzymes located at charcoal surfaces occur. This cannot be resolved within the present study.

The C-1/C-2,3-ratio reveals that C-1-oxidation by glycolysis occurred faster and with higher intensity than the C-2 and C-3 oxidation by the citric acid cycle. The initially exchanged alanine is immediately taken up by the microorganisms (Dippold and Kuzyakov, 2013; Fischer et al., 2010) and the C-1 is metabolized very fast via glycolysis (Fig. 7). With a temporal delay the oxidation via the citric acid cycle starts. The glycolysis peaks much less for the alanine sorbed to iron minerals and does not occur for that sorbed on activated charcoal (Fig. 4 and Fig. 5). Thus, highly available free alanine is metabolized by a different intensity of metabolic pathways than sorbed alanine. Kinetics of desorption versus kinetics of microbial uptake determines the relative availability of alanine in soil solution, which determines the C allocation in microbial metabolism

(Dippold and Kuzyakov, 2013). If alanine has to be removed from sorbents by microorganisms this occurs by time- and energy-consuming mechanisms. Two possible reasons might explain the shifts in alanine metabolization if it requires microbial induced desorption: Dashman and Strotzky (1982) already discussed that the more intensive a substrate is bound to a sorbent, the less attractive it is for catabolism because the energy efficiency of that substrate decreases. If, however, C- or N-demand for the anabolism exists, then these substrates are nevertheless desorbed and used, but mainly by anabolic pathways. It has to be considered that in addition to sorbed alanine further C sources were available from the added soil – which might be preferentially used the more inaccessible the alanine is. If we compare the ratio of the kinetic constants for mineralization and incorporation k_{resp}/k_{inc} of the five sorbents investigated in this study, clay minerals ($3.4 < k_{resp}/k_{inc} < 3.8$) exceed iron minerals ($2.3 < k_{resp}/k_{inc} < 2.8$), with the lowest value shown by activated charcoal (1.2). This is consistent with the concept of Dashman and Strotzky (1982) and reflects the increasing portion of anabolic C utilization with increasing sorption strength.

In contrast, Jones and Edwards (1998) argued that cell metabolism might change if cells are attached to surfaces: They have an increasing demand in structural cellular components needed to attach to the surface or for the formation of biofilms like extracellular polysaccharides (Chenu and Stotzky, 2002). This would cause a high demand for gluconeogenesis products and, in turn, dampen the opposite process – glycolysis (Fig. 4). This phenomenon was observed for charcoal for the entire 78 h; it was less expressed with the iron oxides and was not visible for microorganisms using alanine bound on clays.

Our approach cannot definitively distinguish between the potential explanations given by Dashman and Strotzky (1982) or Jones and Edwards (1998). Answering this question would require a metabolite tracing, i.e. characterization of the newly formed microbial products from alanine C. It has to be considered that data based on the modeling approach e.g. the kinetic constants k_{inc} and k_{resp} have to be considered carefully: the model strongly simplifies reality e.g. not considering backflux from stable microbial products towards fast cycling microbial metabolites. This simplification can cause a worse fit of the model especially for the last time points where slower processes, not considered here, become more relevant. Thus, slow processes (e.g. the further mineralization observed at late time points) might be underestimated by this approach. Nevertheless, DI and modeling revealed that with decreasing bioavailability of a substrate due to sorption, an increasing relative portion of this substrate is incorporated into microbial C and this microbial C remains partially associated with the sorbents' surface.

2.5.4.4 Stabilization of amino acid C by sorption

Clearly, interactions with sorbents stabilize DOC and also LMWOS compounds in soils (Kaiser and Kalbitz, 2012). In a similar experiment with lysine, 3.6% of the lysine sorbed on soil was respired (Gonod et al., 2006). This rate was 10 times lower than the decomposition of free lysine, and also 6.6 times lower than the average alanine mineralization observed in this experiment (23.7% of added U-alanine). This can be explained by the two-times-higher sorption of positively charged lysine (two NH_2^+groups) compared to dipolar ions such as glycine or alanine (Jones and Hodge, 1999). Indeed, Jones and Hodge (1999) observed a maximally 3 times higher sorption of positively charged compared to dipolar ion amino acids. Thus, an additional mechanism preventing microbial utilization must have occurred in Gonod's experiment (2006), e.g. spatial inaccessibility within the intact aggregates or enhanced sorption due to a multilayer sorption with additional sorption sites and mechanisms present within soils. The pure minerals used in this study do not allow clear conclusion about sorption mechanisms on partially saturated soil surfaces. In addition, spatial inaccessibility in aggregates and micropores is of minor relevance for this experiment, as we used fine powdered minerals in a shaken soil suspension. However, binding of microbial cells or exoenzyms to strong sorbents may contribute to a reduced utilization of alanine in treatments with strong sorbents. Stabilization by sorption might even be more pronounced in natural soils than in individual clean sorbents because additional stabilization mechanisms like spatial inaccessibility might have synergistic effects there.

Our study shows that a remarkable percentage of sorbed alanine is still microbially available and that direct interactions are not the only mechanisms explaining how LMWOS-C can be stabilized by sorbents. The DI of the bound alanine after 78 h clearly demonstrates some of the sorbed alanine C is already microbially transformed (Fig. 6). Presumably, anabolic cellular as well as extracellular components accumulate on the sorbents' surface and sorption sites (Dashman and Stotzky, 1982; Jones and Edwards, 1998; Miltner et al., 2011). This corresponds to an increase in microbial polysaccharides and proteins associated on the mineral surfaces with increasing soil development (Duemig et al., 2012). Similar results were observed for aging charcoal (Lehmann et al., 2011). Thus, the direct stabilization of LMWOS-C by sorbents is potentially less relevant for the stabilization of C by mineral interactions: Interestingly, the microbially desorbable LMWOS may contribute even more to C stabilization than the irreversibly bound ones if initiating the accumulation of microbial products on the mineral surfaces (Miltner et al., 2007). These products are probably more recalcitrant than the initial LMWOS because they are polymeric polysaccharides, proteins or larger lipids. Nonetheless, they exhibit multiple sorption sites which allow a more intensive binding to the mineral surfaces. They

may also initiate a more diverse sorption surface (Sollins et al., 1996) that may have a self-enhancing effect on further stabilization by sorption.

2.5.5 Conclusions and Outlook

This study demonstrates that stabilization of LMWOS-C by sorption is a complex process: amino acids will be sorbed as whole molecules, but by various sorption mechanisms to the individual sorbents. Clay minerals show a combination of exchangeable, weak binding of alanine as well as stronger interaction with Al-OH-groups, protecting alanine from microbial degradation. Iron oxides sorbed a higher amount of alanine presumably by ligand exchange with the carboxyl group. Especially for goethite, a low portion of sorbed alanine was available for microorganisms. Highest sorption capacity as well as sorption strength was measured for activated charcoal. Modeling reflected that between 22% (activated charcoal) and 44% of sorbed alanine C can be microbially used, but microbial transformation products can be further stabilized by the sorbents.

We conclude that the stronger the sorption by the individual sorbent, the lower the microbial utilization. The fate of individual molecule positions showed that, at least for the four mineral phases, the alanine is used by the classical biochemical pathways: deamination, decarboxylation of C-1 and further oxidation of C-2 and C-3 in the citric acid cycle. The ratio of C-1 oxidation in glycolysis versus oxidation of C-2 and C-3 in the citric acid cycle depends on the microbial availability of alanine: high availability of sorbed DOC and alanine due to fast cation exchange causes an initial peak in C-1 oxidation by glycolysis and an abrupt shift to oxidation via the citric acid cycle – i.e. high amount of energy production by catabolism. Low microbial availability of sorbed alanine, in contrast, leads to a slow, parallel oxidation of all three positions by glycolysis and the citric acid cycle and a large transfer of C toward maintenance anabolism. The DI of the alanine C remaining in soil after three days reflects a mixture of untransformed sorbed alanine ($DI_{1,2,3} = 1$) and microbial transformation products ($DI_1 < 1$ and $DI_{2,3} > 1$): The higher the microbial availability, the higher the portion of bound alanine C present as microbial transformation products (Apostel et al., 2013).

Activated charcoal shows a deviating behavior, with preferential stabilization of C-3 and oxidation of C-1 and C-2. This indicates that the hydrophobic C-3 is preferentially stabilized by charcoal and that, in addition to basic microbial mechanisms, further pathways of alanine transformation occur (e.g. by exoenzyms).

Details on the pathways and the newly formed microbial products can be further deepend combining position-specific labeling with compound-specific isotope analysis of microbial products. In addition, studies with a broader range of LMWOS with deviating

sorption properties as well as sorption under natural soil conditions are needed. This will yield a more mechanistic understanding of the processes leading to a stabilization of C by sorption.

Acknowledgments

We thank the Deutsche Forschungsgemeinschaft for funding and DAAD for support of the exchange visit of Mikhail Biryukov. In addition we thank the technical assistants of the Department of Soil Science of temperate ecosystems and Leopold Sauheitl from the Soil Science Department of the University of Hanover for performing analysis of basic soil parameters.

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Supplementary Data

Supplementary Table A1: Nested ANOVA between single Ala positions nested in sorbent treatment for sorbed, dissolved and respired alanine C. Degrees of freedom (df), F-values and significance level (p) are shown for the five concentration treatments.

factor	sorbed Ala-C			dissolved Ala-C			respired Ala-C		
	df	F	p	df	F	p	df	F	p
sorbent	4	108.92	***	4	25.87	***	4	105.20	***
Ala-C position	10	90.30	***	10	1.67	n.s.	10	91.70	***

n.s. not significant
* significant with $p < 0.05$
** significant with $p < 0.01$
*** significant with $p < 0.001$

Supplementary Table A2: ANOVA, calculated according to Cohen (2002), for the divergence index DI for respired and sorbed alanine C. Degrees of freedom (df), F-values and significance level (p) are shown for the three alanine positions and the two sterilization treatments.

Divergence Index DI_i	respired Ala-C			sorbed Ala-C		
	df	F	p	df	F	p
Ala-1	4	4.191	**	4	5.802	**
Ala-2	4	0.341	n.s.	4	6.436	***
Ala-3	4	0.788	n.s.	4	1.506	n.s.

n.s. not significant
* significant with $p < 0.05$
** significant with $p < 0.01$
*** significant with $p < 0.001$

2.6 Study 6: Biochemistry of hexose and pentose transformations in soil analyzed by position-specific labeling and ^{13}C-PLFA

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Abstract

Microbial transformations are key processes of soil organic matter (SOM) formation, stabilization and degradation. Combining position-specific ^{13}C labeling with compound-specific ^{13}C-PLFA analysis is a novel tool to reconstruct metabolic pathways. This combination was used to determine short-term transformations (3 and 10 days after tracer application) of two monosaccharides – glucose and ribose – in soil under field conditions. Transformations of sugars were quantified by the incorporation of ^{13}C from individual molecule positions in bulk soil, extractable microbial biomass (by CFE) and in cell membranes of distinct microbial groups classified by ^{13}C-PLFA.

As the incorporation of ^{13}C was higher by one order of magnitude in the Gram negative bacteria compared to all other microbial groups, this group was considered to have a great influence on sugar utilization and transformation in soil. All of the ^{13}C recovered in bulk soil on day 3 was allocated in microbial biomass. On day 10 however, a part of the ^{13}C was recovered in soil, revealing either incorporation into non-extractable microbial cell components or the excretion of microbial products. As sugars cannot interact with soil particles due to a lack of functional groups, their quick mineralization is generally expected. However, our results showed that microorganisms transformed sugars into metabolites with a slower turnover. In bulk soil and extractable microbial biomass, the tracer ^{13}C incorporation from the individual glucose positions showed that the two main glucose utilizing pathways in organisms –glycolysis and the pentose phosphate pathway – exist in soils in parallel. However, the pattern of tracer incorporation from individual glucose positions into PLFAs showed additionally and intensive recycling of the added ^{13}C via gluconeogenesis and an intermingling of both glucose utilizing pathways. This shows that glucose – as a ubiquitous substrate - is used by various metabolic pathways and glucose C is intensively recycled in microbial biomass. Nevertheless, glucose can be used as a metabolic tracer in short-term experiments, especially for tracing carbohydrate metabolism (i.e. glycolysis and pentose phosphate pathway and their side branches).

Analyzing the fate of individual C atoms by position-specific labeling allows strong improvement of our understanding of the mechanisms and kinetics of microbial utilization of hexoses and pentoses by various microbial groups and so, of soil C fluxes.

2.6.1 Introduction

Soil organic carbon (SOC) plays a major role within the global C cycle as soils can function as a source or sink of atmospheric C. Plant residues and rhizodeposits are the main sources of organic matter in soils (Rasse et al., 2005). Therefore, many studies have focused on decomposition, microbial utilization and stabilization processes of C from these sources in soils.

The low molecular weight organic substances (LMWOS) play a crucial role within the C cycle in soil. Although their portion of SOC is quite low, they represent the SOC pool with the highest turnover and a huge gross flux of C passes through this pool (Fischer et al., 2007). LMWOS are defined as the lightest components of dissolved organic carbon (DOC) with a molecular weight lower than 250 Da (Boddy et al., 2007). Their main sources are exoenzymatic depolymerization of above- and belowground litter as well as rhizodeposition. Microorganisms determine the fate of LMWOS in soil because they are able to either produce them, decompose them to CO_2 (catabolism) or incorporate them in cellular compounds (anabolism). Microbial incorporation and transformation of LMWOS are key processes in stabilizing soil organic carbon (SOC) (Miltner et al., 2011; Simpson et al., 2007). Therefore, microbial transformation pathways of LMWOS represent a crucial step of soil C and N fluxes, and a molecular-level knowledge of these processes is needed (van Hees et al., 2005).

Besides amino acids and carboxylic acids, sugars are a main component of LMWOS (van Hees et al., 2005). Sorption and other interactions with soil organic matter (SOM) are nearly irrelevant for sugars, as they have neither charged functional groups nor hydrophobic molecule parts. Thus, their fate is mainly determined by microbial utilization. They can occur as monosaccharides with a five C backbone (pentoses) or a six C backbone (hexoses) or as di- or oligosaccharides within the LMWOS. Individual monosaccharide concentrations in soil solutions typically range from 1-10 µM (Fischer et al., 2007). Within this class, glucose is most abundant monomer, deriving from the decomposition of plant residues as well as rhizodeposition (Kuzyakov, 2010), and is known to be a ubiquitous substrate for microorganisms (Macura and Kubatova, 1973). The main sources of pentoses in soil are plant hemicelluloses (Cheshire et al., 1971; Koegel-Knabner, 2002), but ribose in particular is actively formed in soils (Murayama, 1988), e.g. for the biosynthesis of ribonucleotides and their polymers (DNA and RNA).

Sugar monomers are the building blocks of different polysaccharides (e.g. cellulose, starch), and are also precursors of the ribonucleotide backbone and cell wall polymers. Microorganisms can degrade all of these polymers to monosaccharides or *vice versa*, build them up from monosaccharides. However, sugars are not only anabolic sub-

strates but also a preferred source for energy production in catabolism. For glucose, it was known that an average of 60% is incorporated in cellular compounds and 40% is oxidized to gain energy (Fischer et al., 2010), but there is no information whether this ratio is similar for other monosaccharides. This ratio can be influenced by many factors like the nutritional state of microorganisms or the supply by further LMWOS. In addition, individual functional groups of the microbial community are expected to use LMWOS-C in different pathways and produce various metabolites from them. To date, neither the metabolic pathways of monosaccharides nor the specifics of individual functional groups of the soil microbial community are investigated in soils. The combination of position-specific labeling with compound-specific isotope analysis is a unique approach, which enables tracing the transformations of LMWOS within the microbial community of soils (Apostel et al., 2013). Position-specific labeling – a tool that is commonly used in biochemistry to investigate metabolism pathways - has recently reached an increasing consideration in soil science (Apostel et al., 2013; Dijkstra et al., 2011a; Dippold and Kuzyakov, in press; Fischer and Kuzyakov, 2010). It overcomes the limitations of uniform labeling because it allows differentiation between the incorporation of molecule fragments vs. the incorporation of entire intact molecules and thus enables the reconstruction of metabolic pathways. PLFA analysis not only allows a reconstruction of the microbial community composition (Zelles, 1999; Zelles et al., 1995), but in combination with ^{13}C labeling – i.e. ^{13}C-PLFA analysis - also enables tracing of substrate incorporation and reconstruction of sugar metabolism of individual microbial groups (Glaser, 2005).

This study aimed to trace C transformations of monosaccharides in soil. The hexose glucose and the pentose ribose were applied position-specific ^{13}C labeled to undisturbed soil cores and the ^{13}C incorporation in microbial biomass and PLFA was traced over 10 days. As sugars possess no functional groups with which they could interact with the soil matrix, we hypothesize complete uptake into the microbial biomass. In addition, we state the hypothesis that the incorporation of glucose into microbial biomass and bulk soil reflects the oxidation pattern of glycolysis i.e. preferential oxidation of C-3 and C-4 positions to CO_2. In contrast, the pentose phosphate pathway may dominantly affect ribose utilization and lead to a preferential loss of ribose C-3, C-1 and C-2 as CO_2. If glucose is utilized in this pentose phosphate pathway, we will detect a loss of the glucose C-1 and C-4 positions. Furthermore, we hypothesize that pathways of eukaryotes and prokaryotes differ, which will be reflected in the different incorporation of glucose and ribose C positions into the specific PLFAs of these microbial groups.

2.6.2 Material and Methods

2.6.2.1 Sampling Site

Sampling site

The field experiment is located on an agriculturally used loamy Luvisol in northern Bavaria (49°54' northern latitude; 11°08' eastern longitude, 500 a.s.l.) with a mean annual temperature of 7°C, and a mean annual precipitation of 874 mm. The last crop was *Triticale.* The soil had a pH_{KCl} of 4.88, a pH_{H_2O} of 6.49, a TOC and TN content of 1.77% and 0.19%, respectively. CEC was 13 $cmol_C\ kg^{-1}$.

Experiment design

Field experiment is in detail described in Apostel et al (2013). Briefly, the 12 × 12 m field was divided into four quadrants, representing the four replications. PVC-tubes (diameter: 10 cm, height: 13 cm) were installed 10 cm deep in the soil. Application dots were marked with five wooden rods 5 days prior to applying the sugars. 10 ml tracer-solution per column were applied with a multipette (Eppendorf, Hamburg, Germany) with concentrations of ^{13}C-labeled sugars according to Table 1. A 7 cm long needle with lateral holes enabled homogeneous distribution of the tracer solution within the column. The solution was only injected in the upper 2/3 of the column to avoid leaching and rainfall was blocked by a roof. In each of the quadrants and for both sampling times, glucose and ribose were applied once as 1) non-labeled background, 2) uniformly ^{13}C-labeled, and 3) as four and two position-specific ^{13}C-labeled isotopomeres of glucose and ribose, respectively (see Table 1) with a random distribution of the isotopomere within the block.

Table 1 Locations of ^{13}C in position specifically labeled glucose and ribose and their amounts added to soil in the field experiment.

	Glucose					Ribose		
	^{13}C-1	^{13}C-2	^{13}C-4	^{13}C-6	U-^{13}C	^{13}C-1	^{13}C-5	U-^{13}C
C concentration (µmol ml^{-1})	23.83	24.29	24.16	23.95	23.22	62.65	62.43	26.25
C amount (µmol g $soil^{-1}$)	0.38	0.39	0.38	0.38	0.63	0.45	0.45	0.66
Atom% ^{13}C	38.17	38.21	38.54	38.08	43.04	14.84	14.84	37.22

Sampling and sample preparation

Sampling occured 3 and 10 days after labeling and the complete columns from one set (background, uniformly and position-specifically labeled) of four replications were dug out. Height of the soil inside the column was determined to calculate soil volume. Afterwards, the soil was weighted; a subset was sieved to 2 mm for further analysis and

stored at 5°C for not longer than one week until chloroform-fumigation extraction and at -20°C for PLFA-extraction.

2.6.2.2 Analytical methods

Bulk soil measurements

For bulk soil C content and $\delta^{13}C$-analysis, the samples were freeze dried, ground in a ball mill and 5-6 mg per sample was filled into tin capsules. Measurement was performed with an Euro EA Elemental Analyzer (Eurovector, Milan, Italy) coupled by a ConFlo III interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). Incorporation of ^{13}C from the applied sugars into the soil was calculated by a mixing model (Eq. 1 and 2), where the C content of the background in Eq. 1 was substituted according to Eq. 2.

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C-BG} + [C]_{appMS} \cdot r_{appMS} \quad (5)$$

$$[C]_{soil} = [C]_{BG} + [C]_{appMS} \quad (6)$$

with:

$[C]_{soil/BG/appMS}$	C content of sample/background/applied monosaccharide	$(mol \cdot g_{soil}^{-1})$
$r_{soil/BG/appMS}$	^{13}C atom%-excess of sample/background/applied monosaccharide	(at%)

Chloroform fumigation extraction

Microbial biomass and its $\delta^{13}C$ values was determined by Chloroform fumigation extraction according to Apostel et. al (2013): Briefly, two subsets of 15 g of soil were taken per sample and one subset was directly extracted as described below; the other was first fumigated with chloroform for 5 days in an exsiccator to lyse microbial cells and afterwards extracted.

The samples were extracted twice with 22.5 ml of 0.05 M K_2SO_4. They were shaken on a horizontal shaker, 1 h on the first, 0.5 h on the second extraction. After shaking, the samples were centrifuged (10 min, 2000 rpm) and the supernatant was filtered (Rotilab® round filters, type 15A, cellulose, membrane 70 mm).

The C content of the K_2SO_4 extracts was measured on the TOC analyzer multi C/N® 2000 (Analytik Jena, Jena, Germany). For $\delta^{13}C$ measurements, the samples were freeze-dried and then measured on the Euro EA Elemental Analyzer (Eurovector, Milan, Italy) unit with a ConFlo III interface (Thermo-Fischer, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). ^{13}C uptake into fumigated and

unfumigated extracts was calculated according to the mixing model (Eq. 1 and 2) and microbial biomass C and ^{13}C incorporation was determined with an extraction factor of 0.45 according to Wu et al. (1990).

PLFA-Analysis

Phospholipid analysis was performed analogue to Apostel et al (2013): Extraction and purification is a modified method by Frostegard et al. (1991). Modifications included using 6 g of soil for extraction and elution of polar lipids from the silica column occurred four times with 5 ml of water-free methanol. 25 µl of the internal standard 1 (IS 1), phosphatidylcholine dinonadecanoic acid, was added before extraction. For gas chromatography (GC) measurements, the fatty acids were saponified to free fatty acids and derivatized into fatty acid methyl esters (FAME) according to Knapp (1979). 15 µl of the internal standard 2 (IS 2), tridecanoic acid methyl ester, was added before transferring the samples to sampler vials. External standards consisting of the 27 fatty acids given in Supplementary Table 1 and internal standard 1 were prepared with total fatty acid contents of 1, 4.5, 9, 18, 24 and 30 µg, respectively, and derivatized and measured together with the samples.

FAME-contents were measured on a GC-MS (GC 5890 with MS 5971A, Agilent, Waldbronn, Germany) with a 30 m DB1-MS column, in the selected ion mode. The relation between the area of each FAME and the area of the IS 2 was calculated and quantification occurred via a linear regression calculated from the six external standards. The recovery rate for every sample was determined from the area of the initially added 25 µg of IS 1, and applied against the quantified masses of the FAMEs.

$\delta^{13}C$-values were measured by a GC-C-IRMS, consisting of the autosampler unit AS 2000, the Trace GC 2000 by ThermoQuest, the combustion unit Combustion Interface III and the isotope-ratio mass spectrometer Delta^{Plus} (all units from Thermo Fisher, Bremen, Germany). Volumes of 1.5 µl were injected into a liner (Type TQ(CE) 3 mm ID TAPER) at a injector block temperature of 250°C in splitless mode (splitless time: 1 min). Gas chromatography was performed with a combination of two capillary columns: a 30 m DB5-MS and a 15 m DB1-MS (both: internal diameter 0.25 mm, film thickness 0.25 µm; Agilent), a constant He-flux (99.996% pure) of 2 ml · min^{-1} and the temperature program presented in Supplementary Table 2. CO_2 reference gas (99.995% pure) was injected for 20 s into the detector several times throughout the measurement to identify any drift of the $\delta^{13}C$-values. The $\delta^{13}C$ of the second reference gas peak was defined as -40‰ and all other $\delta^{13}C$ values were calculated by comparison. $\delta^{13}C$ of all PLFA samples was measured four times.

The chromatograms were evaluated with ISODAT NT 2.0 and $\delta^{13}C$ value in ‰ was computed from the isotopic ratio $^{13}C/^{12}C$.

Linear regressions were calculated from reference gas peaks two and three, and three and four to correct for any drift during measurements: Eq. 3 was applied to the $\delta^{13}C$ value of FAMEs that were detected before reference gas peak three; Eq. 4 was applied to those that were detected after reference gas peak three.

$$C_{FAME-DK}(at\%) = C_{FAME-0}(at\%) - (m_{RG} \cdot (t_{FAME} - t_{RG})) \quad (3)$$

$$C_{FAME-DK}(at\%) = C_{FAME-0}(at\%) - (m_{RG} \cdot (t_{FAME} - t_{RG})) - \Delta C_{RG}(at\%) \quad (4)$$

with: $C_{FAME\text{-}DK}$(at%) drift-corrected ^{13}C amount of the FAME (at%)

$C_{FAME\text{-}0}$(at%) measured ^{13}C amount of the FAME (at%)

m_{RG} slope of regression between the reference gas peaks enveloping the FAME (s^{-1})

t_{FAME} retention time of FAME (s)

t_{RG} retention time of reference gas peak prior to FAME (s)

ΔC_{RG}(at%) difference between reference gas peaks three and two (at%)

To correct for amount-dependent ^{13}C isotopic fractionation during measurements (Schmitt et al., 2003), and for the addition of C during derivatization, linear and logarithmic regressions of the external standards $\delta^{13}C$-values to their area were calculated. If both regressions were significant, that with the higher significance was applied. As the $\delta^{13}C$-value for the derivatizing agents was unknown, the correction was performed according to Glaser and Amelung (2002) (Eq. 5).

$$C_{FS}(at\%) = \frac{N(C)_{FAME}}{N(C)_{FS}} \cdot (C_{FAME-DK}(at\%) - (m_{lin/\ln} \cdot A_{FAME} + t_{lin/\ln})) + C_{EA-FS}(at\%) \quad (5)$$

with: C_{FS}(at%) corrected ^{13}C amount of the fatty acid [at%]

C_{FAME}(at%) drift-corrected ^{13}C amount of the FAME [at%]

$m_{lin/ln}$ slope of linear/logarithmic regression [at% · Vs^{-1}]

$t_{lin/ln}$ y-intercept of linear/logarithmic regression [at%]

A_{FAME} area of FAME [Vs]

$N(C)_{FAME}$ number of C atoms in FAME

$N(C)_{FS}$ number of C atoms in fatty acid

$C_{EA\text{-}FS}$(at%) measured ^{13}C-value of fatty acid [at%]

2.6.2.3 Divergence Index

Discrimination of C from individual positions of a molecule during uptake and incorporation was assessed. In addition, the extent of discrimination between pools, microbial groups and sampling times was also compared. Therefore, the differences in absolute uptake into C pools or microbial groups had to be relativized which was done by the divergence index (DI) according to Dippold and Kuzyakov (2013):

$$DI_i = \frac{n \cdot C_i}{\sum_{1}^{n} C_i} or = \frac{n \cdot C_i}{C_u} \quad (6)$$

with: n number of C atoms in molecule

C_i relative incorporation of tracer C [mol · mol^{-1}]

C_u relative incorporation of uniformly labeled tracer C [mol · mol^{-1}]

The DI can be calculated from the relative incorporation of tracer per bulk soil, microbial biomass, single PLFA or Σ–PLFA, which are specific for a microbial groups. It compares the incorporation of C from each position with the mean C incorporation from all positions of a molecule. As not all positions of glucose or ribose were applied in this study, the mean uniformly labeled tracer C incorporations were entered in the calculation instead. The DI reflects the result which the experiments would have generated if uniformly labeled tracers had been used. A DI of 1 indicates no discrimination between positions, values above 1 indicate preferential incorporation, and values below 1 show preferential degradation.

2.6.2.4 Statistical analysis

A Nalimov outlier test with significance levels of 95% (when four repetitions were available) or 99% (when three repetitions were available) was performed for the replication analyses of $\delta^{13}C$-values. According to a factor analysis of PLFA amounts of the entire dataset, the PLFA were classified into corresponding microbial. Fatty acids with a loading of more than 0.5 (absolute value) on the same factor were categorized taking additionally previous studies with pure cultures into account (Zelles, 1999; Zelles et al., 1995). All data were tested with a one-way analysis of variance (ANOVA); significance was determined with the Tukey Honest Significance Difference (Tukey HSD) post-hoc test with a significance level of 99.9%. All positions were tested for significant differences between ^{13}C incorporation in soil, microbial biomass and PLFAs. For every microbial group and soil pool, the difference in DI for the seven specific ^{13}C-labeled positions was

also tested for significance. All statistical tests were performed with R version 3.0.0 (17.04.2009).

2.6.3 Results

2.6.3.1 Incorporation of uniformly labeled monosaccharides

Extractable microbial biomass C accounted for 3.4% of the soil C stock and 0.29% was PLFA C (Table 2). The incorporation of ^{13}C from uniformly labeled glucose and ribose remained stable in soil between days 3 and 10. ^{13}C from uniformly labeled glucose in extractable microbial biomass decreased by 50% between days 3 and 10, but the ^{13}C from ribose remained nearly constant. This suggests that ribose was incorporated into different cell constituents to glucose.

Table 2 Total C content and ^{13}C incorporation of uniformly labeled monosaccharides into soil, microbial biomass and sum of PLFA (Σ-PLFA).

		TOC	C_{mic}	Σ PLFA
Pool Size	day 3	14.533 ± 2.616	0.493 ± 0.052	0.042 ± 0.003
(mg C g^{-1} soil)	day 10	16.332 ± 1.695	0.455 ± 0.111	0.050 ± 5.95
Glucose ^{13}C recovery	day 3	1113.53 ± 34.73	576. 96 ± 36.19	16.81 ± 0.54
(ng glucose-^{13}C g^{-1} soil)	day 10	1198.14 ± 120.61	312.48 ± 75.29	17.33 ± 0.66
Ribose ^{13}C recovery	day 3	546.34 ± 23.42	335. 24 ± 47.14	11.02 ± 0.16
(ng glucose-^{13}C g^{-1} soil)	day 10	649.71 ± 58.18	307. 04 ± 22.52	13.61 ± 0.10

2.6.3.2 Incorporation of position-specifically labeled monosaccharides

The application of position-specific ^{13}C-labeled sugars enabled tracing of individual positions in the three soil C pools: total soil, microbial biomass C and PLFA (Fig. 1). On day 3, there were no significant differences in the incorporation of any glucose or ribose position in soil. However, a trend for lower recovery of glucose ^{13}C-1 and ^{13}C-4 and ribose ^{13}C-1 in soil could be perceived on day 3. This trend became significant on day 10: 1) glucose ^{13}C-1 was recovered significantly less in soil than glucose ^{13}C-6 and 2) ribose ^{13}C-1 was incorporated significantly less than the ^{13}C-5 tracer. However, there was no significant difference in tracer-C recovery in soil of the sugar positions between day 3 and 10. All of the tracer-C that was stabilized in soil at day 3 was still remaining at day 10. In microbial biomass, we observed an equal recovery of glucose ^{13}C-1 and ^{13}C-4, which was lower than the equally high recovery of glucose ^{13}C-2 and ^{13}C-6 tracer on day 3. There was also more than twice as much ribose ^{13}C-5 recovered than ^{13}C-1 on day 10. In contrast to bulk soil, in microbial biomass, we saw a significant decrease of tracer C recovery

from glucose C-2, C-4 and C-6 and also ribose C-5 between day 3 and 10. Additionally, not only did the overall recovery decrease, but also the pattern of incorporation of individual positions changed, as not all positions' C recovery in microbial biomass decreased equally. We observed an especially high decrease in tracer C recovery from glucose C-2 (-90%) and ribose C-5 (-70%). As there was no corresponding decrease in the soil, these positions were considered to not have been degraded to CO_2 and lost into the atmosphere, but instead stabilized in the soil as microbial residues.

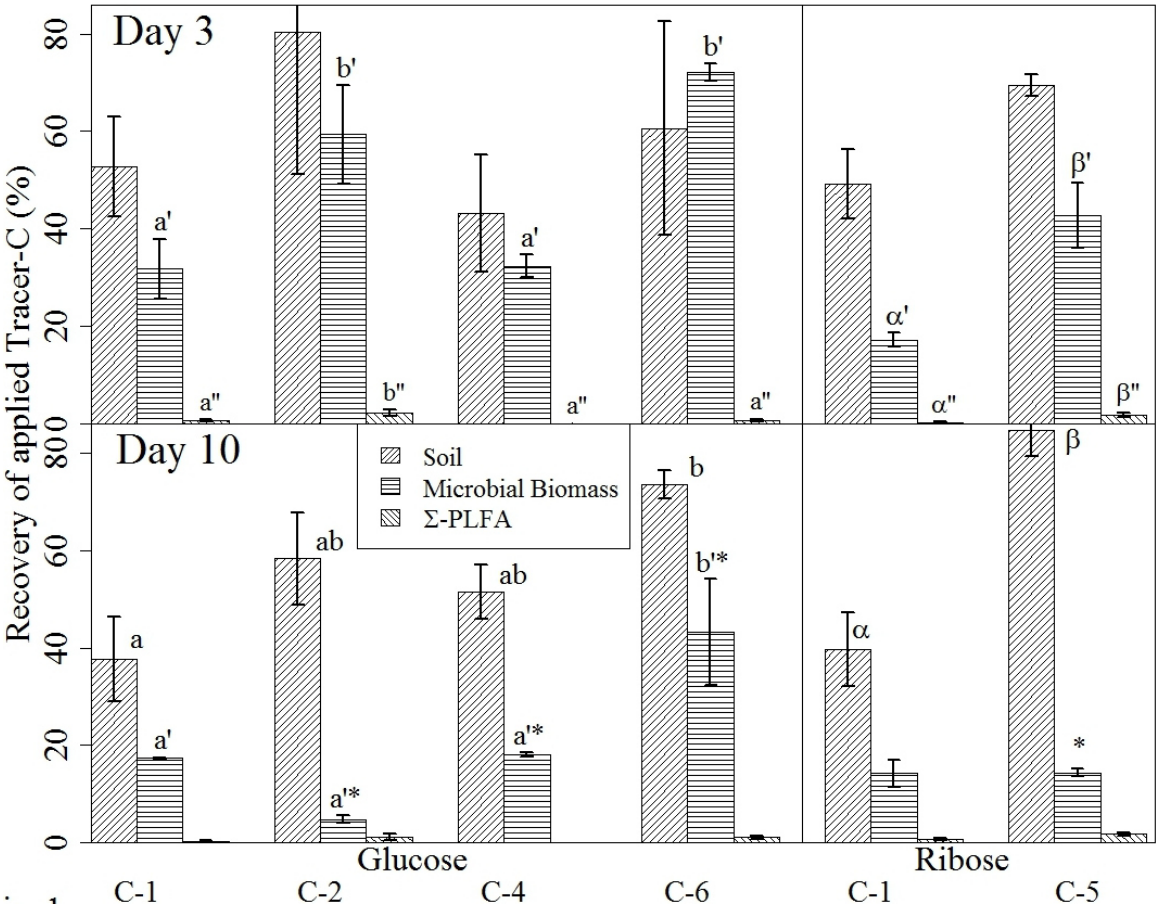


*Fig. 1 Recovery of position-specific ^{13}C labeled glucose and ribose in soil, microbial biomass and Σ-PLFA, three (top) and ten days (bottom) after application. Letters indicate significant differences ($p < 0.05$) between recovery in bulk soil (glucose: a, ribose α), microbial biomass (glucose: a', ribose α') and Σ-PLFA (glucose: a'', ribose: α''). * reflects significant differences between day 3 and day 10. Error bars show standard error of the mean from the four field repetitions.*

As absolute incorporation of tracer ^{13}C into ∑-PLFA was much lower than into the other two pools, the DI aids in the observation of the sugar positions' incorporation pattern into ∑-PLFA (see Supplementary, Figure A1). Interestingly, on day 3, the incorporation pattern of glucose positions into ∑-PLFA was different to that of microbial biomass: we found a preferential incorporation of glucose ^{13}C-2 and the highest discrimination against glucose ^{13}C-4. On day 10, the difference in the relative incorporation of individual

positions was no longer significant, although the pattern observed on day 3 was still implied.

2.6.3.3 Tracer uptake of functional microbial groups

By performing PCA on the PLFAs C-contents from both sampling times and comparison of the statistical grouping with literature (Zelles 1999, Zelles et al. 1995), the PLFAs were classified into 7 microbial groups (Supplementary Table A1). The recovery of applied ^{13}C in the microbial groups' PLFAs was calculated based on the mixing model. Recovery of ^{13}C from both sugars was highest in the Gram negative PLFA on both days, with a maximum of 0.8%. Two of the Gram positive groups - Gram positive I and actinomycetes – also showed elevated maximum incorporations of 0.2–0.4%. For most microbial groups, significant differences between the recovery of ^{13}C from individual sugar positions were observed on both days. Glucose ^{13}C-2 and ribose ^{13}C-5 tracer had the highest recovery in most microorganism groups. There was little to no incorporation of the glucose ^{13}C-4 tracer and ribose ^{13}C-1 in all microorganisms. However, due to differences in absolute tracer uptake, comparison of position-specific ^{13}C behavior between the microbial groups is difficult. Therefore, the relative tracer ^{13}C incorporation (divergence index (DI)) is better suited for detailed comparison.

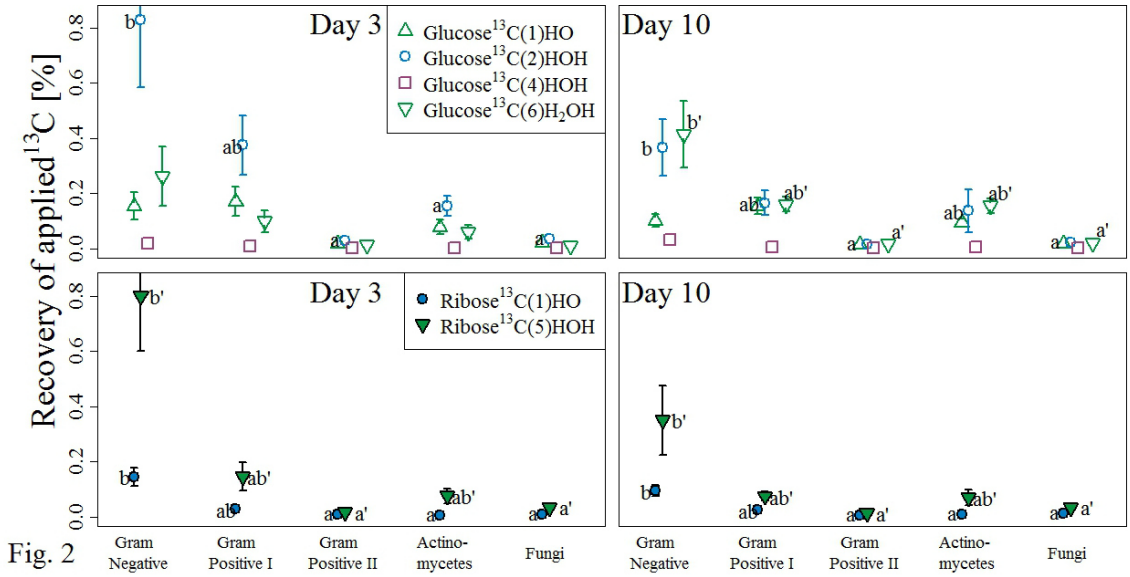


Fig. 2

Fig. 2 Recovery of applied ^{13}C from molecule positions of glucose (top) and ribose (bottom) in microbial groups after three and ten days. Letters indicate significant differences ($p < 0.05$) between the individual C positions of glucose and ribose. Error bars show standard error of the mean from the four field repetitions.

2.6.3.4 Divergence Index

The divergence index calculates the deviation of incorporation of one position from the mean of all positions, which corresponds to the ^{13}C incorporation from the uniformly labeled molecule. As DI is a relative value, the pattern of ^{13}C incorporation into individual soil pools (e.g. PLFAs of microbial groups) can be directly compared.

On day 3, the pattern of ^{13}C incorporation from the glucose and ribose positions into PLFAs was very similar for all microbial groups. Glucose ^{13}C-4 was always the most discriminated against and the DI of glucose ^{13}C-2 was nearly always significantly higher. Glucose ^{13}C-1 and ^{13}C-6 showed a slight preferential degradation; their DI lay mostly around 0.5. The only exceptions to this pattern of glucose incorporation were the fungal PLFAs – there was no significant difference between discrimination against the four positions. For ribose, we observed a clear pattern on day 3: preferential incorporation of ribose ^{13}C-5, with a significantly lower DI for ^{13}C-1. For both glucose and ribose, the overall divergence in all groups was lower on day 10 than on day 3.

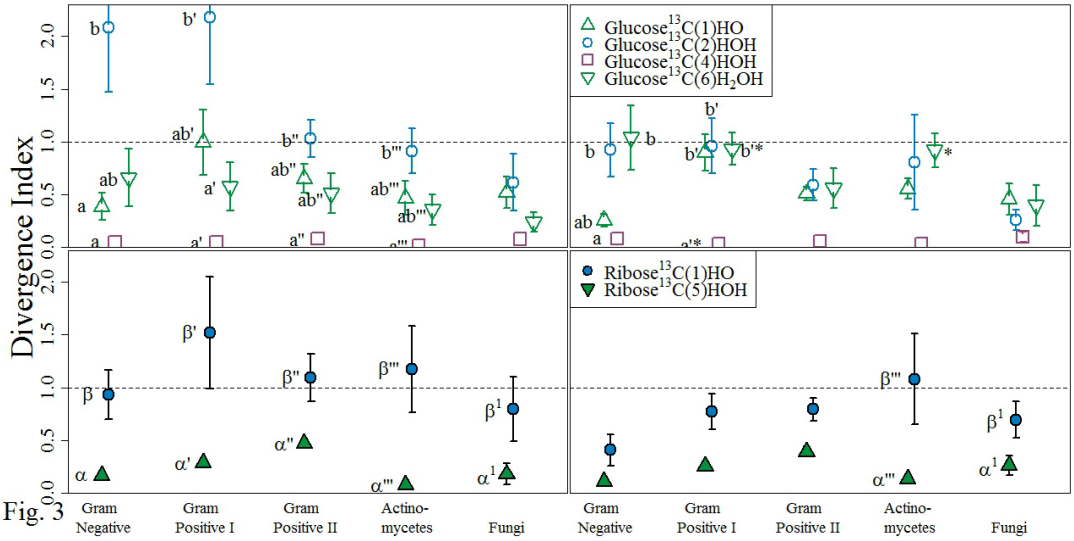


Fig. 3

Fig. 3 Divergence Index (DI), reflecting discrimination between the C positions of glucose and ribose, three (top) and ten (bottom) days after application. Letters indicate significant differences ($p < 0.001$) between the relative incorporation of the C positions into the individual microbial groups. Error bars show standard error of the mean from the four field repetitions.

2.6.4 Discussion

2.6.4.1 Glucose and Ribose incorporation into soil and microbial biomass

The majority of the decomposition of glucose and ribose occurred during the first 3 days (~50%). Thereafter, the amount incorporated in microbial biomass further de-

creased, but the total residue in soil only slightly decreased (Figure 1). This can be attributed to three potential processes: 1) microbial turnover may lead to a release of microbial products into the soil, where it may be stabilized, 2) C may be excreted by microbial cells e.g. for the formation of exoenzymes or extracellular polysaccharides, and 3) C may be continuously transferred from the well extractable, water soluble metabolite pool within the microbial biomass to more complex, e.g. polymeric substances, like proteins or cell walls, which are presumably worse extractable by the CFE method. Turnover of these high-molecular weight pools is significantly lower than of low-molecular weight microbial compounds (Malik et al., 2013). Therefore, incorporation into these pools would be slower and a continued transfer from the water dissolvable to the polymeric, non-dissolvable pool may explain the decrease in extractable microbial biomass ^{13}C on day 10. Transformation of the applied ^{13}C towards polymeric, non-dissolvable compounds will increase the potential for stabilization in soils (Miltner et al., 2007; Miltner et al., 2009).

The average amount of mineralized glucose and ribose was in a similar range, indicating that both substances may have been equal concerning their quality as a substrate for catabolism. A strong coupling of hexose and pentose uptake by microorganisms was proven in several studies (Baumann and Baumann, 1975; Nobre et al., 1999). The similar percentage of mineralized ribose and glucose in this study confirms this similar microbial uptake and the utilization of both substances. Whereas the majority of glucose ^{13}C was in extractable microbial biomass compounds at day 3, a significantly lower portion of ribose ^{13}C could be extracted. This indicates that ribose C is transferred much faster into non-extractable pools of the microbial biomass C. Direct biochemical utilization of ribose C would be the formation of the ribonucleotide backbone of DNA and RNA (Caspi et al., 2008; Keseler et al., 2009). Both ribonucleotide substance classes need optimized extraction procedures with buffers and competitors to yield high extraction efficiencies (Paulin et al., 2013), and a quantitative extraction of ribonucleotides by the potassium sulfate extraction of the CFE method is unlikely. Therefore, the ratio of extractable, microbial ^{13}C to total soil ^{13}C already indicates that glucose C and ribose C are transferred to different compound classes synthesized by their specific pathways.

Incorporation into PLFA was less than 1% for glucose and ribose and even decreased from day 3 to day 10. Malik et al. (2013) stated after studies based on ^{13}C natural abundance of microbial biomass compounds that the turnover of CFE extractable compounds is higher than the turnover of phospholipids. However, we observed a decrease of ^{13}C incorporation for both microbial pools. This confirms the results from labling with acetate (data not presented), which showed that the incorporation of ^{13}C from LMWOS into PLFA after pulse labeling was not homogenous (Blagodatskaya and Kuzya-

kov, in press), but mainly occurred in terminal or specific functional groups of the PLFA, which have a higher turnover then the entire fatty acid molecules.

2.6.4.2 Microbial utilization of individual positions of glucose and ribose molecules

Specific incorporation of ^{13}C from individual positions of the molecules can be used to assess the glucose and ribose metabolism pathways. Glycolysis, the direct hexose metabolism pathway, would lead to the formation of two trioses, where C-3 and C-4 are the terminal C atoms with the highest oxidation number (Caspi et al., 2008). After their transformation from pyruvate to acetyl-CoA, C-3 and C-4 are removed e.g. by the pyruvate dehydrogenase. This is similar to results from Apostel et al. (2013) and Dippold and Kuzyaov (in press) for the highest oxidized position of alanine (which becomes deaminated to pyruvate) and results from Djikstra et al (2011a) after position-specific pyruvate labeling. As a consequence, the labeled C-4 position would show a lower incorporation into microbial products and no incorporation into PLFAs as the product of pyruvate dehydrogenase – acetyl-CoA – is the precursor of fatty acid synthesis (Caspi et al., 2008). Both phenomena can be observed in this study (see figure 1), e.g. by a significantly lower incorporation of C-4 at day 3 compared to C-2 and C-6.

Glycolysis splits hexoses between C-3 and C-4 and results in two symmetric trioses: C-1 to C-3 and C-6 to C-4. If glycolysis is the only pathway to use glucose, this would result in a symmetry of the positions recovery: C-6 ~ C-1 > C-5 ~ C-2 > C-4 ~ C-3 ~ 0 (Scandellari et al., 2009) (Figure 5). However, we also observed a significantly lower incorporation of C-1 than C-6 into extractable microbial biomass products after 3 days. This shows that, in addition to glycolysis, a portion of glucose molecules was transformed by the pentose phosphate pathway. This pathway leads in a first oxidation step to the loss of the C-1 position, whereas the other positions are transferred back to the triose pool (Scandellari et al., 2009) (Fig. 4) The parallel existence of glycolysis and the pentose phosphate pathway for glucose utilization in soil reflects the parallel existence of catabolic, oxidizing and anabolic cellular maintenance pathways. This existence of parallel metabolic pathways is characteristic for soils because of the high microbial diversity and various habitat properties, causing a wide spectrum of various metabolic states.

The loss of pathway-specific, classical fingerprint from day 3 to day 10 is in accordance with glucose uptake studies from Dungait et al. (2011), which suggest fast uptake and incorporation of ^{13}C from glucose and other LMWOS and continued metabolization of the incorporated ^{13}C latest 120 h after labeling. This suggests that basic C metabolism, with the parallel existence of decomposing and constructing pathways, leads to a mixing

of C positions in microbial metabolism within several days (Scandellari et al., 2009). This convergence may even be pronounced, if universal substrates like glucose (Macura and Kubatova, 1973), which can be spread over each metabolic pathway in cells, are used for labeling compared to less-preferred substrates like glycine (Dungait et al., 2011, 2013).

A similar behavior was observed for the microbial products formed from ribose: a clear preference for C-5 incorporation at day 3 is until day 10 in the extractable microbial products. This reflects the fast mixing of monosaccharide C positions within the pool of fast cycling cytosolic compounds. The preference of C-5 incorporation at day 3 reflects the classical pentose metabolizing pathway – the pentose phosphate pathway. Pentoses, like ribose, enter this pathway after the oxidation step (in which hexoses are oxidized to pentoses by the loss of C-1) and the upper half of the molecule gets mixed up. Conversion of C-1 and C-2 of ribose leads to a preferential oxidation of C-1 if this C is allocated to glycolysis afterwards by the pyruvate dehydrogenase oxidation step (Caspi et al., 2008; Keseler et al., 2009).

The observation that C that passes downwards from glucose towards the catabolic citric acid cycle and afterwards gets located upwards into anabolic pathways (i.e. the so called backflux: Scandellari et al. (2009)), is in accordance with bulk soil ^{13}C data: the backflux via gluconeogenesis can lead to various anabolic pathways like carbohydrate synthesis (e.g. for extracellular polysaccharides), protein synthesis (e.g. for exoenzymes) or amino sugar formation (for cell walls). All of those pathways result in non-extractable compounds of the microbial biomass, which are later likely to be stabilized in soils (Kindler et al., 2006; Miltner et al., 2007; Miltner et al., 2009) and would explain why ^{13}C recovery from glucose in soil did not further decrease from day 3 to day 10.

To summarize, both classical monosaccharide metabolizing pathways – glycolysis and the pentose phosphate pathway – could be proven to exist in soils in parallel. However, their position-specific fingerprint – at least in mixed pools of metabolites, like the extractable microbial biomass – gets lost within several days of continued metabolism due to C recycling and backflux in the metabolism. This causes a decrease of DI with continued microbial turnover.

2.6.4.3 Specific pathways of glucose and ribose utilization by individual microbial groups

Several reviews aimed to discuss the potential of coupling stable isotope labeling with compound specific isotope analysis of microbial biomarkers (Boschker and Middelburg, 2002; Frostegard et al., 2011). Coupling ^{13}C biomarker analysis with uniform ^{13}C labeling enables tracing of utilization and C partitioning within microbial cells and microbial com-

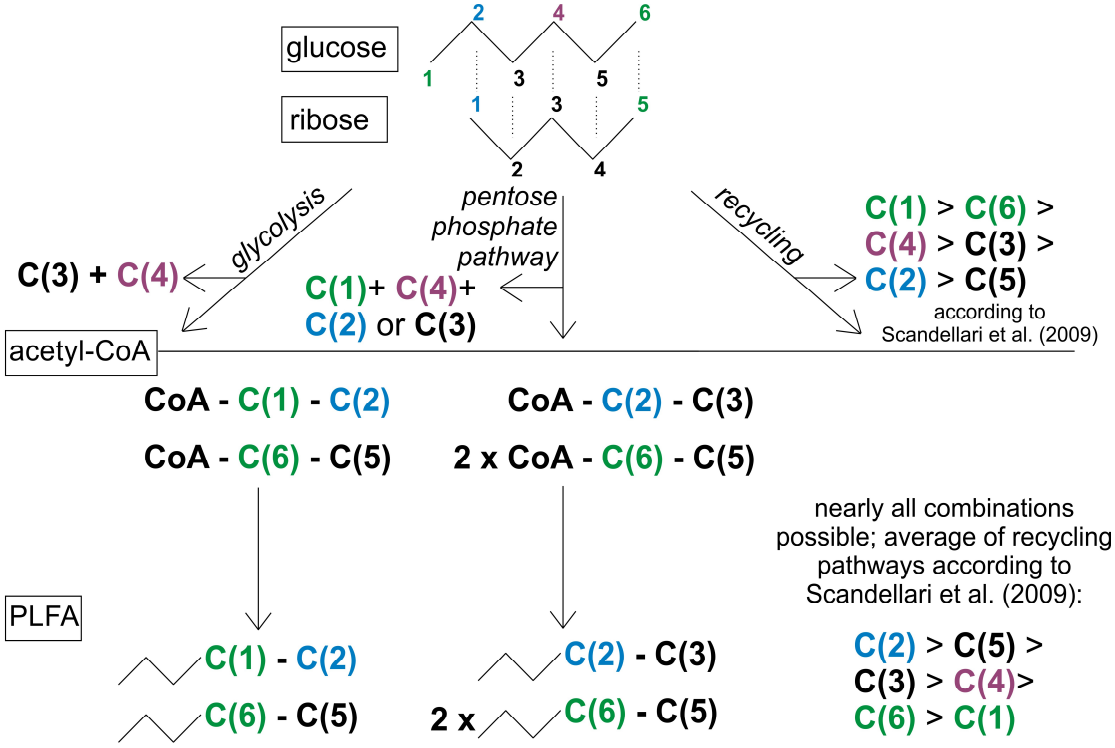
munities. However, only position-specific labeling allows the identification of the transformation steps of the added substrate and the reconstruction of pathways for their metabolism (Dijkstra et al., 2011a; Dippold and Kuzyakov, in press).

Uptake and incorporation of ^{13}C (Figure 2) clearly reflect the highest competition of Gram negatives for the utilization of monosaccharides for formation of PLFA. This is in accordance with previous studies using further LMWOS (Apostel et al., 2013); Gunina et al., submitted) and DNA studies on glucose ^{13}C uptake (Padmanabhan et al. 2003). In addition, Gram negatives are known to dominate in the rhizosphere and the uptake of low molecular weight rhizodeposits (Tian et al., 2013; Treonis et al., 2004). Considering the whole microbial community in soil, Gram negatives are can be considered r strategists. In contrast, Gram positives are known to mainly live from old SOM (Kramer and Gleixner, 2006) and have properties of K strategists. Consequently, they are less competitive for LMWOS, like easily available monosaccharides, which is confirmed in this study.

In general, C flux from both added monosaccharides to the PLFAs was in a similar range for each of the microbial groups (Figure 2). This observation suggests that uptake and also C allocation into the fatty acid formation pathway were equivalent for hexoses and pentoses. However, position-specific ^{13}C labeling, especially DI (Figure 3), reveal a deviating picture: utilization of ribose C for PLFA formation is clearly affected by the pentose phosphate pathway, which leads to a preferential incorporation of C-5 and a loss of C-1 (as this C-1 is in parts transformed into the C-3 position during the formation of hexoses from pentoses) (Figure 5). This general pathway (Caspi et al., 2008; Keseler et al., 2009) dominated the position-specific incorporation of ribose in each of the microbial groups, whereas the intensity seemed to be different for the individual microbial groups. In general, a decrease of divergence (the DI) between ribose C-1 and C-5 from day 3 to day 10 reflects that further transformations of the ^{13}C incorporated in PLFA occurs and thus the original pattern of the basic C metabolic pathway is less visible with increasing metabolization time.

The situation gets more complex if glucose metabolism to fatty acids is reconstructed. A detailed picture of the possible pathways transforming glucose C to PLFA and consequences for the position-specific incorporation was given in Scandellari et al. (2009) and is presented in figure 4. The lack of C-4 incorporation in PLFA clearly indicates the effect of glycolysis with the oxidation of C-3 (could not be proven in this study) and C-4 by pyruvate dehydrogenase (Figure 4). However, simple straightforward glycolysis would lead to a similar incorporation of C-1 and C-6 (Scandellari et al., 2009)) and a similar or lower incorporation of C-2 (Apostel et al., 2013), which was only observed for fungi. Higher incorporation of C-2 than C-1 and C-6 – as observed for any of the prokaryotic groups on day 3 – can only be explained by a complex network of glycolysis, the

pentose phosphate pathway and the triose triangle (Scandellari et al., 2009) (Figure 4), which leads to a re-ordering of the hexose C backbone. Therefore, a significant part has to flow through these reversible pathways more than once in the prokaryotic groups (Figure 5). This is in accordance with the observations of Dippold et al. (data not shown) for amino sugar synthesis from position-specific labeled glucose: 1) catabolic, oxidizing pathways occur simultaneously to anabolic, constructing pathways in soils (Derrien et al., 2007) and 2) bacteria have a higher C turnover due to more intensive backflux processes. Consequently, after the addition of position-specific labeled glucose, the intensive intracellular C recycling could be seen in the bacterial PLFA as well as the amino sugar pool, which supports the concept of slow-cycling, fungi-based and fast-cycling, bacteria-based branch of the food web (Moore et al., 2005).



Theoretically expected C-recovery:

Initially:	C(1) ~ C(6) ~ C(2) ~ C(3) > C(4) ~ C(5) ~ 0	C(6) ~ C(5) > C(2) ~ C(3) > C(4) ~ C(1) ~ 0
After PLFA transformation:	C(1) ~ C(6) > C(2) ~ C(3) > C(4) ~ C(5) ~ 0	C(6) ~ C(2) > C(5) ~ C(3) > C(4) ~ C(1) ~ 0

Fig. 4 Theoretical fate of individual glucose and ribose C positions of single pathways. Left: glycolysis; middle pentose-phophate-pathway and right mixing of glycolysis, backflux via triose-triangle and pentose-phophate-pathway: results for the right branch are experimental data (for fungi) taken from Scandellari et al (2009).

2.6.4.4 Metabolic tracing by position-specific labeling of monosaccharides

Monosaccharides, especially the basic ubiquitous substrate glucose (Macura and Kubatova, 1973), are classical substrates for metabolic tracing analysis (Hobbie et al., 2004; Scandellari et al., 2009), as they are spread throughout all metabolic pathways and can be found in each product. However, recent studies tracing citric acid cycle activity mainly chose pyruvate or the pyruvate-precursor alanine as a metabolic tracer (Apostel et al., 2013; Dijkstra et al., 2011b; Dijkstra et al., 2011c; Dippold and Kuzyakov, in press; Wegener et al., 2010). Fatty acid synthesis pathways branch off from basic C metabolism after the pyruvate dehydrogenase step forming acetyl-CoA (Figure 5). Therefore, pyruvate is a more direct precursor for metabolic tracing in lipids, which causes some advantages for pathway reconstruction: Less reversible, C mixing steps are located between educt and product and, consequently, a mixing of the C backbone just by reversible processes does not occur. This enables more specific metabolic tracing: in the case of alanine and glutamate incorporation into PLFAs, specific pathways of distinct microbial groups could be identified (Apostel et al., 2013).

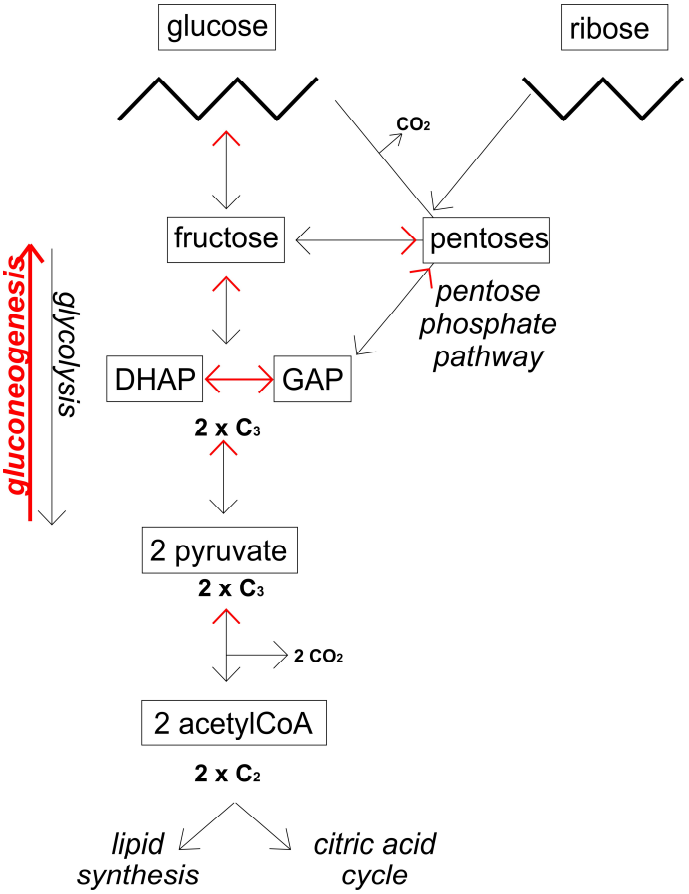


Fig. 5 Microbial transformation pathways of glucose and ribose. Black arrows indicate catabolic pathways, transferring C towards the oxidizing steps of pyruvate dehydrogenase reaction and citric acid cycle. Red arrows indicate anabolic pathways transferring C "up" for the construction of new microbial biomass compounds.

However, if glucose is used as a tracer, as in this study, the reversibility of glycolysis and interaction of the pentose phosphate pathway and glycolysis causes an intensive mixing of the C backbone. This averaging of positions is overprinting microbial group-specific pathways, which are located in the citric acid cycle or fatty acid formation (Apostel et al., 2013). However, glucose may be a good metabolic tracer for the formation of amino sugars, as intact incorporation could be distinguished from C allocation through glycolysis or the pentose phosphate pathway. In general, these applications of position-specific labeling reveal that - to fully trace the range of microbial products - a combination of metabolic tracers is needed: 1) Products of citric acid cycle and fatty acid synthesis, (can be traced by substances entering the citric acid cycle or the pyruvate pool), 2) direct products of sugars like amino sugars or polysaccharides (can be traced by pentoses and hexoses), 3) products of the pentose phosphate pathway, like ribonucleotides (traced by pentoses), and 4) products of processes branching off glycolysis like some amino acid formation. Products of various pathways as well as biosynthetic intermediates (i.e. in the case of PLFA the fatty acid precursor acetyl-CoA) need to be measured compound-specifically to fully identify the C allocation through metabolic pathways. In combination, the tool of position-specific labeling has the unique ability to reconstruct the microbial metabolism in soils and predict C allocation within microbial biomass compounds.

2.6.5 Conclusions and Outlook

This study has shown that position-specific ^{13}C labeling and compound-specific ^{13}C-PLFA analysis are a valuable combination to gain new insights into microbial transformations of sugars in soil. Three days after labeling we found nearly all ^{13}C remaining in soil was taken up by microorganisms for both glucose and ribose. This can be explained by the fact that sugars have no functional group to interact with the soil matrix and thus are taken up very efficiently. On day 10, however, we observed a decrease in ^{13}C recovery in the extractable microbial biomass but not in bulk soil. We explained this either by a) the incorporation of ^{13}C into less extractable microbial cell components between day 3 and 10, b) the excretion of sugar metabolites into the soil, or c) incorporation into SOM after cell death.

The two most common glucose utilizing pathways, glycolysis and the pentose phosphate pathway, could be detected by a lower incorporation of glucoses C-4 and C-1 in both soil and microbial biomass. Consequently, C decomposing and C reconstructing pathways can be traced in parallel in soils. This is likely to arise from cells in different physiological states existing in parallel in soil (Blagodatskaya and Kuzyakov, in press).

Preferential incorporation of ribose C-5 and preferential degradation of ribose C-1 shows that pentoses are preferentially used by the pentose phosphate pathway.

The incorporation of glucose into PLFA revealed the highest recovery of C-2 and the equal recovery of C-1 and C-6. This indicates the parallel pathways of 1) glycolysis, 2) mixing of the trioses and 3) gluconeogenesis, followed by a repetition of these processes with the newly formed glucose molecules. This shows that not only do the anabolic and catabolic pathways exist in parallel in soil microbial communities, but they are also used in short steps by the same organisms. This overprinting of single pathway patterns complicates the reconstruction of individual pathways, especially if time intervals after tracer application become too long. Thus for monosaccharide metabolite tracing, time intervals have to be shortened and “metabolic distance” between educt and product decreased.

None of these findings could have been achieved without using position-specific labeled substances. The method of coupled position-specific ^{13}C labeling and compound-specific isotope analysis helps to identify the transformation steps of LMWOS, and determine C allocation within microbial biomass and the consequent stabilization processes of microbial compounds in soil. This will greatly improve our knowledge about soil C fluxes.

Acknowledgements

We thank the DFG for funding for the funding of this work (KU1184 19/1), Stefanie Boesel from the GC-EA-IRMS laboratory of the Department of Soil Biogeochemistry (Halle) for the reliable measurement of all bulk isotope samples and Evgenia Blagodatskaya for her helpful discussions.

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Supplementary Data

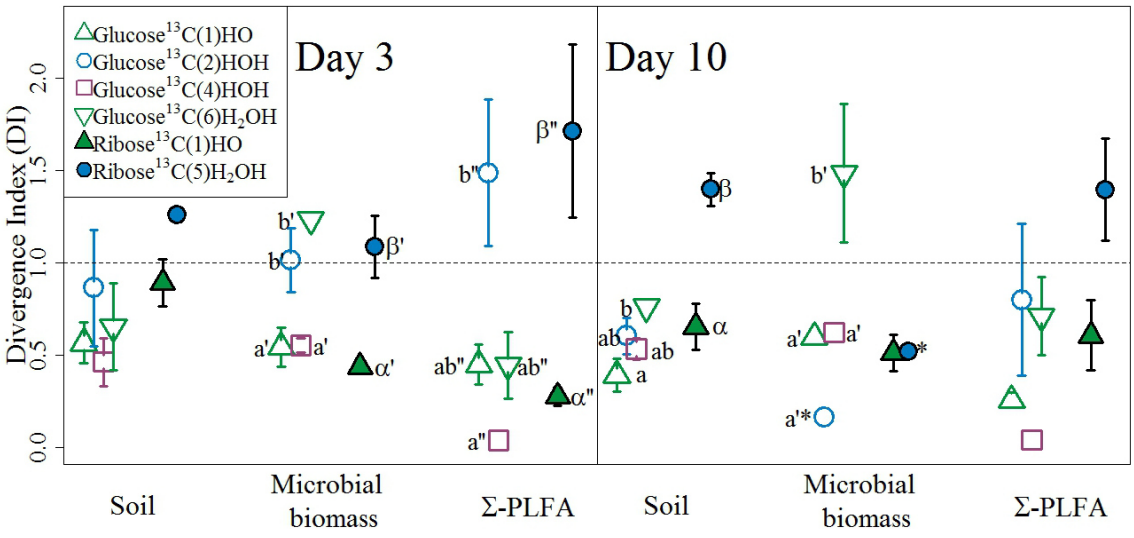
Supplementary Table A1: Fatty acids in the external standard

FA-type	Name	Common name	Abbreviation	Retention time (s)*
Saturated	Tetradecanoic acid	Myristic acid	14:0	150
	Pentadecanoic acid	-	15:0	355
	Hexadecanoic acid	Palmitic acid	16:0	630
	Heptadecanoic acid	Margaric acid	17:0	1010
	Octadecanioc acid	Stearic acid	18:0	1520
	Eicosanoic acid	Arachidic acid	20:0	2930
Branched chain	11-Methyltridecanoic acid	Anteisomyristic acid	a14:0	100
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	90
	12-Methyltetradecanoic acid	12-Methylmyristic acid	a15:0	290
	13-Methyltetradecanoic acid	13-Methylmyristic acid	i15:0	270
	13-Methylpentadecanoic acid	Anteisopalmitic acid	a16:0	545
	14-Methylpentadecanoic acid	Isopalmitic acid	i16:0	525
	14-Methylhexadecanoic acid	14-Methylpalmitic acid	a17:0	890
	15-Methylhexadecan acid	15-Methylpalmitic acid	i17:0	860
Cyclo-propane	cis-9,10-Methylenhexadecanioc acid	cis-9,10-Methylpalmitic acid	cy17:0	940
	cis-9,10-Methylenoctadecanoic acid	Dihydrosterculic acid	cy19:0	2025
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic acid	10Me16:0	780
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	1745
Mono-unsaturated	9-Tetradecaeoic acid	Myristoleic acid	14:1w5c	130
	cis-11-Hexadecenoic acid	-	16:1w5c	595
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1w7c	570
	cis-Octadecenoic acid	Cis-Vaccenic acid	18:1w7c	1400
	cis-9-Octadecenoic acid	Oleic acid	18:1w9c	1375
	11-Eicosenoic acid	Eicosenoic acid	20:1w9c	2700
Poly-unsaturated	cis,cis-9,12-Octadecadienoic acid	Linoleic acid	18:2w6,9	1320
	6,9,12-Octadecatrienoic acid	g-linolenic acid	18:3w6,9,12	1355
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4w6	2320

*for 45 m ± 0.5 m column lengths, deviations of ± 15 s possible

Supplementary Table A2: Results of factor analysis

Fatty acid	Factor 1	Factor 2	Factor 3	Factor 4	Microbial group
i16:0	**0.76**	-0.12	-0.48	0.19	Actinomycetes
10Me16:0	**0.83**	-0.26	0.26	0.17	
a17:0	**0.79**	0.19	-0.41	0.06	
10Me18:0	**0.59**	0.62	-0.21	0.28	
18:1w9c	**-0.76**	0.28	0.22	0.15	Gram negatives I
18:1w7c	**-0.73**	-0.61	0.13	0.07	
20:4w6	**0.83**	-0.01	-0.06	0.18	Protozoa
20:1w9	**0.80**	-0.02	0.18	0.11	Fungi
18:2w6,9	**0.79**	-0.05	0.45	0.03	
a15:0	0.50	**-0.57**	0.13	0.07	Gram positives I
16:1w5c	-0.10	**0.93**	0.04	0.15	VA-mycorrhyza
cy17:0	0.55	-0.23	**0.57**	0.06	Anaerobes
i15:0	-0.31	-0.48	**-0.67**	-0.02	Gram positives II
i17:0	0.36	-0.37	**-0.69**	-0.30	
a16:0	0.13	0.35	0.12	**-0.71**	Gram positives III
16:1w7c	-0.50	-0.19	0.24	**0.51**	Gram negatives II
i14:0	-0.38	-0.15	-0.47	0.48	N/A
cy19:0	0.11	-0.46	0.07	-0.20	
18:3w6,9,12	-0.02	0.95	-0.19	0.00	



*Supplementary Fig. A 1: Divergence index (DI) reflecting incorporational discrimination between C-positions into soil, microbial biomass and Σ-PLFA, 3 (left) and 10 (right) days after applying ^{13}C-labeled glucose and ribose. Letters indicate significant differences ($p < 0.05$) in the relative incorporation of the C positions into soil (a), microbial biomass (a'), Σ-PLFA (a''). * indicates significant difference ($p<0.05$) between day 3 and day 10.*

2.7 Study 7: Metabolic pathways of fungal and bacterial amino sugar formation in soil assessed by position-specific ^{13}C-labeling

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Abstract

Amino sugars are the major constituent of microbial cell walls and are an important biomarker for microbially-derived soil organic matter (SOM). Amino sugar formation from low molecular weight organic substances (LMWOS) transforms C from labile to more stable SOM pools. In this study, we investigated the formation steps of fungal and bacterial amino sugars from glucose by coupling position-specific ^{13}C labeling with compound-specific $\delta^{13}C$ analysis.

The 1-^{13}C-, 2-^{13}C-, 4-^{13}C-, 6-^{13}C- and uniformly-^{13}C-labelled isotopomers of glucose were applied to an agricultural soil and their incorporation into microbial biomass was analyzed. Fungal and bacterial metabolic pathways of amino sugars formation were traced by determining the ^{13}C incorporation from individual C positions of glucose into the individual amino sugars by ion chromatography – oxidation – isotope ratio mass spectrometry (IC-O-IRMS).

Only 0.75% of the glucose ^{13}C was incorporated into amino sugars within 10 days. ^{13}C incorporation was highest in glucosamine (the most abundant amino sugar), was slightly lower into galactosamine and much lower into muramic acid (amino sugar with the lowest content). However, considering the amino sugar concentrations, the replacement of muramic acid (a biomarker for bacteria) was highest and the replacement of galactosamine (biomarker for fungi) was lowest. This reflects the higher turnover of bacterial cell walls compared to the turnover of fungal chitin.

Less than 55% of the incorporated ^{13}C in amino sugars was derived from intact, untransformed glucose. The lowest incorporation was observed for C-1 and C-4. C-1 incorporation even decreased from day 3 to day 10, reflecting the production and incorporation of metabolites from the pentose-phosphate pathway. The formation of amino sugars by direct pathways (e.g. intact incorporation of the glucose precursor), oxidation via catabolism and constructing pathways with amino sugar formation from metabolites of basic C metabolism occurred in parallel in this study.

Bacterial muramic acid showed the higher dynamics of ^{13}C replacement and a stronger variation in the incorporation of individual C positions over a period of 10 days e.g. by more intensive glycolysis, pentose-phosphate pathway and gluconeogenesis. This reflects the lower metabolic activity of fungi versus bacteria, at maintenance metabolism, which is in accordance with the differences in C turnover observed for the slow and fast cycling branches of soil food webs.

Analyzing the formation of microbial compounds by position-specific labeling and compound specific ^{13}C analysis allowed conclusions to be drawn about the biochemical mechanisms and pathways driven in soil by two microbial groups: bacteria and fungi.

This combined approach will strongly improve our understanding of soil C fluxes and the formation and stabilization of microbially-derived SOM compounds.

Keywords: position-specific tracers, isotopomers, metabolic tracing, C mineralization and stabilization, microbial-derived soil organic matter, amino sugar formation, labeling approaches

2.7.1 Introduction

Soil organic carbon (SOC) represents a major terrestrial carbon (C) sink. Thus, studies on the transformation of organic substances in soils are important for understanding terrestrial C and N cycles. Plant residues and rhizodeposits are the main sources of organic matter in soils (Kuzyakov and Domanski, 2000; Rasse et al., 2005). Accordingly, many studies have focused on decomposition, microbial utilization and stabilization of C from these sources in soils (Dungait et al., 2012; Rasse et al., 2005; von Luetzow et al., 2006). Within the last decade it has become evident that microbial compounds were an underestimated contributor to soil organic matter (SOM) (Miltner et al., 2011; Simpson et al., 2007).

Within the spectra of microbial compounds, the cell walls are the most relevant for slow cycling SOM. Cell walls are highly polymeric substances (Amelung, 2003), which may accumulate in soil compared to living soil microbial biomass (Glaser et al., 2004; Veuger et al., 2006) because they interact with the surfaces of clay minerals and sesquioxides (Amelung et al., 2001; Miltner et al., 2011). Thus, an increasing interest in the turnover and accumulation of microbial cell wall compounds – e.g. amino sugars (Miltner et al., 2011) in soils has led to their investigation. Beside its contribution to SOM, amino sugars – together with proteins – are the compound class linking C and N cycles in the soil and contribute significantly to the stable soil organic N pool (Amelung, 2003).

Due to the specific amino sugar composition of cell walls of various microbial groups, amino sugars provide information about the microbial community structure. Bacterial cell walls consist of peptidoglycan, a polymer of N-acetylmuramic acid and N-acetylglucosamine with amino acids, whereas fungal cell walls consist of chitin, an N-acetlyglucosamine polymer (Engelking et al., 2007; Glaser et al., 2004). Acid hydrolysis induces the elimination of the acetyl group and the splitting of the polymers and releases monomeric amino sugars. Consequently, bacterial cell walls deliver a glucosamine to muramic acid ratio of 1:1, whereas fungal chitin consists only of glucosamine. Based on this theoretical knowledge of concentrations of amino sugars per dry weight of a microbial biomass, the theoretical biomass of fungi versus bacteria can be calculated (Engelking et al., 2007). While muramic acid is unique for bacteria, galactosamine can occur in trace amounts in some bacterial cell walls but seems to be quantitatively relevant only for fungal cell walls (Engelking et al., 2007; Glaser et al., 2004). The origin of mannosamine, another amino sugar found in hydrolysis extracts of soils, is still being discussed (Glaser et al., 2004). Glucosamine pool in soils seem to be mainly of fungal origin; however, it is unknown whether the active amino sugar pool, which we define here as the amino sugars of living cells, has a completely different fingerprint than the amino sugar necromass. For

many ecosystems, the total glucosamine pool is dominated by fungal chitin. However, the active glucosamine pool, representing the present microbial community, may originate to a higher degree from bacterial peptidoglycan.

In contrast to cell membrane compounds like phospholipids, which have half-life times of less than one week (Kindler et al., 2009; Ranneklev and Baath, 2003; Rethemeyer et al., 2004) amino sugars have a much slower turnover (Lauer et al., 2011) and many acid-hydrolysable amino sugars are derived from necromass. Consequently, the amino sugar fingerprint reflects only the average, long-term microbial input remaining in soil. Amino sugars can only be used for tracing the current state of a microbial community and their activity if the pool of active amino sugars is taken into account.

A promising approach for tracing the fate of C sources in active amino sugars is coupling isotope labeling with ^{13}C or ^{15}N determination of amino sugars (Bode et al., 2013; Glaser and Gross, 2005; Indorf et al., 2012; Said-Pullicino et al., in press). This enables the quantification of C transformation from plant biomass to stable microbial C in soils (Bode et al., 2013; Glaser and Gross, 2005; Indorf et al., 2012). However, mechanisms of the formation and individual role of distinct members of the microbial community can only be reconstructed if labelling of a distinct substrate pool is performed. The most appropriate substances, therefore, are low molecular weight organic substances (LMWOS). They play a crucial role within the SOC cycle as all macromolecular compounds are split by enzymes into LMWOS during litter decomposition (Cadisch and Giller, 1996). Within the microbial biomass, they are precursor of the biosynthetic pathways for any cellular compound. Within the LMWOS, sugars (mainly glucose) are some of the most abundant monomers. Glucose builds up cellulose, but is also an important constituent of many lipopolysaccharides, glycoproteins and many other compounds (Derrien et al., 2006). In addition, glucose is also directly released in soils via rhizodeposition (Derrien et al., 2004; Fischer et al., 2010). Due to the ubiquitous properties of this substance (Macura and Kubatova, 1973) it is taken up and utilized by microbial biomass very rapidly and, therefore, its concentration in soil solution is extremely low, ranging from 0.7 to 2.5 µmol l^{-1} (Meyer et al., 2008). Glucose is one of the most efficient energy sources for catabolism, but is also a direct precursor of many anabolic pathways, like the formation of cell wall polymers. Therefore, glucose C is a key substrate in microbial metabolism, reflecting C partitioning in the microbial biomass.

To elucidate glucose metabolism pathways, we used the approach of position-specific labeling. This tool, derived from biochemistry to investigate metabolism pathways, has rarely been applied in soil science (Fokin et al., 1993, , 1994; Haider and Martin, 1975; Kuzyakov, 1997; Nasholm et al., 2001). However, using position-specific labeling to assess metabolism pathways in soil has arisen within the last few years (Apostel et

al., in press; Dijkstra et al., 2011a; Dijkstra et al., 2011b; Dippold and Kuzyakov, in press; Fischer and Kuzyakov, 2010). This technique enables the fate of individual C positions to be traced through various pools or metabolites, consequently allowing the reconstruction of pathways leading to oxidation or splitting of individual C positions.

To our knowledge, there are no studies investigating the *in-situ* formation of microbial amino sugars in soils. We used position-specific ^{13}C-labeled glucose to trace microbial utilization of amino sugars for formation of cell walls. We hypothesized that glucose is preferentially used intact, as it is a direct precursor for amino sugar synthesis. However, differences in the positions incorporated will enable transformation via glycolysis to be distinguished from transformation via pentose-phosphate pathway or further sugar metabolizing pathways. We hypothesized that metabolic pathways transforming glucose from fungal and bacterial cells differ significantly and that these individual pathways can be identified by the incorporation of individual glucose C positions in the respective amino sugars.

2.7.2 Material and Methods

2.7.2.1 Experimental Site

The experimental site is located in Bavaria near Hohenpölz (49.907 N, 11.152 E, 501 m asl, mean annual temperature 6.7 °C, mean annual precipitation 874 mm). The agriculturally used loamy Luvisol is managed by a rotation of corn, barley, wheat and triticale. The soil had a pH_{KCl} of 4.88, a pH_{H_2O} of 6.49, TOC content of 1.77%, TN content of 0.19% and CEC was 13 $cmol_C\ kg^{-1}$. Triticale was the last crop before the experiment started and all above-ground biomass was removed.

2.7.2.2 Experiment Design

The 12 × 12 m field was divided into four quadrants. PVC-tubes with a diameter of 10 cm and height of 13 cm were installed 10 cm deep into the soil, resulting in a soil sample weight of about 1.5 kg for each column. Column location followed a randomized block design with the four blocks containing the four replications of each treatment. Consequently, the block could be included as a random variable in statistical evaluation to account for the spatial heterogeneity within the field side.

10 ml of tracer-solution were applied per column with a multipette (Eppendorf, Hamburg, Germany). Uniformly ^{13}C-labeled glucose as well as four position-specific labeled isotopomers (1-^{13}C glucose, 2-^{13}C glucose, 4-^{13}C glucose and 6-^{13}C glucose) was

applied. In addition, the identical amount of non-labeled glucose C was applied on background columns. Concentrations of ^{13}C were 100 µmol per column and a total of 0.60 µg glucose C was applied per gram of soil.

A 7-cm-long needle with closed tip and peripheral holes enabled the homogeneous lateral distribution of the tracer solution. Leaching was avoided by injecting the solution only into the upper 2/3 of the column and blocking rainfall by a roof installed above the experimental site.

2.7.2.3 Sampling and Sample Preparation

Sampling was performed 3 and 10 days after labeling by removing the entire column from the field site. At both times, complete columns from one set (background, uniformly and position-specifically labeled) with four replications were dug out and the height of the soil inside the column was measured to determine the labeled soil volume. Then, soil was taken from the column completely, fresh weight was determined and the entire soil sample was homogenized manually. Afterwards, a subsample was taken to estimate water content. Then, the sample was split: one subsample was freeze-dried and ball milled for bulk isotope analysis and amino sugar analysis, while another subsample was 2 mm sieved and stored at <5 °C for chloroform-fumigation-extraction (CFE).

2.7.2.4 Bulk Soil and Microbial Biomass Analysis

For the analysis of C content and $\delta^{13}C$ values, the soil samples were freeze-dried, ground in a ball mill and 5-6 mg per sample was filled into tin capsules. The samples were measured on the Euro EA Elemental Analyzer (Eurovector, Milan, Italy) unit with a ConFlo III interface (Thermo-Fischer, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). Incorporation of ^{13}C from the applied glucose into the soil C pool was calculated according to the mixing model (Eq. 1 and 2), where the C content of the background in Eq. 1 was substituted according to Eq. 2 (Gearing et al., 1991).

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C-BG} + [C]_{appAGlc} \cdot r_{appGlc} \quad (7)$$

$$[C]_{soil} = [C]_{BG} + [C]_{appGlc} \quad (8)$$

with: $[C]_{soil/BG/appGlc}$ C content of sample / background / applied glucose (mol · g_{soil}^{-1})

$r_{soil/BG/appGlc}$ ^{13}C atom%-excess of sample / background / applied glucose (at%)

To determine microbial C and its $\delta^{13}C$ values, two subsets of 15 g of soil were taken from each sample. One sample was directly extracted as described below; the other was first fumigated with chloroform for 5 days in a desiccator to lyse microbial cells. The samples were extracted with 45 ml of 0.05 M K_2SO_4. They were shaken on a horizontal shaker for 1.5 h. After shaking, the samples were centrifuged (10 min, 2000 rpm) and the supernatant was filtered (Rotilab® round filters, type 15A, cellulose, membrane 70 mm).

The C content of the K_2SO_4 extracts was measured on the TOC Analyzer multi C/N® 2000 (Analytik Jena, Jena, Germany). For $\delta^{13}C$ measurements, the remaining extracts were freeze-dried. A subsample of the freeze-dried residue was transferred to tin capsules and measured on the Euro EA Elemental Analyzer (Eurovector, Milan, Italy) unit coupled via a ConFlo III interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). ^{13}C incorporation into fumigated and non-fumigated sample was calculated according to the mixing model (Eq. 1 and 2), and microbial biomass as well as incorporated glucose ^{13}C in the microbial biomass was calculated according to Wu et al. (1990).

2.7.2.5 Amino sugar $\delta^{13}C$ analysis

Chemicals, Reagents and Standard Substances

Amino sugar $\delta^{13}C$ analysis was performed according to Dippold et al. (2014). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) with at least the grade pro analysis (>99.0% purity). Ultra-pure, 50-52% NaOH solution for ion chromatography was purchased from Sigma Aldrich (St. Louis, MO, USA). 0.01 M $NaNO_3$-solution was produced from metal-free sodium nitrate, puratronic (99.999% purity, Alfa Aesar, Karlsruhe, Germany). For oxidation, 0.26 M sodium persulfate and 10% phosphoric acid solutions were produced (St. Louis, MO, USA).

External standards were combined from methylglucamine, glucosamine, mannosamine and galactosamine at concentrations of 5, 14, 1.5 and 20 mg l^{-1} (Sigma Aldrich, Louis, MO, USA) and muramic acid (Toronto Research Chemicals, North York, Canada) at a concentration of 7.5 mg l^{-1} in the stock solution. Five mg ml^{-1} methylglucamine p.a. and 1 mg ml^{-1} fructose p.a. (Sigma Aldrich, Louis, MO, USA) were used as first and sec-

ond internal standards (IS1 and IS2), respectively. IAEA-calibrated $\delta^{13}C$ values of external standards was determined by repeated Elemental analyzer-Isotope Ratio Mass Spectrometer measurement (Flash 2000 HT Plus coupled to a Delta V Advantage Isotope Ratio Mass Spectrometer, Thermo-Fischer, Bremen, Germany) of these substances and calibrated against certified standards (IAEA-CH6: -10.4‰, IAEA-CH7 -31.8‰ and USGS41 37.8‰) versus Pee Dee Belemnite (PDB).

Soil hydrolysis and ion removal

The method of Zhang and Amelung (1996), modified by Glaser and Gross (2005), was used for soil hydrolysis and ion removal were performed. Briefly, hydrolysis was performed with 10 ml of 6 M HCl at 105°C for 8 h. After filtration via a glass fiber filter, the dried filtrate was re-dissolved in water. 50 µg of the IS1 methylglucamine were added. With 0.6 M KOH, the pH was adjusted to 6.6-6.8, and precipitated iron was removed by centrifugation. Freeze-dried supernatant was redissolved in 5 ml dry methanol and salt precipitations were removed by centrifugation. Dried supernatant was stored frozen until column purification.

Purification by cation exchange column

Cation exchange column (AG 50W-X8 Resin, H+ form, mesh size 100-200, Biorad, Munich, Germany) was adapted from Indorf et al. (2013): a glass wool layer was installed at the bottom of the glass columns (inner diameter: 0.8 cm). Then, 4 cm of cation exchange resin was placed into the column and preconditioned with ~10 ml of 0.1 M HCl solution to ensure the H^+-form of the sorbent. Afterwards, 5 ml of water was added to reach the neutral pH of the mobile phase. Dried extracts were re-dissolved in ~1 ml of water with one drop of 0.1 M HCl to convert muramic acid to the cationic form. Neutral and anionic compounds were eluted with 8 ml water. The cationic fraction, containing the amino sugars, was eluted using 15 ml 0.5 M HCl, thereafter freeze-dried and transferred with 5 ml of dried methanol into 5 ml glass vials. After drying, samples were stored frozen (-20 °C) until analysis. For subsequent measurements, the samples were re-dissolved in 200 µl water with the addition of 50 µl of IS2 solution and measured within 24 hours after re-dissolving.

Measurement by IC-O-IRMS

All measurements were performed by a Dionex ICS-5000 SP ion chromatography system coupled by an LC IsoLink to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo-Fischer, Bremen, Germany). 9 µl sample was injected via a 25 µl injection loop. Chromatography was performed by a CarboPacTM PA 20 analytical anion ex-

change column (3 x 150 mm, 6.5 µm), which was preceded by a PA 20 guard column (Bode et al., 2009), both from Dionex (Amsterdam, The Netherlands). The elution sequence included 25 min preconditioning time before injection, and a total chromatogram duration of 35 min, which was performed at a constant temperature of 30 °C. Flow rate, gradients and eluents are described in detail in Supplementary Table 1 and Dippold et al. (2014).

We measured external standards in four concentrations (e.g. 50, 100, 175 and 250 µl of the stock solution) at least once before and once after each sample batch. A sample batch consisted of 4-6 samples and each sample was at least measured in 4 replications.

Quantification and $\delta^{13}C$ determination of amino sugars

The relation between the area of each amino sugar and the area of the IS2 was calculated and a calibration line was adapted by linear regression to the area ratio of the four external standards. The recovery for each sample was determined based on the result of the initially added IS1 and each peak in the chromatogram was corrected for that recovery.

Measured $\delta^{13}C$ values were drift-corrected based on the reference gas drift according to GC-C-IRMS methods (Apostel et al., in press). Thereafter, correction for off-set and amount dependence were performed according to Glaser and Amelung (2002). Linear, exponential and partial linear amount dependence functions, as well as a constant function correcting for the offset (Dippold et al, 2014), were fitted to the plot of measured at% value against peak area. The function with the best fit was used to correct the measured $at\%_{measured}$ values of the sample dependent on the area of this peak. Therefore, the difference of amount-dependent correction value $at\%_{corrected}$ (Ai) and the measured and calibrated value of the substance $at\%_{EA}$ was subtracted from the measured value to gain the IAEA-calibrated ^{13}C enrichment ($at\%_{sample}$):

$$at\%_{sample} = at\%_{measured} - (at\%_{EA} - at\%_{corrected}(A_i)) \quad (3)$$

Each substance and sample batch was corrected by its individual correction function which best described the behavior of the external standards. Incorporation of ^{13}C into each amino sugar was performed according to the mixing model (equations 1 and 2).

2.7.2.6 Divergence Index

According to Dippold and Kuzyakov (2014), the transformation of C from individual intramolecular positions can be expressed by the Divergence Index, DI_i:

$$DI_i = \frac{n \cdot [C\text{-}i]}{\sum_{i=1}^{i=n} [C\text{-}i]} = \frac{6 \cdot [Glc - C\text{-}i]}{\sum_{1}^{3} [Glc - C\text{-}i\ ]} \quad (4)$$

This index reflects the fate of individual C atoms from the position i relative to the mean transformation of the n total number of C atoms within a transformation process. Thus, a DI_i of 1 means that the transformation of this position in the investigated pool corresponds to the transformation of uniformly labeled substance (average of all C atoms). DI_i ranges from 0 to n, and values between 0 and 1 reflect reduced incorporation of the C into the investigated pool, whereas values between 1 and n show increased incorporation of the C atom into this pool as compared to the average.

2.7.2.7 Statistics

All experiments were done with four replications, and the values present mean ± standard error of mean (± SEM). SEM of the divergence index was gained by Gaussian error propagation. Measured variables were screened for outliers using the Nalimov test (Gottwald, 2000), normal distribution was tested using the Kolmogorov Smirnoff test and homogeneous variances were assessed using Levene's test. ANOVA with the factors C position and amino sugar were performed using Statistica (version 7.0, Statsoft GmbH, Hamburg, Germany). If assumptions such as normal distribution or homogeneous variances were not met, the result of the ANOVA was confirmed by non-parametric Kruskal-Wallis ANOVA before performing a Tukey HSD post-hoc test for unequal sample size.

2.7.3 Results

2.7.3.1 Glucose ^{13}C incorporation into soil and microbial C pools

Amino sugars amounted for 3.7% of the TOC pool and thus contributed significantly to the stored C in this soil. None of the investigated microbially-derived C pools changed significantly from day 3 to day 10 (Table 1). Not only microbial C but also the sum of microbial-derived phospholipids fatty acids (PLFA) did not change from day 3 to day 10 (day 3: 0.96 ± 0.21 ng g^{-1} soil, day 10: 0.76 ± 0.17 ng g^{-1} soil: data provided by Apostel C. (submitted)). Consequently, both active microbial pools reflect microbial communities under steady state conditions without active growth for at least 10 days.

Tracing ^{13}C shows that similar amount of glucose-C remained in soil between 3 and 10 days; e.g. that loss of glucose mainly occurred during the first 3 days. Glucose mineralization was similar to rates observed by Nguyen and Guckert (2001) for bare soil. From the glucose ^{13}C bound in the soil at day 3, around 57% was in the microbial biomass C,

which decreased significantly from day 3 to day 10. Less than 0.75% of the ^{13}C remaining in the soil was incorporated into the amino sugar pool. Within the amino sugars, glucosamine was the most abundant monomer and had the highest glucose ^{13}C incorporation (Table 1). Abundance of galactosamine was lower by a factor of 5-10 and ^{13}C incorporation was also lower by a factor of 10 compared to glucosamine. Muramic acid had the lowest abundance: it was found to be 10-fold lower than galactosamine in this soil. However, glucose ^{13}C incorporation was only 2-3-fold lower reflecting the higher formation or metabolism rate of this acidic amino sugar compared to basic amino sugars.

Table 1 Amount and glucose ^{13}C recovery in total organic C (TOC), microbial biomass C (C_{mic}) and the total amino sugars ($\Sigma_{AminoSugars}$) as well as the three individual amino sugars

		TOC	C_{mic}	$\Sigma_{AminoSugars}$	Galactosamine	Glucosamine	Muramic acid
Pool Size (mg C g^{-1} soil)	day 3	14.53 ± 2.62	0.49 ± 0.05	0.61 ± 0.08	0.08 ± 0.01	0.53 ± 0.08	0.009 ± 0.001
	day 10	16.33 ± 1.70	0.46 ± 0.11	0.54 ± 0.05	0.09 ± 0.01	0.43 ± 0.06	0.009 ± 0.001
Glucose ^{13}C recovery (ng glc-^{13}C g^{-1} soil)	day 3	1134.9 ± 77.0	653.0 ± 40.9	8.52 ± 6.19	0.83 ± 0.35	8.00 ± 0.31	0.36 ± 0.14
	day 10	1225.9 ± 812.7	336.9 ± 12.9	6.19 ± 0.31	0.65 ± 0.34	6.25 ± 0.24	0.28 ± 0.19

2.7.3.2 Incorporation of C from various positions of glucose molecule into individual amino sugars

Carbon from individual positions of the glucose molecule was incorporated differently into each of the three identified amino sugars (Figure 1). Thus, glucose was not fully incorporated as an intact molecule, but at least in parts split into fragments i.e. metabolites, which were incorporated. Some trends are similar for each of the amino sugars: 1) glucose C-4 was incorporated by day 3 at least from all glucose molecule positions, and 2) the majority of the C-positions remained constant between days 3 and 10. However, the incorporation of glucose C-1 strongly decreased for each of the amino sugars from day 3 to day 10. The expected symmetry of the fingerprint e.g. C-1 ~ C-6, C-2 ~ C-5 and C-3 ~ C-4 may exist for day 3 but cannot be clearly seen for day 10 (Figure 1). This reveals that there are pathways other than glycolysis and gluconeogenesis which are responsible for the glucose transformation for cell wall formation.

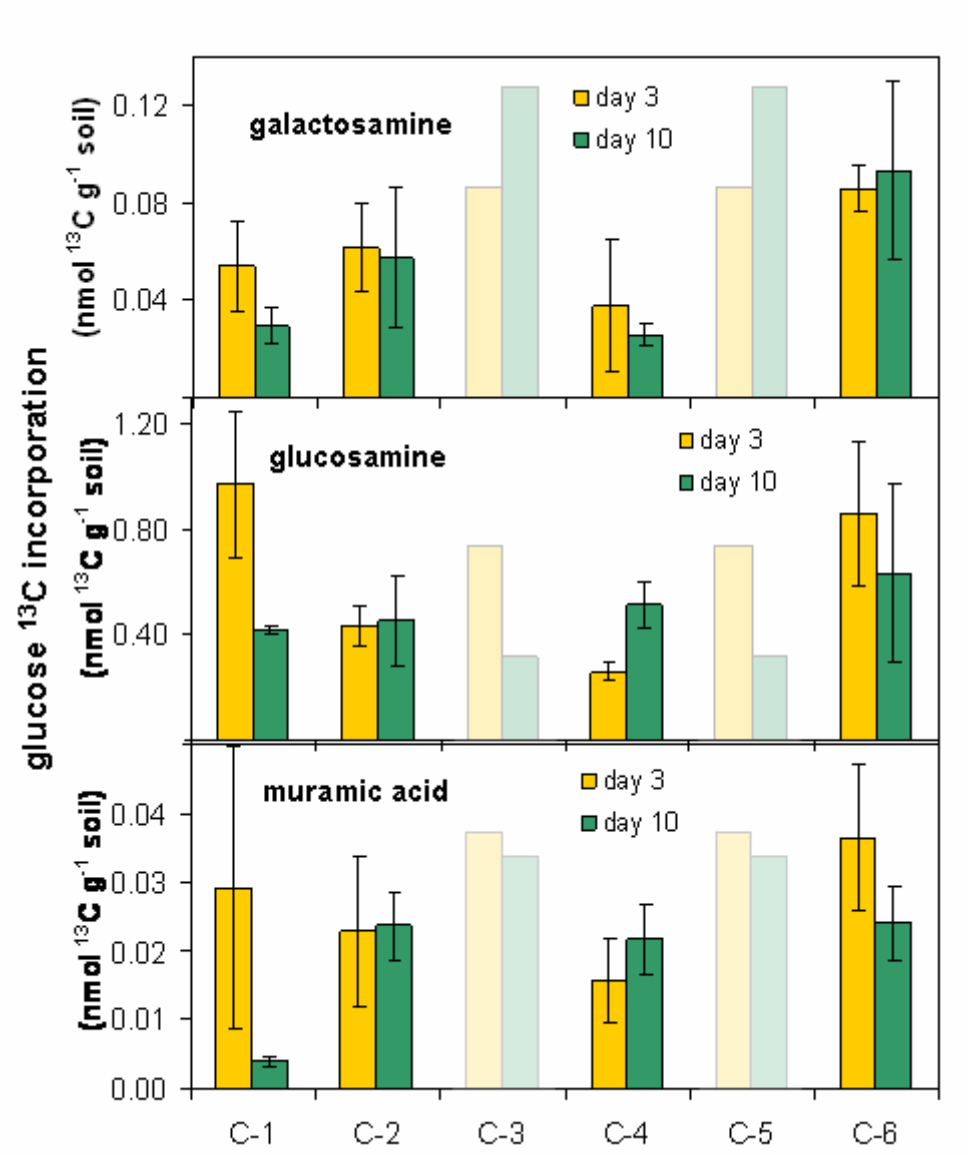

Fig. 1 Incorporation of ^{13}C from individual glucose into the three amino sugars in % of applied ^{13}C. Values represent means (± SEM, N=4). Yellow bars show incorporation 3 days after labelling, green bars show 10 days after labeling.

Intact glucose incorporation would lead to the identical incorporation of all glucose C positions. Consequently, the position with the lowest incorporation reflects the maximum intact glucose C incorporation. Calculating the ratio of the minimum position to average glucose C incorporation shows that for galactosamine and glucosamine, a maximum of 55% and 39% of glucose-C, respectively, were incorporated directly. This calculation is no longer possible for muramic acid, as an additional pyruvate is condensed to the hexose ring in this amino sugar monomer (Richmond and Perkins, 1962). However, the basic amino sugars already show that a large percentage of cell walls were not built up from untransformed glucose taken up from soil solution.

A better comparison of the specifics of individual positions in the formation of amino sugars can be gained by the divergence index (Figure 2), as this index does not consider

the absolute pool size, which is strongly different for the individual amino sugars. Figure 2 shows that the relative incorporation is highest for C-6 and lowest for C-4. Transformations from day 3 to day 10 show clear similarities between glucosamine and muramic acid: both amino sugars show a decrease in C-1 incorporation and an increase in C-2 and C-4 incorporation, whereas the relative portion of C-6 remained constant (Figure 2). In contrast, galactosamine showed only a slight decrease in C-1 from day 3 to 10, while all other positions remained nearly constant.

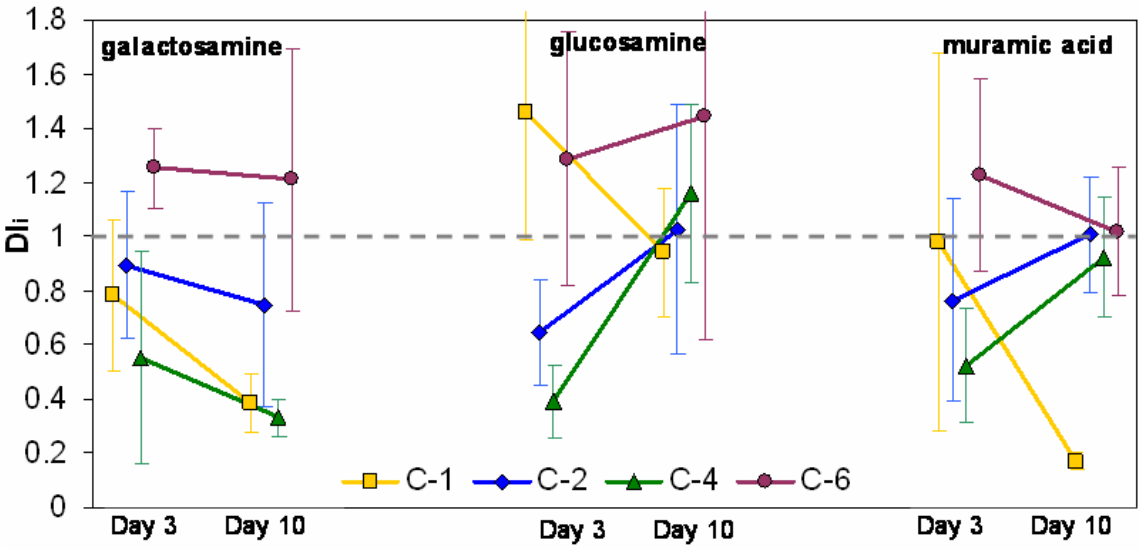

Fig. 2 Divergence Index (DI), reflecting discrimination between C positions of glucose three (left) and ten (right) days after ^{13}C application for galactosamine, glucosamine and muramic acid.

2.7.3.3 Replacement of cell wall pool by glucose ^{13}C

In contrast to the absolute incorporation of glucose ^{13}C, which shows the activity of the newly formed cell walls, the replacement of amino sugar C by ^{13}C (Figure 3) reflects the turnover of an existing soil pool. Whereas glucosamine has the highest absolute ^{13}C incorporation (Figure 1), its replacement is intermediate between galactosamine and muramic acid. Galactosamine showed the lowest values, whereas muramic acid showed the highest replacement with 0.006% of the muramic acid pool 3 days after labeling. Replacement slightly decreased for the basic amino sugars and significantly for muramic acid from day 3 to day 10.

However, as position-specific ^{13}C incorporation was not equal for all glucose molecule positions (Figure 1), the replacement was also strongly dependent on the labeled position (Figure 3). Turnover varied by a factor of six depending on the C positions in the molecule and the highest range between maximum and minimum incorporation was observed for muramic acid.

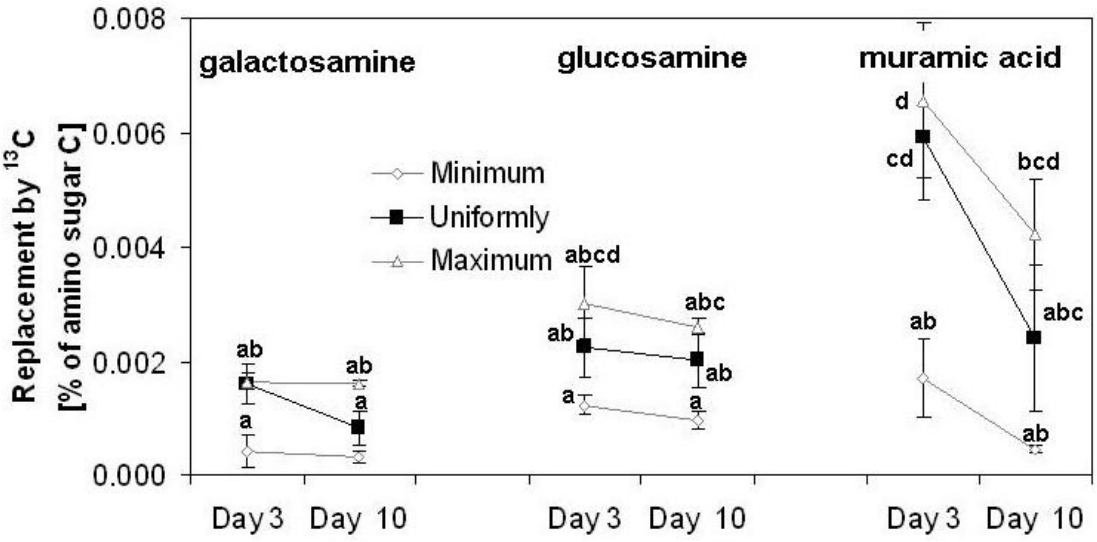

Fig. 3 Replacement of amino sugar pool by newly formed amino sugars from glucose ^{13}C. Experimental points (means ± SEM, N=4) are presented. Filled symbols represent means and open symbols show the position with lowest (minimum) and highest (maximum) replacement. Letters indicate significant differences of means according to ANOVA with Tukey HSD for unequal N Post-Hoc Test and always refer to the points below the letter.

2.7.4 Discussion

2.7.4.1 Fungal versus bacterial contribution to the amino sugar fingerprint and glucose utilization

Glucosamine is the quantitatively most relevant amino sugar in soils. The review from Engelking et al. (2007) showed that glucosamine from mixed microbial communities, as in soils, is mainly derived from fungal cell walls. This can be attributed to the fact that, in the case of fungi, the cell wall comprises a higher percentage of the cellular dry weight compared to bacteria, and that fungal chitin consists of a higher proportion of glucosamine than bacterial peptidoglycan. Consequently, glucosamine was frequently used to calculate the fungal biomass in soils. However, peptidoglycan – the bacterial cell wall polymer – also consists of up to 50% glucosamine and an interpretation of glucosamine as a fungal biomarker only has to be considered critical. In contrast to glucosamine, galactosamine is found only in trace amounts in bacterial cell walls (Engelking et al., 2007; Glaser et al., 2004) and thus seems to be the better biomarker for fungal cell walls (Engelking et al., 2007). Also, the ratio of galactosamine to muramic acid was significantly higher for fungal (59) than bacterial (3) biomass (Glaser et al., 2004).

The first results on amino sugar formation from ^{13}C-labeled plant litter reflected that glucosamine and galactosamine in soils had different dynamics and formation kinetics

and were likely produced from different microorganisms (Bode et al., 2013). It was assumed that galactosamine is a better fungal biomarker than glucosamine. However, they could not prove this assumption and the bacterial contribution to newly formed glucosamine pool as they were not able to detect newly formed ^{13}C-enriched muramic acid (Bode et al., 2013). Our results (Figure 3) clearly reflect that the turnover of glucosamine is intermediate to that of galactosamine and muramic acid. This confirms the theory that the active glucosamine pool in soils, which is part of living cells and consequently incorporates added ^{13}C, is a mixture of fungal and bacterial cell walls; e.g. chitin and peptidoglycan. It was considered that the main contributor to the glucosamine pool of entire SOM might be fungal cell walls, i.e. chitin, due to the high recalcitrance of this crystalline polymer (Beckham et al., 2011). However, this is not feasible if the active pool of amino sugars, e.g. the cell walls of living, active microbial biomass in soils is considered, because the bacterial contribution to the active glucosamine pool gets relevant or even dominates the fungal contribution, which is in contrast to the necromass. This was observed for the formation of new amino sugars under microbial growth (Bode et al., 2013) or for the incorporation of substrates under maintenance conditions into cell walls (Figure 1 and 3) in this study. Our data confirm this as the behaviors of the divergence index changes from day 3 to day 10 are similar between glucosamine and muramic acid but not between glucosamine and galactosamine. This can be interpreted as similar pathways for amino sugar formation or transformation between muramic acid and glucosamine, in contrast to galactosamine. Consequently, similar source organisms for muramic acid and glucosamine are likely. This result confirms the data of PLFAs analyses of Apostel et al. (2013) of the same field site, which showed by ^{13}C-PLFA analysis that bacteria are the most active group in the utilization of LMWOS-^{13}C.

Consequently, although the soil glucosamine pool might be mainly fungal-derived, the newly formed ^{13}C glucosamine was mainly from bacterial sources. Thus, the amino sugar fingerprint alone is unlikely to reflect the current microbial community structure and cannot be used to draw conclusions about the activity of fungi and bacteria, as already stated by Glaser et al. (2004). However, the newly formed ^{13}C amino sugars or the replacement by ^{13}C reflect the activity of fungi versus bacteria in substrate utilization enable a quantitative and specific conclusion about the activity of microbial community members.

2.7.4.2 Pathways of amino sugar formation

According to biological principles, microbial cells produce their biomass by the most economic pathways, e.g. they tend to use pre-formed building blocks as substrates for macromolecular synthesis, if these direct precursors are available (Lengeler et al., 1999).

Acetylated amino sugars, used for cell wall formation, are directly produced from the glycolysis intermediate fructose-6-phosphate by transamination, isomerization and acetylation, and afterwards become activated (Kotnik et al., 2007; Milewski et al., 2006). In the case of muramic acid, an additional condensation with activated pyruvate (Richmond and Perkins, 1962) occurs in the periplasm (Figure 4). Consequently, amino sugars should be built-up mainly by direct glucose precursors, especially if glucose is available and cells are only in maintenance metabolism and do not need huge amounts of newly synthesized cell walls. Constant levels of microbial biomass C reflect the steady state of the microbial community in this experiment (Table 1). The lack of easily available C sources in this bare soil experiment (without plants, rhizodeposits and litter inputs) supports the suggestion that the microbial community is in a non-growing state.

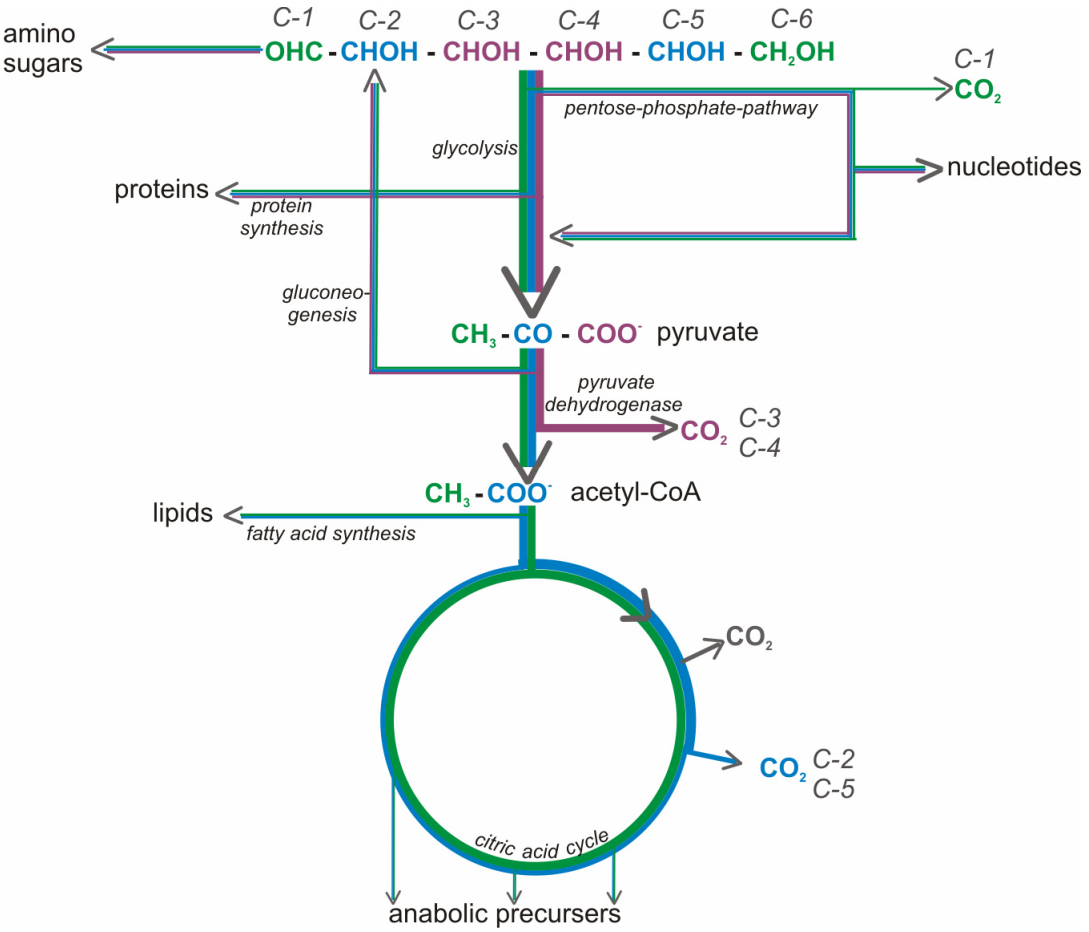

Fig. 4 Metabolic pathways of amino sugar formation from glucose precursor. Colours represent the opposite positions of glucose: green = C-1 and C-6, blue = C-2 and C-5 and purple = C-3 and C-4.

Nevertheless, a maximum of 40 to 55% of untransformed, intact glucose was used as a direct building block for cell wall (re)synthesis. This reflects that: 1) glucose is a perfect substrate for many pathways and is distributed over catabolism and anabolism, and 2) metabolic pathways cannot be considered as one-way; there is always the parallel

existence of catabolic, oxidizing pathways and anabolic, constructing pathways. This is in accordance with previous results for microbial carbohydrate synthesis (Derrien et al., 2007): they showed that the formation of microbial sugars from glycine occurred in parallel to direct formation from glucose and in parallel to glucose oxidation.

The direct pathways for glucose oxidation and new formation would be glycolysis and gluconeogenesis (Caspi et al., 2008; Keseler et al., 2009). The lowest incorporation of C-4 into amino sugars corresponds with the parallel existence of these pathways, as glycolysis with subsequent pyruvate-dehydrogenase oxidation would lead to a preferential oxidation of C-3 and C-4 to CO_2 (Scandellari et al., 2009) (Table 2). If C-1-C-2 or C-5-C-6 acetyl-CoA or metabolites of citric acid cycle are redistributed via gluconeogenesis into the glucose pool, the observed lack of C-4 incorporation could be explained. However, if these were the only pathways, symmetry in the incorporation of glucose C positions would be observed: C-1 ~ C-6, C-2 ~ C-5 and C-3 ~ C-4 (Scandellari et al., 2009) (Table2). We can only directly compare C-1 and C-6, as C-3- and C-5-labeled isotopomers were not commercially available. As the incorporation of C-1 and C-6 is not equal, this directly shows that further pathways are involved in the formation of glucose precursors for cell wall synthesis. There are two further main monosaccharide metabolizing pathways, which have been described for microorganisms that are common in soils, and can explain this result: the pentose-phosphate pathway and oxidative glucose degradation (with Entner-Douderoff pathway) (Caspi et al., 2008; Keseler et al., 2009). The first leads to a loss of C-1 position in the first, immediate step transferring the hexose glucose into the pentose ribulose (Scandellari et al., 2009). The remaining C backbone, if transformed back to pyruvate, would show the preferential oxidation of C-4 and lower oxidation of C-2 and C-3. In contrast, the oxidative pathway, occurring extracellularly in gram-negative bacteria, has no direct decarboxylation, and leads to metabolites which preferentially miss C-1 and C-4 (Caspi et al., 2008; Keseler et al., 2009). The decrease in C-1 for each of the amino sugars from day 3 to day 10 strongly supports the interpretation of a high incorporation of pentose-phosphate pathway products. This is characteristic for maintenance conditions as the pentose-phosphate pathway provides precursors for many anabolic pathways, like ribonucleotide or amino acid synthesis. Therefore, a high flux of glucose C into this pathway is in accordance with the expected metabolic state of the microbial community.

To summarize, even if simple direct pathways are considered, and labeling occurs with direct precursors of these pathways, the intact incorporation of this precursor molecule cannot be postulated. In contrast, the new formation of amino sugars via direct pathways, oxidation via catabolism and formation from metabolites of basic C metabolism

occur in parallel. Consequently, if uniform labeling is used to assess the turnover of a pool, non-equal incorporation of the glucose C positions has to be considered.

Table 2 Theoretic C pattern of newly formed amino sugars after simple pathway combinations of basic C metabolism

Glucose utilizing pathway	Intermediate	Glucose forming pathway	Relative incorporation of glucose positions			
			C-1	C-2	C-4	C-6
intact utilization for amino sugar formation	-	-	1	1	1	1
glycolysis	acetyl-CoA	gluconeogenesis	1	1	0	1
pentose-phosphate-pathway	glycerine-alde-hyd-3-phosphat	gluconeogenesis	0	1	1	1

2.7.4.3 Specific pathways of fungi and bacteria

Incorporation of C from individual molecule positions in galactosamine and glucosamine reflected a higher portion of intact glucose in the fungal biomarker galactosamine compared to the more bacteria-dominated newly formed glucosamine (figure 1). This indicates that the glucose splitting pathways like glycolysis as well re-synthesizing pathways like gluconeogenesis (i.e. catabolic and anabolic pathways, respectively) are more intensive in bacteria than in fungi. If the replacement of existing amino sugar pools is compared (figure 3), the higher turnover of the peptidoglycan, e.g. of bacterial cell walls compared to fungal cell walls, also becomes obvious. This can be a result of the shorter generation time (bacteria 20 min versus fungi 4-8 h to complete a life cycle under optimal conditions) and consequently higher cellular turnover of bacterial cells in soil (bacteria 2-3 times and fungi 0.75 times biomass turnover per year) (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). It is known that bacteria recycle up to 60% of their peptidoglycans during cellular life (Park and Uehara, 2008; Uehara and Park, 2008). These observations in pure cultures are in accordance with the high turnover of bacterial muramic acid observed in this experiment. To our knowledge there are no similar studies about intracellular chitin turnover of fungal cells. However, it is known that a wide spectra of taxa possess the enzymes needed for chitin degradation (Caspi et al., 2008; Merzendorfer, 2011) and that chitin has a rapid turnover in soils (Fernandez and Koide, 2012; Keiluweit et al., 2013). Our results suggest that the turnover of bacterial peptidoglycan is significantly faster than of fungal chitin in soils, but nevertheless a certain monomer exchange and turnover takes place during the short-term dynamics of chitin.

The position-specific labeling enables a more detailed look at the metabolic pathway of precursor formation used for cell wall turnover: whereas fungal galactosamine shows only slight changes in the relative incorporation of positions from day 3 to day 10,

the bacterial muramic acid and glucosamine showed an increase in C-2 and C-4 and a significant decrease in C-1 incorporation. This shows 1) that the bacterial peptidoglycan is more dynamic than fungal chitin and that renewing of that pool is higher, and 2) that the pentose-phosphate pathway, which shows active maintenance metabolism, seems to be more active in bacteria than in fungi. In addition, further C-1 oxidizing pathways, like the gram-negative, periplasmatic oxidative glucose oxidation may enhance the observed low C-1 oxidation in bacterial amino sugars. The contribution of this pathway can only be confirmed by investigating specific, anabolic products of gram-negative bacteria like PLFAs. However, this study took place under microbial maintenance conditions, and clearly reflected that individual members of the microbial community use specific pathways even in anabolic maintenance metabolism. At least for highly polymeric cell walls, the turnover of bacterial biomass seems to be higher and maintenance metabolism more active for bacteria.

2.7.5 Conclusions and Outlook

This is the first study which has coupled position-specific labeling with compound-specific ^{13}C analysis of amino sugars in soils and thus could observe the *in-situ* formation of amino sugars from its precursor glucose. Comparison of galactosamine with glucosamine and muramic acid confirmed former conclusions that galactosamine is the better fungal biomarker than glucosamine. Glucosamine, at least the active, newly formed pool, is derived from both fungal chitin and bacterial peptidoglycan. If bacteria are dominating in microbial turnover, as in agricultural soil with neutral pH which was investigated here, the main contributor to newly formed glucosamine appears to be bacterial cell wall formation.

Although glucose is a direct precursor for amino sugar synthesis, it is not directly used for amino sugar formation; however, up to 55% of the incorporated ^{13}C is derived from glucose metabolites. This reflects that glucose, as the preferred substrate for many catabolic and anabolic pathways, is spread over microbial metabolism and that oxidizing, catabolic pathways occur in parallel to constructing, anabolic pathways. Consequently, if uniform labelling is used to assess the turnover of a pool, a non-equal incorporation of the glucose-C positions has to be considered. However, this intensive metabolism and re-incorporation of metabolites enables metabolic tracing based on glucose for almost every biosynthetic pathway.

In addition, this study proved the lower metabolic activity of fungi versus bacteria in maintenance metabolism. This is not only proven by the higher dynamics of ^{13}C replacement of the cell walls, but also by the strong variation in the incorporation of individual C

positions over a period of 10 days. This reflects a more active maintenance metabolism based on glycolysis, pentose-phosphate pathway and gluconeogenesis in bacteria, which leads to an increasing incorporation of glucose metabolism fragments within 10 days. Therefore, this study proved for the first time that not only the C turnover in the slow and fast cycling branches of soil food webs differ, but also that the processes transforming C within these branches are strongly different and lead to specific C partitioning over various SOC pools.

Therefore, a more detailed understanding of fluxes of LMWOS C through the microbial network is needed. Systematic studies based on position-specific labeling and compound-specific isotope measurements of various microbial metabolites are needed to reveal which pathways are driven under which soil conditions. This knowledge will enable us to obtain a mechanistic understanding of SOC fluxes.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for funding (KU1184/19-1 and KU1184/13-2), Katharina Leiber-Sauheitl, Janine Sommer and Ilse Thaufelder for supporting the field work and Carolin Apostel for providing PLFA data and her helpful comments on the manuscript.

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Supplementary Data

Supplementary Table A1: Solvent gradients and flow conditions of the IC-O-IRMS measurment

time	20 mM NaOH	200 mM Na-OH	H_2O	0.01 M $NaNO_3$	flow (ml min^{-1})
-25 min	0%	100%	0%	0%	0.400
-10 min	8%	0%	92%	0%	0.325
11 min	40%	0%	35%	25%	0.400
15 min	45%	0%	45%	10%	0.400
18 min	25%	25%	50%	0%	0.380
35 min			standby		

2.8 Study 8: Formation and transformation of fatty acids in soil assessed by position-specific labeling of precursors

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Abstract

Fatty acids are frequently used as plant and microbial biomarkers to trace the pathways of C stabilization and soil organic matter (SOM) formation. Whereas microbial and plant fatty acid fingerprints are well investigated, their transformations in soils remain unclear. However, knowledge of the transformation pathways in soils is crucial for the interpretation of fatty acid fingerprints, especially because the formation and decomposition processes are simultaneously ongoing. Therefore, we analyzed the formation of microbial fatty acids from their precursor acetate and the transformation of palmitate in soil by coupling position-specific ^{13}C labeling with compound-specific ^{13}C analysis.

Position-specifically and uniformly ^{13}C-labeled acetate and palmitate were applied in an agricultural Luvisol. Pathways of fatty acids were traced by analyzing microbial utilization of C from individual molecule positions of acetate and palmitate and their incorporation into phospholipid fatty acids (PLFAs).

Acetate ^{13}C incorporation into microorganisms and that remaining in the soil were characterized by basic microbial metabolism: C-1 is preferentially oxidized to CO_2 in the citric acid cycle, whereas C-2 is preferentially incorporated into microbial compounds. If palmitate was used in basic C metabolism, it was split into C2-units (acetyl-CoA), and odd and even positions of palmitate were transformed in a manner similar to acetate. However, as palmitate is the preferred precursor for PLFA formation, more than 6% of the added palmitate was incorporated into microbial cell membranes. Newly formed fatty acids were first, on day 3, dominated by basic, straight chain fatty acids. With increasing time, the pattern of newly formed PLFA approached the fingerprint of the microbial community. Therefore, the C backbone of palmitate was not split, but modified (e.g. desaturated, elongated or branched) according to the fatty acid demand of the soil microbial community. If acetate ^{13}C was used for PLFA formation, the construction of new C backbones of fatty acids rarely occurred. However, acetate ^{13}C was incorporated into microbial PLFAs by elongations or branchings of already existing fatty acids. Therefore, the previous assumption, that fatty acids are generally newly formed from the added substrates has to be discarded and future PLFA studies have to consider the reuse of existing plant and microbial-derived fatty acids.

Discrimination of acetate positions by PLFA formation was lowest in the microbial groups with the highest competitiveness for acetate uptake. In contrast, palmitate uptake and transformations were highly specific for the individual microbial groups in soil. For both substrates, it could be concluded that more direct, less complex metabolic pathways are characteristic of fast-growing microbial groups with high turnover.

This study proves the fast microbial turnover of the free fatty acid pool in soils, as well as the high turnover and transformation of cellular PLFAs. Knowledge about these microbial transformations of fatty acids in soils is crucial for the interpretations of microbial as well as plant-derived fatty acid fingerprints. Furthermore, tracing the formation and transformation of lipids in soils improves our understanding of C fluxes and the stabilization of microbial as well as plant-derived lipids in soils.

Keywords: position-specific tracers, metabolic tracing, biomarker approaches, fatty acid formation and transformation, phospholipids, lipid stabilization, paleoenvironmental reconstructions

2.8.1 Introduction

Soil organic matter (SOM) is the largest active carbon (C) pool (1462-1548 Pg, (Batjes, 1996)) within the global carbon (C) cycle, but the genesis and transformation processes are poorly understood. The main input of C into soils occurs via plant litter or rhizodeposition (Rasse et al., 2005). Litter is composed of macromolecules such as cellulose, hemicellulose, lignin or proteins (Crawford et al., 1977; Sorensen, 1975); in addition, rhizodeposits contain low molecular weight organic substances (LMWOS) (Farrar et al., 2003). Along with water soluble low molecular weight organic compounds and their polymers, lipids are important constituents of plant biomass. They comprise around 3-10% of aboveground and 0.5-5% of belowground plant biomass and are thus an essential compound of plant C input into soils (Bliss, 1962; Ohlrogge and Browse, 1995; Wiesenberg et al., 2004). In addition, microbial biomass contains around 10% of lipids, mainly in their cell membranes and cell walls (Lengeler et al., 1999; Zelles et al., 1995) and significantly contribute to the lipidic SOM pool.

Lipids comprise a higher percentage of SOM (Almendros et al., 1991; Rumpel et al., 2004) than their source material i.e. plant and microbial biomass. This accumulation of lipids already indicates their selective preservation in soils (Lichtfouse et al., 1998a). Moreover, lipids are assumed to play an even more important role in SOM formation and stabilization: lipids are stabilized by hydrophobic interactions with SOM and with each other, leading to a decreased wettability and subsequent hampering microbial decomposition (Lichtfouse et al., 1998a; von Luetzow et al., 2006). In addition, functional groups of lipids can covalently bind further molecules (Allard, 2006; Berthier et al., 2000) or encapsulate smaller molecules, leading to their preservation (Lichtfouse et al., 1998b; Piccolo, 2002; Sutton and Sposito, 2005).

The long-term preservation of some lipid classes qualifies them as important biomarkers (Otto et al., 2005; White et al., 1997). N-alkanes are commonly assumed to be plant-derived (Kuhn et al., 2010; Lichtfouse, 1998) and are used as plant biomarkers to differentiate vegetation types (Bush and McInerney, accepted 2013; Schwark et al., 2002; Zech et al., 2012); long-chain fatty acids are used in a similar way (Wiesenberg and Schwark, 2006). More complex cutin-suberin-derived hydroxylated or poly-carboxylic acids enable aboveground litter input to be differentiated from belowground litter input (Mendez-Millan et al., 2011; Spielvogel et al., 2010). Sterols and other isoprenoid lipids, like terpenoids, are indicative for animal or plant-derived SOM (Otto and Wilde, 2001).

Application of all these biomarkers tacitly assumes that they remain unmodified in soils over long periods. However, microorganisms are able to use lipids as substrates and decompose them to metabolites as well as build up their own lipids, therefore signifi-

cantly contributing to the lipid pool of SOM and the modification of initial lipidic compounds (Lichtfouse et al., 1995; Otto et al., 2005). This indicates already that the concept of generally untransformed lipid biomarkers is likely to be too simplified. Recent studies indicate that plant-derived biomarkers can be modified and overprinted by rhizomicrobial activity (Gocke et al., in press). For some biomarker classes like sterols microbial modifications of plant or animal-derived lipids are specifically used to trace the microbial community impact (Arima et al., 1969; Bull et al., 2002; Bull et al., 1999). For others, like alkanes, approaches used to correct for microbial overprint of plant-derived signals have been developed (Buggle et al., 2010; Zech et al., 2013). However, current knowledge on the microbial transformation of lipids in soils is rare and new experimental studies are needed.

^{14}C age of microbial lipid biomarkers revealed that they are not indicative for the age of this substance class, as microorganisms obviously use old lipidic substrates to build up their new lipids, e.g. phospholipid fatty acids (PLFA) (Rethemeyer et al., 2004). However, it is not yet clear whether microorganisms prefer the new synthesis of their lipids from low molecular weight precursors like acetate or whether they prefer to use available lipidic compounds, e.g. fatty acids, and simply modify them. According to the biochemical principle of the most economic pathways, cells tend to use preformed building blocks for biomass synthesis (Lengeler et al., 1999). Consequently, we hypothesize that lipids released by the decomposition of plant or microbial biomass should be the preferred substrates for further lipid synthesis by microorganisms. However, it is known that microbial polar lipids, like PLFA, are degraded to fatty acids after cell death (Lichtfouse et al., 1995) and, consequently, re-contribute to the free lipid pool in soils. The knowledge gap concerning the soil lipid cycle has caused an intensive discussion concerning the contribution of plant versus microbial lipids (Lichtfouse, 1998; Lichtfouse et al., 1995; Otto et al., 2005). Therefore, microbial modification of lipid biomarkers in soil, as well the transformation processes, are crucial for the application of biomarker fingerprints as well as isotope data from lipid biomarkers.

Therefore, we traced the microbial formation of membrane lipids, PLFA, from their low molecular weight organic precursor – the acetate (Caspi et al., 2008; Keseler et al., 2009; Rock et al., 1981). In addition, we traced the utilization of the most abundant fatty acid – palmitic acid – as a microbial substrate for PLFA. Palmitic acid is a key compound for plant and microbial fatty acid metabolism. Investigating its microbial utilization and transformation pathways reveals a general view of the microbial modification of soil lipids.

To elucidate the metabolic pathways of microbial fatty acids, we used the approach of position-specific labeling. This tool was originally derived from biochemistry to investigate metabolism pathways and has rarely been applied in soil science (Fokin et al., 1993,

, 1994; Haider and Martin, 1975; Kuzyakov, 1997; Nasholm et al., 2001). However, within the last years, increasing interest in the use of position-specific labeling to assess metabolic pathways in soil has arisen (Apostel et al., 2013; Dijkstra et al., 2011a; Dijkstra et al., 2011b; Dippold and Kuzyakov, 2013; Fischer and Kuzyakov, 2010b). This is because this approach is the only one which enables the fate of individual C positions to be traced through various pools or metabolites and consequently allows the reconstruction of individual transformation steps.

Knowledge about fatty acid synthesis by microorganisms is mainly derived from experiments with pure cultures (Lennarz, 1970; Rock et al., 1981; Zelles et al., 1995). They can be newly synthesized from precursors like acetate or available lipid precursors can be modified by them (Lennarz, 1970; Rethemeyer et al., 2004; Rhead et al., 1971). If palmitate is given as a substrate, there are three possible mechanisms by which palmitate C can be used for PLFA synthesis: 1) the resynthesis pathway i.e. the complete degradation of the molecule to acetyl-CoA units and the following reconstruction of new fatty acids from C2-mojieties (Rhead et al., 1971); 2) Partial step-by-step degradation of the C2-units without total breakdown of palmitate: subsequently, only parts of the molecule are incorporated into newly formed fatty acids (Rhead et al., 1971); and 3) the untransformed utilization of palmitate as it is the most abundant fatty acid in microorganisms (Rhead et al., 1971; Zelles et al., 1995). Position-specific ^{13}C labeling enables these three pathways to be distinguished and to evaluate the transformation of the straight chain, unsaturated palmitate. This approach will deepen the understanding of the transformation of fatty acids and other lipidic biomarkers and improve the interpretation of fatty acid fingerprints in soils.

2.8.2 Material and Methods

2.8.2.1 Experimental Site

The field experiment is located in Bavaria, close to Hohenpölz (49.907 N, 11.152 E) with 501 m.a.s.l, mean annual temperature 6.7 °C and mean annual precipitation of 874 mm. The agriculturally used field site is managed by a rotation of corn, barley, wheat and triticale. Soil type is a loamy Luvisol which has a pH_{KCl} of 4.88, a pH_{H_2O} of 6.49, a TOC content of 1.77%, a TN content of 0.19% and a CEC of 13 $cmol_C\ kg^{-1}$. Before the experiment started in August 2010 triticale, the last crop, was harvested, and the field site was grubbed for soil homogenization.

2.8.2.2 Experiment Design

The 12 × 12 m field was subdivided into four quadrants. PVC-tubes (diameter: 10 cm; height: 13 cm) were installed 10 cm deep in the soil, resulting in a soil sample weight between 1 and 1.5 kg for each column. Column location was randomized within the blocks and each of the four blocks represented one of the four repetitions of each treatment. Consequently, the block could be included as a random variable in statistical evaluation to account for the spatial heterogeneity within the field site.

Tracer-solution was applied with a multipette (Eppendorf, Hamburg, Germany) at 5 injection points per column, each of 2 ml. Uniformly ^{13}C-labeled acetate and palmitate as well as position-specific labeled isotopomers (1-^{13}C acetate, 2-^{13}C acetate, 1-^{13}C palmitate, 2-^{13}C palmitate and 16-^{13}C palmitate) were applied. In addition, on non-labeled background columns, an identical amount of acetate and palmitate-C was applied. Concentrations of ^{13}C were 100 µmol acetate and 50 µmol palmitate per column. The amount of C applied was constant for each treatment and the backgrounds.

A 7-cm-long needle with closed tip and peripheral holes allowed homogeneous lateral distribution of the tracer solution. Leaching was avoided by injecting the solution only into the upper 2/3 of the column and excluding rainfall by installing a roof above the plots of the experiment.

2.8.2.3 Sampling and Sample Preparation

The sampling occurred by harvesting the entire soil column three and ten days after labeling. At both times, height of the soil inside the column was measured to determine the labeled soil volume. Then, soil was pressed out from the column, fresh weight was determined and the entire soil sample was homogenized manually. Afterwards, a subsample was taken to determine water content and the sample was split: one subsample was freeze-dried and ball milled for bulk isotope analysis, and another subsample was 2 mm sieved and stored at <5 °C for chloroform-fumigation-extraction (CFE). The remaining soil was sieved to 2 mm and stored frozen for analysis of microbial phospholipid fatty acids.

2.8.2.4 Bulk Soil and Microbial Biomass Analysis

For the analysis of bulk soil C content and $\delta^{13}C$-values, the samples were freeze-dried, ground in a ball mill and 5-6 mg per sample were filled into tin capsules. The samples were measured on the Euro EA Elemental Analyzer (Eurovector, Milan, Italy) coupled with a ConFlo III interface (Thermo Fisher, Bremen, Germany) and the Delta V Ad-

vantage IRMS (Thermo Fisher, Bremen, Germany). Incorporation of ^{13}C from the applied carboxylic acids into soil was calculated according to the mixing model (Eq. 1 and 2), where the C content of the background in Eq. 1 was substituted according to Eq. 2 (Gearing et al., 1991).

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C-BG} + [C]_{appCA} \cdot r_{appCA} \quad (9)$$

$$[C]_{soil} = [C]_{BG} + [C]_{appCA} \quad (10)$$

with: $[C]_{soil/BG/appCA}$ C content of sample / background / applied carboxylic acids (mol · g_{soil}^{-1})

$r_{soil/BG/appCA}$ ^{13}C atom%-excess of sample / background / applied carboxylic acid (at%)

Two subsets of 15 g of soil were taken to determine microbial C and its $\delta^{13}C$ values. One subsample was directly extracted, whereas the other was first fumigated with chloroform for 5 days in an exsiccator to lyse microbial cells. The samples were extracted with 45 ml of 0.05 M K_2SO_4 by shaking on a horizontal shaker for 1.5 h: after shaking, the samples were centrifuged (10 min, 2000 rpm) and the supernatant was filtered (Rotilab® round filters, type 15A, cellulose, membrane 70 mm).

The C content of the extracts was determined on a TOC analyser multi C/N® 2000 (Analytik Jena, Jena, Germany). After that, the remaining extracts were freeze-dried for $\delta^{13}C$ measurements. 30-35 µg of the freeze-dried salt were transferred to tin vessels and measured on the same instrument where bulk soil $\delta^{13}C$-value was determined. ^{13}C incorporation into fumigated and unfumigated samples was calculated according to the mixing model (Eq. 1 and 2). Microbial biomass and carboxylic acid ^{13}C incorporated into microbial biomass was calculated according to Wu et al (1990) with an extraction factor of 0.45.

2.8.2.5 PLFA $\delta^{13}C$ analysis

Phospholipids were extracted and purified by a modified method of Frostegard et al. (1991) which are described in detail in Gunina et al. (submitted). Modifications included the use of 6 g of soil for extraction, a doubled liquid-liquid extraction and a very slow elution of polar lipids from the activated silica column with four times 5 ml methanol. Before extraction, 25 µl of a 1 M solution of phosphatidylcholine-dinonadecanoic acid was added as internal standard 1 (IS 1). For measurements on a GC, the fatty acids were saponified to free fatty acids and derivatized into fatty acid methyl esters (FAME) following the description by Knapp (1979). Before transferring the samples to autosampler vials, 15 µl of tridecanoic acid methyl ester (1 µg μl^{-1} in toluene) was added as internal

standard 2 (IS 2). External standards consisting of the 27 fatty acids listed in Supplementary Table 1 together with the phospholipid IS 1 were prepared with fatty acid contents of 1, 4.5, 9, 18, 24 and 30 µg, respectively, and derivatized and measured together with each sample batch.

FAME-contents were measured on a GC-MS (GC 5890 with MS 5971A, Agilent, Waldbronn, Germany) with a 30 m DB1-MS column, in the selected ion mode. The relation between the area of each FAMEs and the area of the IS 2 was calculated and a linear regression based on the six external standards was used for quantification. The recovery of each sample was determined based on the area of the initially added 25 µg of IS 1, and the amount of each fatty acid was corrected by the recovery.

$\delta^{13}C$-values were analyzed on a GC-C-IRMS; consisting of the autosampler unit AS 2000, the Trace GC 2000 by ThermoQuest, the Combustion Interface III combustion unit and the isotope-ratio mass spectrometer Delta^{Plus} (Thermo Fisher, Bremen, Germany). Volumes of 1.5 µl were injected in splitless mode (splitless time: 1 min) into a liner (Type TQ(CE) 3 mm ID TAPER) at a temperature of 250 °C. Gas chromatography was accomplished with a combination of two capillary columns: a 30 m DB5-MS and a 15 m DB1-MS (both: internal diameter 0.25 mm, film thickness 0.25 µm; Agilent); a constant He-flux (99.996% pure) of 2 ml · min^{-1} and the temperature program presented in Supplementary Table 2. CO_2 reference gas (99.995% pure) was injected for 20 s into the detector four times throughout the measurement to identify any detection drift. The $\delta^{13}C$-value of the second reference gas peak was defined as -40‰ and all other $\delta^{13}C$-values were calculated by comparison. $\delta^{13}C$-values of all PLFA samples was measured four times.

The chromatograms were integrated and $\delta^{13}C$-value was generated by the software ISODAT NT 2.0.

Linear regressions were calculated from reference gas peaks surrounding the fatty acid peaks For drift correction within the chromatogram (Apostel et al., 2013) and chromatographic drift was corrected according to the slope of this regression.

To correct for amount-dependent ^{13}C isotopic fractionation during measurements (Schmitt et al., 2003) and for the addition of C during derivatization, linear and logarithmic regressions of the external standard $\delta^{13}C$-values to their area were calculated. If both regressions were significant, that with the higher significance was applied. As the $\delta^{13}C$-value for the derivatizating agents was unknown, the correction was performed according to Glaser and Amelung (2002a) (Eq. 5).

$$C_{FS}(at\%) = \frac{N(C)_{FAME}}{N(C)_{FS}} \cdot (C_{FAME-DK}(at\%) - (m_{lin/\ln} \cdot A_{FAME} + t_{lin/\ln})) + C_{EA-FS}(at\%) \quad (5)$$

with:	C_{FS}(at%)	corrected ^{13}C amount of the fatty acid	[at%]
	C_{FAME}(at%)	drift-corrected ^{13}C amount of the FAME	[at%]
	$m_{lin/ln}$	slope of linear/logarithmic regression	[at% · Vs^{-1}]
	$t_{lin/ln}$	y-intercept of linear/logarithmic regression	[at%]
	A_{FAME}	area of FAME	[Vs]
	$N(C)_{FAME}$	number of C atoms in FAME	
	$N(C)_{FS}$	number of C atoms in fatty acid	
	C_{EA-FS}(at%)	measured ^{13}C-value of fatty acid	[at%]

2.8.2.6 Fatty acid grouping

For the evaluation of biochemical transformations, fatty acids were grouped into biochemical classes. The 16 carbon chain palmitic acid was taken as a border case as this is the key fatty acid in bacterial metabolism. 16 carbon fatty acids, shorter fatty acids (14 and 15 carbon) and longer fatty acids (17 to 20 carbons) were grouped into straight chain even, straight chain odd, desaturated and branched fatty acids.

For the evaluating of the microbial groups, the fatty acids fingerprint of all samples was grouped by a principal component analysis with varimax standard rotation. Fatty acids with a loading of more than 0.5 (absolute value) on the same factor were categorized in one group if in accordance with previous studies on pure cultures (Zelles, 1999; Zelles et al., 1995).

2.8.2.7 The Divergence Index DI_i

According to Dippold and Kuzyakov (2013) the transformation of C from individual molecule positions was presented by the Divergence Index DI_i:

$$DI_i = \frac{n \cdot [C - i]}{\sum_{i=1}^{i=n} [C - i]} = \frac{2 \cdot [Ac - C - i]}{\sum_{1}^{2} [Ac - C - i]} \quad \text{equation 4}$$

This index reflects the fate of individual C atoms from the position i relative to the mean transformation of the n total number of C atoms within a transformation process. Hence, a DI_i of 1 means that the transformation of this C position in the investigated pool corresponds to the transformation of uniformly labeled substance (average of all C atoms). DI_i ranges from 0 to n, and values between 0 and 1 reflect lower incorporation of

the C into the investigated pool, whereas values between 1 and n show higher incorporation of the C atom into this pool as compared to the average.

2.8.2.8 Statistics

The values presented show mean ± standard error of mean (± SEM) of the four field repetitions of each sample. The SEM of the divergence index was calculated by gaussian error propatation. Measured values were tested for normal distribution using the Kolmogorov Smirnoff test, for homogeneous variances using Levene's test and screened for outliers using the Nalimov test (Gottwald, 2000). Factorial ANOVA was calculated using Statistica (version 6.0, Statsoft GmbH, Hamburg, Germany). If assumptions such as normal distribution or homogeneous variances were not met, the result of the ANOVA was confirmed by non-parametric Kruskal-Wallis ANOVA before performing a Tukey HSD post-hoc test for unequal sample size.

2.8.3 Results

2.8.3.1 Incorporation of ^{13}C in soil and microbial biomass

Decomposition of acetate and palmitate until day 3 was similar: 60-70% of the added ^{13}C remained in soil (Figure 1, pools see Table 1). However, at day 10, palmitate and acetate behaved very different: around 80% of ^{13}C acetate was decomposed to CO_2, but this was true for only 50% of the palmitate. Position-specific pattern reflects that the C-1 position of both carboxylic acids is preferentially oxidized. However, the preferential C-1 oxidation was the most pronounced for acetate at day 3 and for palmitate at day 10. Whereas both acetate positions were continuously decomposed from day 3 to day 10, the ^{13}C loss from palmitate between day 3 and day 10 could mainly be attributed to an oxidation of the one terminal position of palmitate only.

Table 1 Total organic C (TOC), microbial biomass C (C_{mic}) and the sum of all measured PLFAs (list of fatty acids see Supplementary, Table A1) in mg C per g soil (dry weight)

		TOC	C_{mic}	Σ_{PLFA}
Pool Size (mg C g^{-1} soil)	day 3	15.60 ± 0.60	0.521 ± 0.022	0.064 ± 0.005
	day 10	16.39 ± 0.30	0.463 ± 0.038	0.051 ± 0.003

Incorporation of ^{13}C into microbial biomass reflected a similar fate of acetate and palmitate positions and the preferential oxidation of the terminal carboxylic groups. In general, ^{13}C incorporation of acetate and palmitate into microbial biomass was similar.

However, a clear preference for the ^{13}C incorporation from palmitate compared to acetate was found for the PLFAs, i.e. the pool of polar microbial membrane lipids. The strong decrease of ^{13}C content from acetate and palmitate from day 3 to day 10 (Figure 1 and Figure 2) reflects the short half-life time of PLFAs in soils.

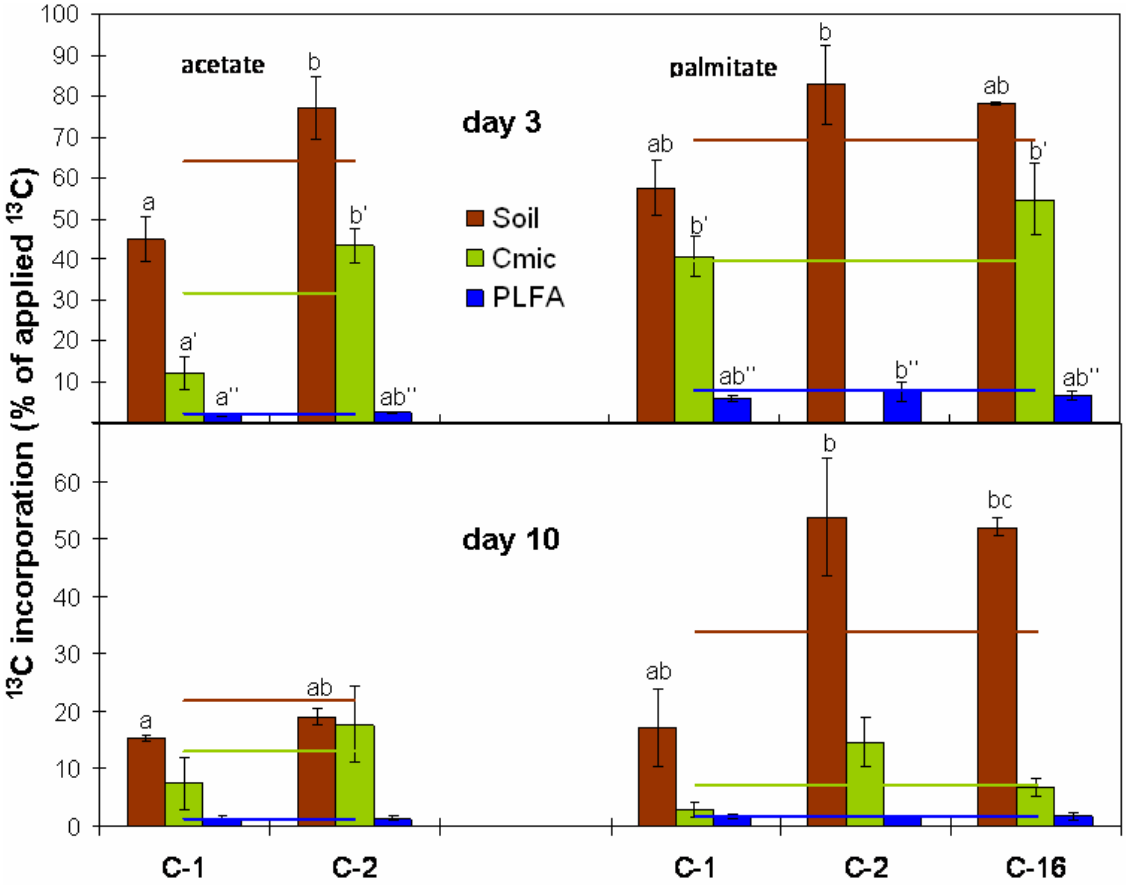

Fig. 1 Recovery of position-specifically ^{13}C-labeled acetate and palmitate in soil, microbial biomass and the sum of PLFA (Σ-PLFA) 3 (top) and 10 days (bottom) after substance application. Bars represent means ± SEM (N=4) for the individual positions and lines represent the mean of the uniformly labeled substances. Letters indicate significant differences ($p < 0.05$) between recovery in bulk soil (a), microbial biomass (a') and in Σ-PLFA (a").

2.8.3.2 Incorporation of C from various positions of acetate and palmitate into individual PLFAs

Acetate and palmitate were used for the formation of microbial PLFAs. This reveals the existence of both pathways: the new formation of fatty acids from acetate precursors as well as modification of existing fatty acids. In each case, the newly formed fatty acids were first, at day 3, dominated by basic, straight chain fatty acids (Figure 2). With increasing time, the newly formed fatty acids approached the fingerprint of the microbial community (Figure 2). Not only new synthesis but also the modification of existing PLFAs occurred according to the demand of the present microbial community (Figure 2).

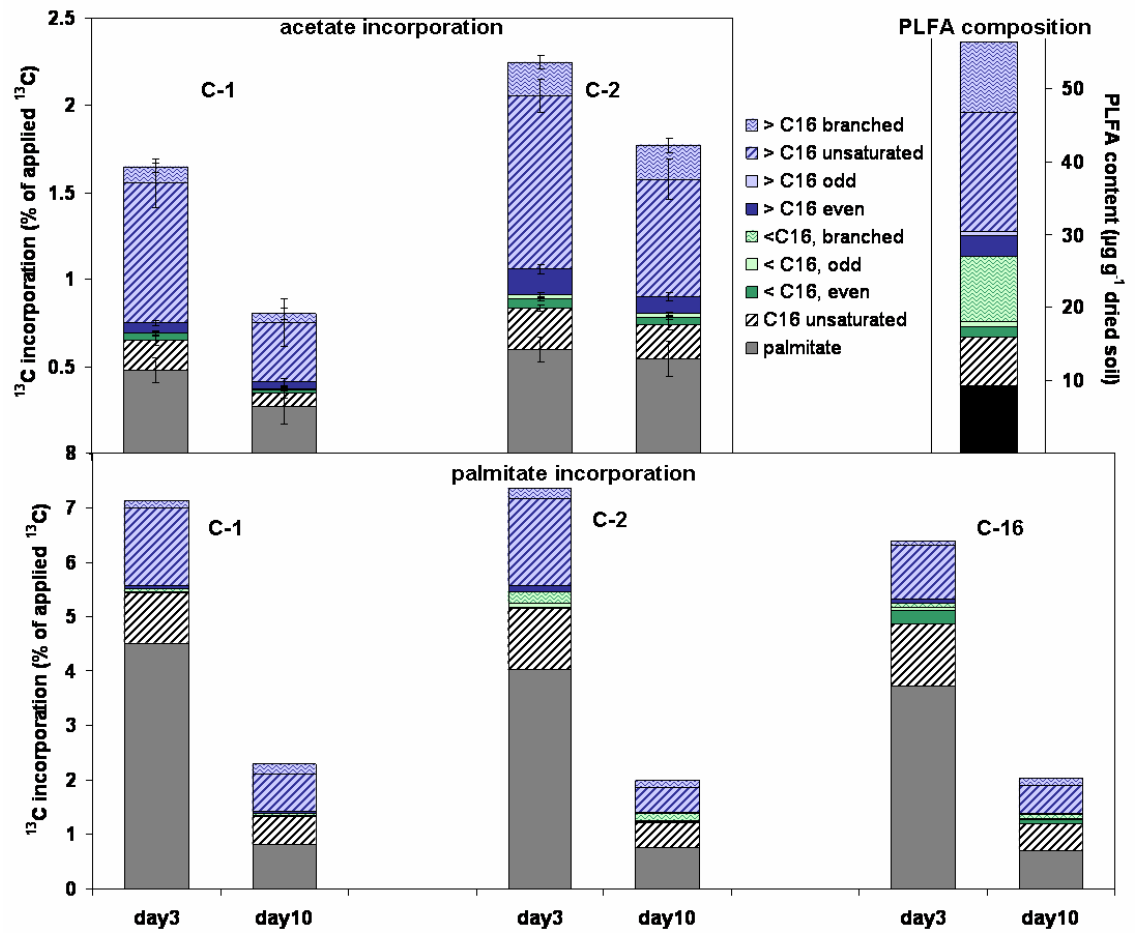

Fig. 2 Classes of phospholipid-derived fatty acids (top left) extracted from soil and recovery of position-specifically ^{13}C-labeled acetate (top right) and palmitate (bottom) in the different fatty acid classes 3 and 10 days after ^{13}C application. Experimental points (means ± SEM, N=4) are presented.

Figure 2 shows that acetate was an appropriate precursor for the new formation of fatty acids: e.g. at day 3, the pattern of newly formed fatty acids from acetate C-2 was already quite similar to the PLFA profile in soils (Figure 2). Only branched fatty acids smaller than 16 C atoms were not formed from acetate, not even from acetate C-2. In general, acetate C-1 incorporation into each fatty acid was lower than C-2, reflecting that not only intact acetate was used as a precursor in fatty acid synthesis. Especially in branched fatty acids, a preferential incorporation of C-2 was observed (Figure 2). The C-1 from acetate was not incorporated in any of the odd-numbered fatty acids (Figure 2).

Palmitate ^{13}C incorporation into phospholipid-bound palmitate is higher than acetate ^{13}C incorporation (Figure 2). C-1 of palmitate is incorporated only in negligible amounts into fatty acids which were shorter than 16 carbons (Figure 2 and Figure 3). In addition, the terminal C-1 position of palmitate was not incorporated into odd-numbered PLFAs. This indicates the preference to use palmitate as a direct precursor for microbial phospholipid synthesis.

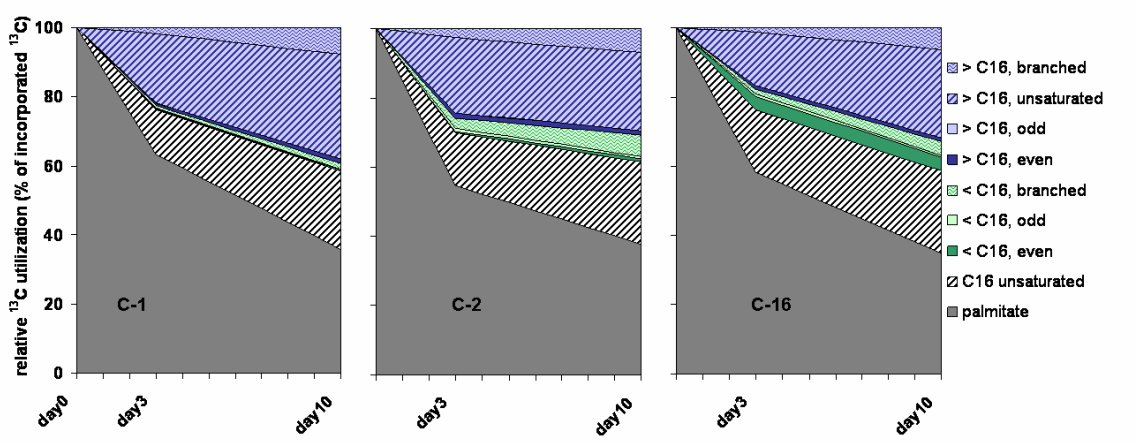

Fig. 3 Fingerprint of phospholipids-derived fatty acids in soil (top left) and relative recovery of individual positions from palmitate ^{13}C in fatty acid classes. Experimental points (means ± SEM, N=4) are presented.

Figure 3 presents the transformations of incorporated palmitate by microbial PLFA formation. The portion of desaturated fatty acids was already high at day 3 and did not increase significantly from day 3 to day 10 (Figure 3). This shows a fast desaturation of the palmitate precursor. However, incorporation of palmitate ^{13}C into elongated and even more into branched fatty acids significantly increased from day 3 to day 10 for each of the C positions, reflecting slower kinetics of these processes.

In general, a modification of palmitate according to the demand of the microbial community could be observed in this study. After 10 days the fingerprint of newly formed fatty acids already closely approached the PLFA distribution of the present microbial community. However, individual transformation steps occurred with different kinetics.

2.8.3.3 Incorporation of acetate and palmitate ^{13}C into PLFAs of individual microbial groups

Preference for acetate and palmitate strongly differed for individual microbial groups. The PCA, based on the amounts of fatty acids, revealed two groups of gram negatives: whereas gram-negative 1 (18:1w9c, 18:1w7c, 14:1w5c) showed the highest incorporation of acetate, gram-negatives 2 (16:1w7c, cy19:0) reflected the highest uptake of palmitate (Figure 4). In general, the low molecular weight acetate was a better substrate for prokaryotic groups than for eukaryotic fungi or protozoa. A similar pattern was not observed for palmitate, which was preferentially used by prokaryotic gram-negatives and eukaryotic fungi and protozoa (Figure 4). The amount of incorporated ^{13}C decreased from day 3 to day 10 for each of the microbial groups and both carboxylic acids (Figure 4, the only exception is incorporation of palmitate into actinomycetes).

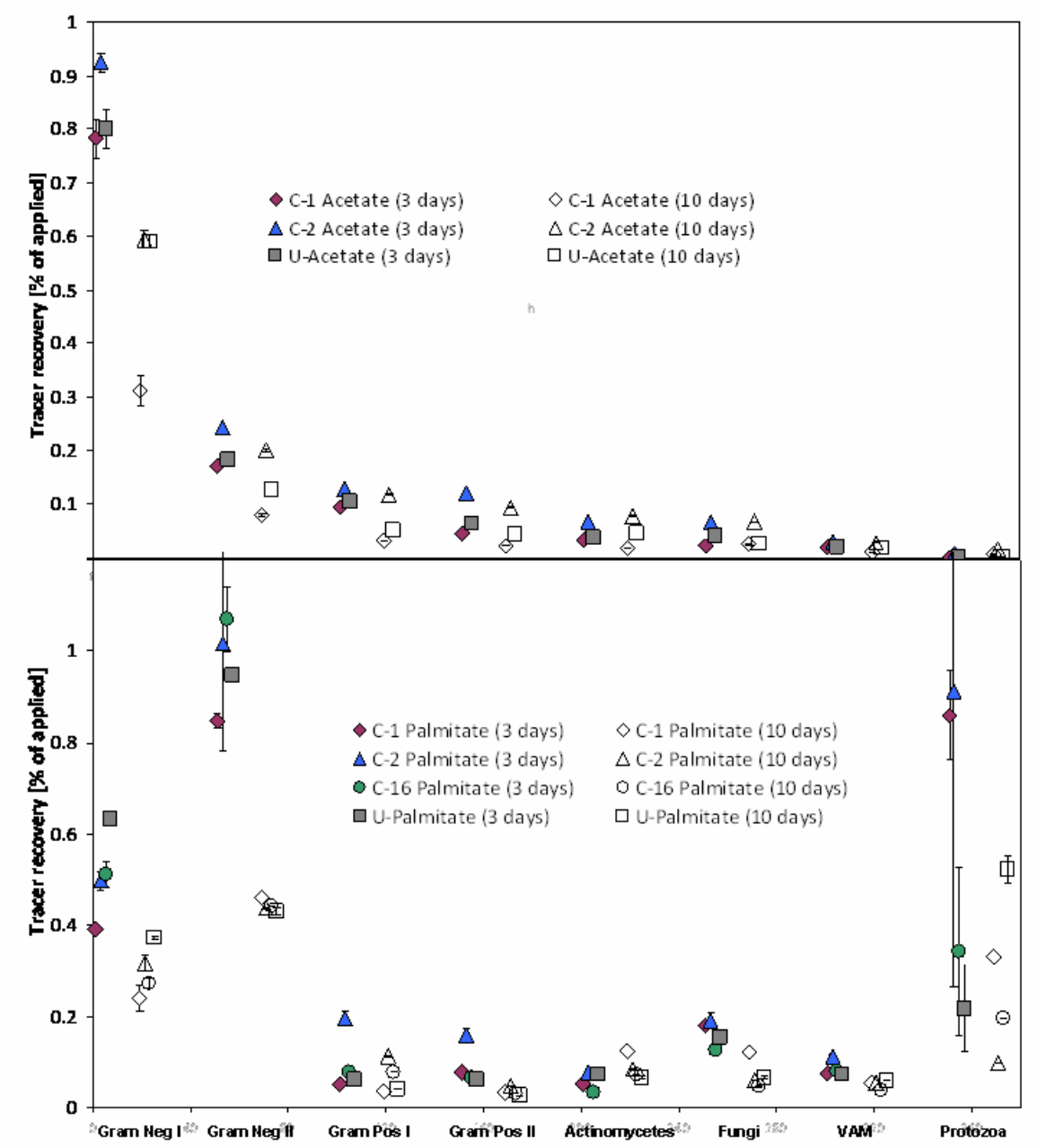

Fig. 4 Recovery of applied ^{13}C from positions of acetate (top) and palmitate (bottom) in microbial groups after 3 and 10 days. Experimental points (means ± SEM, N=4) are presented. Significant differences of incorporation of individual positions and incorporation between the days, calculated by nested ANOVA, are presented in Supplementary, Table A4

Specifics in the acetate and palmitate transformations are more visible if the divergence index (DI) is considered rather than the absolute ^{13}C incorporation (Figure 5). For acetate, each of the microbial groups showed the preferential incorporation of C-2. However, the discrimination between C-1 and C-2 was lowest for gram-negative groups (who had the highest absolute incorporation) and highest for eukaryotic groups (who had the lowest acetate ^{13}C incorporation) (Figure 5). The DI of palmitate did not show similar trends: each microbial group had individual preferences for incorporation of palmitate

positions into their PLFAs. Similar to acetate, the discrimination between positions was lowest for the two gram negative groups. For many microbial groups, the position-specific preferences and discrimination between positions strongly changed from day 3 to 10. This reflects an intensive turnover of palmitate ^{13}C, even if incorporated into PLFA.

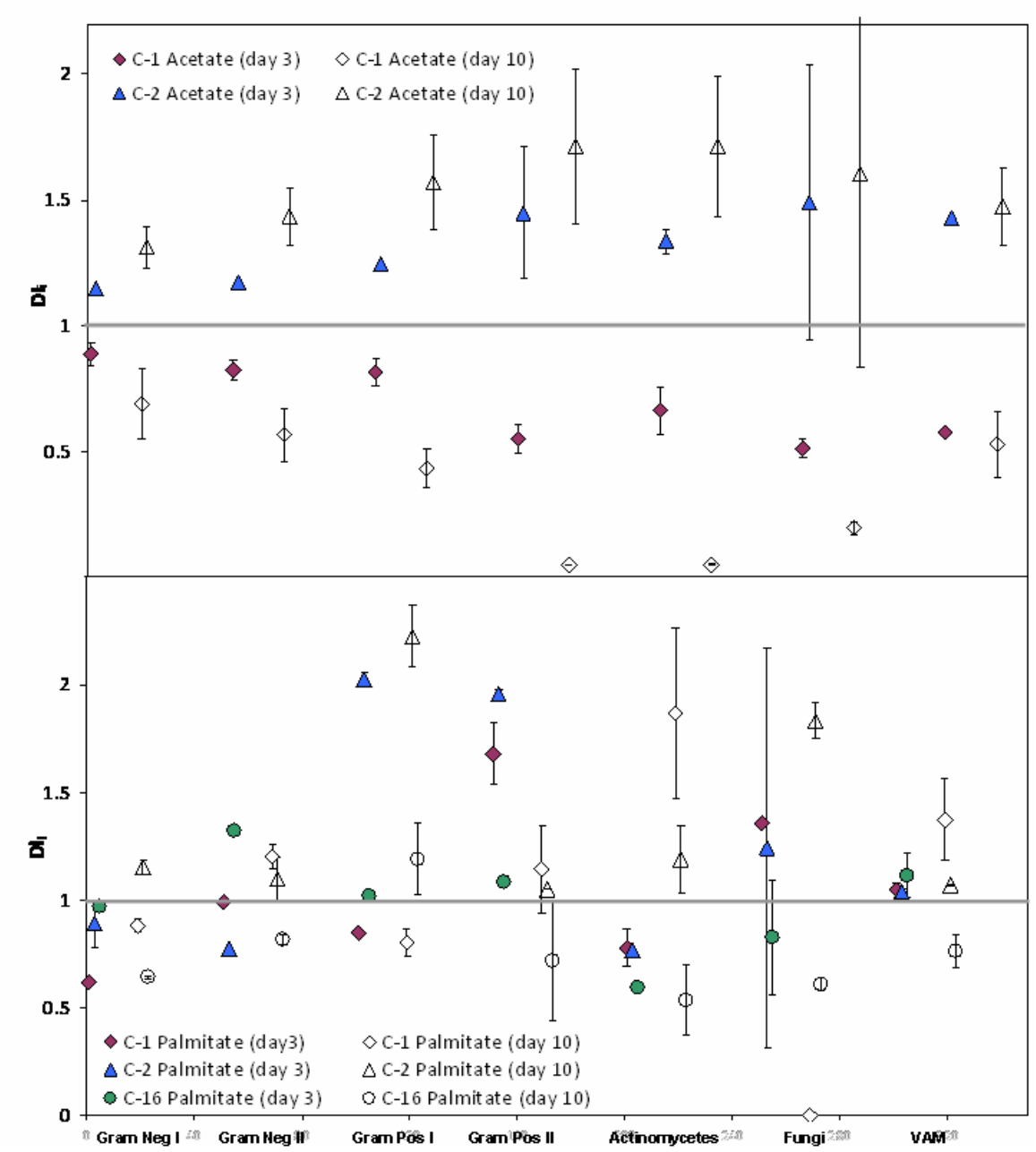

Fig. 5 Divergence index (DI) reflecting discrimination between C positions by incorporation into individual microbial groups 3 (left) and 10 (right) days after application of ^{13}C-labeled acetate (top) and palmitate (bottom). Experimental points (means ± SEM, N=4) are presented. Significant effects of C position and day on DI, calculated by nested ANOVA, are presented in Supplementary Table A5. Letters indicate significant differences ($p < 0.05$ derived from HSD post-hoc test) in the relative incorporation of the C positions into one group.

2.8.4 Discussion

2.8.4.1 Utilization and turnover of acetate and palmitate by soil microbial community

The short-chain low molecular weight organic acids are a well-used microbial substrate (Jones et al., 2003). Our study shows that long chain carboxylic acids like palmitate are also good substrate in soils and are used in similar proportions by the microbial community (Figure 1). In specific pathways, such long chain carboxylic acids can function as direct precursors for lipid formation, e.g. that of PLFA. Then, their incorporation into microorganisms can even exceed those of low molecular weight substances (Figure 1).

The preferential oxidation of C-1 of acetate is in accordance with previous studies and can clearly be linked to microbial metabolism, i.e. the oxidation of acetate in the citric acid cycle (Dippold and Kuzyakov, 2013; Fischer and Kuzyakov, 2010b) (Figure 6). A similar preferential oxidation of C-1 was observed for palmitate, especially after 10 days. This reflects that if palmitate is used in basic C metabolism, e.g. as an energy source, it is successively oxidized by fatty acid β-oxidation to acetyl-CoA (2 C atoms) units (Caspi et al., 2008; Keseler et al., 2009). Consequently, terminal C-1 and C-2 from palmitate form an acetate unit, and are transformed similarly to acetate in basic C metabolism.

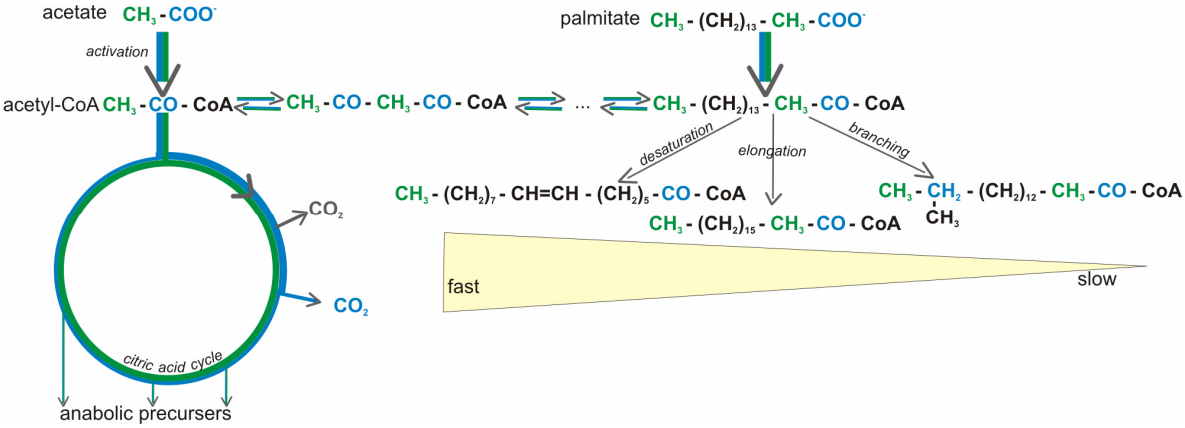

Fig. 6 Metabolic pathways of fatty acid formation from acetate and fatty acid transformations of palmitate in soil.

At day 10, a higher portion of the ^{13}C remaining in soil is found in microbial biomass for acetate than for palmiate ($p<0.05$). A higher portion of the ^{13}C in microbial biomass for acetate than for palmiate ($p<0.05$) clearly shows the lower microbial availability of palmitate. Consequently, a higher relative proportion of the added ^{13}C remained extracellular, e.g. as SOM-associated palmitate. Nevertheless, a high discrimination between C-1 and C-2 was observed, which was even higher for palmitate than for acetate at day 10. This indicates that in addition to the microbial transformed palmitate also palmitate stabilized in soil gets transformed by terminal oxidation. This terminal oxidation of carboxylic acids

to odd and even alkanes has been previously described for plants and microorganisms (Dennis and Kolattukudy, 1992; Ladygina et al., 2006; Park, 2005), and specific as well as unspecific decarboxylases contribute to the decarboxylation of carboxylic acids in soils (Hofrichter et al., 1998). Extracellular transformations are known to be less relevant for well available, low molecular weight organic substances (Dippold and Kuzyakov, 2013). However, their quantitative relevance for hydrophobic substances, such as palmitate, which could be stabilized by hydrophobic interactions in soils and are consequently less available for microbial uptake, still remains open. To finally identify extracellular, terminal oxidation, selective inhibition of microbial, intracellular processes coupled with position-specific lipid ^{13}C labeling has to be performed (Dippold and Kuzyakov, 2013).

2.8.4.2 Pathways of fatty acid formation from acetate in soil

Whereas in microorganisms the C-2 from acetate is preferentially incorporated compared to C-1, this clear pattern is less expressed if acetate C is used for PLFA synthesis (Figure 1). This shows that many of the microbial compounds in cytosol (released by the chloroform-fumigation-extraction) are small water soluble products like carboxylic acids or nucleotides derived from citric acid cycle metabolites. During the citric acid cycle the C-1 of acetate gets oxidized in an early step, whereas several cycles are needed until acetate C-2 gets oxidized (Figure 6). However, acetate is a direct precursor for fatty acid synthesis, which is built up from the C2-unit acetyl CoA (Caspi et al., 2008; Keseler et al., 2009; Lengeler et al., 1999). Therefore, the direct formation of fatty acids from acetate would lead to an identical incorporation of both positions (Figure 5). This identical incorporation was not observed in this study. Instead, there are clear specifics for C-1 and C-2 incorporation into individual PLFAs (Figure 2b): The lower incorporation of C-1 into basic, straight chain fatty acids like palmitate can be explained by the utilization of already partially oxidized fragments of acetyl CoA for fatty acid synthesis. This is similar to the metabolism of glucose (Dippold et al., submitted). Although glucose is a direct precursor for amino sugar synthesis, glucose molecules were transferred into basic glucose C metabolism and only fragments of the partially oxidized and split molecule were subsequently used for amino sugar synthesis. Similar bidirectional pathways were observed for carbohydrate synthesis: formation of microbial sugars from glycine occurred parallel to direct formation from glucose and in parallel to glucose oxidation (Derrien et al., 2007). Such bidirectional pathways can also explain the C positions used for fatty acid formation in this study: acetate was partially oxidized by the citric acid cycle and fragments, only containing C-2, were transferred back from citric acid cycle metabolites towards acetyl-CoA for new fatty acid synthesis.

An alternative explanation would be that the majority of fatty acids are not newly formed from acetyl-CoA (equal incorporation of C-1 and C-2). Instead, acetate ^{13}C was only used to perform transformation and modifications at already existing fatty acids, e.g. elongations of partially degraded fatty acids or the introduction of branching points into molecules. Figure 2b shows that no acetate C-1 is incorporated in any odd fatty acid: this is rather unlikely, if fatty acids with 15, 17 or 19 C are newly formed from acetyl-CoA (using 7, 8 or 9 units of acetyl-CoA). However, if only a terminal acetyl-CoA is added and afterwards the terminal carboxylic group is split to reach an odd fatty acid, this would cause a low incorporation of C-2 and the absence of C-1 incorporation into odd fatty acids (Figure 2b). For non-growing microbial communities under maintenance conditions, like in this study (see Table 1), internal recycling of fatty acids is likely to occur: the utilization of direct precursors contributes to save energy and C (Lengeler et al., 1999). However, this can only finally be proven, if not only position-specific labeling but also position-specific detection of the isotopic label in PLFAs is performed.

2.8.4.3 Pathways of fatty acid transformations in soils

The higher absolute incorporation of palmitate C compared to acetate C for the formation of PLFA showed that the more complex and direct precursor palmitate is preferred for synthesis and acetate is preferred for catabolism. This suggests already that palmitate was not fully degraded to acetyl-CoA and new fatty acids were built up from acetyl-CoA according to the resynthesis pathway (Rhead et al., 1971). Instead it is likely that modification of intact palmitate occurred. Figure 4 shows that the initially added ^{13}C palmitate is successively transformed to more diverse spectra of fatty acids. Comparing those transformed fatty acids with the PLFA fingerprint of the soil (Figure 3) shows that over a period of 10 days the newly transformed fatty acids are approaching the composition and consequently the demand of the microbial community. However, different kinetics of transformations are clearly shown by figure 3: Simple desaturation of palmitate occurred rapidly during the first 3 days after labeling. Thereafter, the proportion of desaturated fatty acids only marginally increased. More complex, biochemical processes, like elongations or even more branchings, occurred more slowly and therefore the proportion of these fatty acids strongly increased after day 3 .

However, the use of intact palmitate and the following modifications are in accordance with high recycling of existing fatty acids in soil microorganisms observed by acetate ^{13}C (Figure 2). This is confirmed by the fate of individual palmitate positions: 1) there is almost no C-1 and C-2 incorporated in even numbered fatty acids smaller than palmitate, e.g. C14 fatty acid (tetradecanoic acid). This suggests that the terminal acetate (C-1

and C-2) of palmitate is just split off to form the C14 fatty acid, whereas the basic C skeleton containing C-16 remained intact. 2) There is no palmitate C-1 in odd numbered fatty acids, suggesting that the terminal C-1 is only oxidized during the formation of odd numbered fatty acids from even numbered palmitate. 3) C-1, C-2 and C-16 are incorporated in similar amounts in desaturated fatty acids (Figure 2c). This suggests that the unsaturated, straight chain palmitic acid is just desaturated – or elongated and desaturated – for the formation of desaturated C16 and C18 fatty acids.

Position-specific ^{13}C labeling cannot distinguish whether these modifications occur as free fatty acids or bound to the PLFA. Whereas elongations and shortenings need to occur with a free, non-esterified terminal carboxylic group (Caspi et al., 2008), modifications like 10-methyl branching, cyclization or desaturation are known to be possible if fatty acids are bound to a PLFA (Aguilar et al., 1998; Lennarz, 1970). To distinguish transformations of free fatty acids from those occurring bound in PLFAs within the membrane, the measurement of isotopic label in intact phospholipids and free fatty acids has to be performed with much shorter time intervals than those chosen for this study. However, irrespective of the detailed biochemical mechanism, this study proved: 1) an intensive modification and use of intact fatty acids taken up from soil, and 2) an intensive recycling of the microbial fatty acid pool (which can occur intracellular or intercellular after cell death). Therefore, the previous assumption that fatty acids are generally newly formed from the added substrates have to be discarded and future PLFA studies, because they have to consider the reuse of existing plant and microbial-derived fatty acids (see section 4.5).

2.8.4.4 Pathways of specific microbial groups in soils

For the example of acetate, Figure 3 reflects the classical use of LMWOS by individual microbial groups in soils: gram-negatives are known to be the dominating group in the rhizosphere (Soderberg et al., 2004; Tian et al., 2013) and are most competitive for LMWOS (Apostel et al, 2013, Gunina et al, submitted). In contrast, gram-positives prefer old SOM (Kramer and Gleixner, 2006) and are less competitive for LMWOS. In general, the more complex organisms are structured, the lower their turnover and their competitiveness for fast uptake of LMWOS is: Bacteria have a shorter generation time (bacteria 20 min versus fungi 4-8 h to complete a life cycle under optimal conditions) and consequently higher cellular turnover (bacteria 2-3 times and fungi 0.75 times biomass turnover per year under soil conditions) (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). The turnover is even slower for higher levels of the nutritional net, e.g. the protozoa. Consequently, the results for acetate utilization in this study confirm that fast growth

is based on the utilization of readily available substrates and is closely associated with a fast turnover of the respective microbial groups in the soil. Microbial groups with a faster turnover are commonly more competitive for LMWOS, like acetate, even if the microbial community in general is under maintenance conditions (Gunina et al., submitted).

However, such a general rule is not valid for more complex, not ubiquitous substrates like palmitate: Figure 3 shows that, even within the gram-negatives, there is a clear preference of palmitate utilization by the gram-negatives 2. Separation of the two groups of gram-negatives was based on different groupings of the respective gram-negative fatty acids by explorative statistical tools (here: loadings of fatty acid contents on different factors in a principle component analysis). This tool, commonly used to characterize fatty acid fingerprints, supported here the separation and identification of two ecophysiologically different groups of gram-negatives: gram-negative 1 with a preference for LMWOS and gram-negative 2 with a preference for more complex, hydrophobic carboxylic acids (Figure 4).

Not only the absolute uptake but also the metabolism was specific for the investigated microbial groups. The Divergence Index revealed the preference for acetate C-2 incorporation for each of the microbial groups. However, discrimination between C-1 and C-2 increased significantly for those groups with high LMWOS, i.e. acetate, uptake. (Figure 4 and Figure 5). This can have two possible reasons: 1) Fast growing microbial groups with rapid turnover are characterized by a more direct metabolismn using precursors without further transformation, and 2) The fast growing gram-negatives are mainly characterized by straight chain, monounsaturated C16 and C18 fatty acids (Zelles, 1999), which are formed by simple desaturation without complex metabolic processes like methylations or branchings (Lennarz, 1970) leading to discriminations between C-1 and C-2. In both cases, it can be concluded – at least for the PLFA formation pathway - that more direct, less complex metabolic pathways are characteristic for fast growing microbial groups with high turnover. This is also confirmed for palmitate incorporation into PLFA, where the gram-negatives showed a comparatively low discrimination between the palmitate positions (Figure 5). However, to prove these general or microbial group-specific transformation steps, a combination of position-specific labeling with position-specific analysis of the microbial transformation products is needed. Nevertheless, the strong difference in DI from day 3 to day 10 for palmitate confirms that a high, internal turnover e.g. by recycling and transformation of the fatty acids, took place after ^{13}C incorporation.

2.8.4.5 Consequences for the application of fatty acids as biomarkers

The observed transformation of free fatty acids in soil by microorganisms causes consequences for the application of fatty acids as microbial and plant biomarkers. Alkanes can more easily be distinguished between plant- and microbial-derived n-alkanes. This enables the alkane fingerprint to be corrected for microbial contribution (Buggle et al., 2010; Zech et al., 2013). In contrast, the differentiation between microbial and plant-derived fatty acids is not as sharp: Vegetation type as well as microbial community affect the fatty acid sources in soils (Otto et al., 2005) and, in many cases, a reconstruction of the original source is not possible (Gocke et al., 2014). Furthermore, it is not clear, whether microbial enzymatic systems modifying n-alkanoic acids like palmitate are highly specific enzymes, which work only intracellularly or whether unspecific modification of plant-derived free fatty acids can occur. This would strongly limit the application of fatty acid fingerprints (Zhou et al., 2005) as well as their isotope signatures (Li et al., 2011) for paleo-environmental reconstructions. Therefore, further investigations, e.g. position-specific labeling of long-chain plant-derived fatty acids and investigation of their microbial transformations, is needed.

The transformation and internal recycling of fatty acids within microbial cells has important consequences for its application as microbial biomarkers. Changes within the fatty acid fingerprint in soils are commonly assumed to be related to changes in the microbial community structure (Zelles, 1999). However, acetate as well as palmitate labeling showed in this study that fatty acids are transformed and modified very fast in soils. Pure culture studies confirm that these modifications of fatty acids occur within living cells, if environmental conditions surrounding a living organism are changing, e.g. by temperature changes (Aguilar et al., 1998). These modifications of existing fatty acids can even occur in intact PLFA within the membranes (Aguilar et al., 1998; Lennarz, 1970). Therefore, further knowledge about the impact of internal fatty acid turnover for the interpretation of the PLFA fingerprint is needed (Frostegard et al., 2011). However, the high internal turnover of fatty acids within living microbial cells explains the discrepancy between the turnover of PLFA and of that of microbial biomass. PLFAs are assumed to have a half-life between one day and one week (Kindler et al., 2009; Ranneklev and Baath, 2003; Rethemeyer et al., 2004). In contrast, the turnover of the bacterial microbial community is assumed to occur 2-3 times per year (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). An intensive intracellular turnover of PLFA explains the much faster turnover of PLFA. This is similar to observations for the turnover of microbial cell walls (Dippold et. al, submitted): for *E. coli* it was even shown that they recycle 60% of their peptidoglycan during cellular life (Park and Uehara, 2008; Uehara and Park, 2008). Malik et al. (2013) showed that the turnover of microbial biomass compounds de-

creased with increasing molecular size. In contrast to cell wall amino sugars, PLFA do not need to be depolymerized to get modified and, therefore, a higher turnover of this microbial compound class is likely. This has consequences for the interpretation of isotope label incorporation into PLFAs: The PLFA will not be labelled homogeneously after the pulse addition of ^{13}C (or ^{14}C) compounds. The ^{13}C label is mainly located in newly introduced branchings, functional groups or terminal elongations of the fatty acids. Therefore, turnover times gained from this approach are not representative for the entire PLFA pool as these terminal endings and functional groups are subjected to a higher turnover than the basic skeleton of fatty acids.

2.8.5 Conclusions and Outlook

This is the first study investigating the formation and transformation of fatty acids by the microbial groups in soil. Both substances – acetate and palmitate – were used in diverse metabolic pathways: They were fully and partially oxidized in the citric acid cycle, but were also used for the formation of microbial biomass or PLFAs. The transformations of the basic microbial C metabolism dominated the position-specific pattern in soil: Acetate C-2 was preferentially incorporated into microbial biomass whereas acetate C-1 was preferentially oxidized.

Both carboxylic acids were used as precursors for PLFA formation. However, acetate was rarely used to form completely new fatty acids, but was mainly utilized as a precursor for the transformations of existing fatty acids, e.g. was introduced by elongations or branchings into the PLFAs.

Palmitate incorporation into PLFA was higher than that of acetate. This supports the preferential use of the existing fatty acid pool for PLFAs formation. Position-specific transformations reflect that palmitate was mainly used as an intact precursor for PLFAs. After the uptake it is successively modified according to the fatty acid demand of the microbial community, whereas desaturations are performed faster than elongations or branchings. Uptake and transformations of fatty acids are specific for individual microbial community members. In summary, ^{13}C labeling of palmitate and acetate reveals a high internal turnover of fatty acids, presumably even within living cells.

This causes consequences for the utilization of fatty acids as plant and microbial biomarkers: if the uptake and transformation of long-chain plant-derived fatty acids occur in a similar range as for palmitate, then the interpretation of plant-derived fatty acid fingerprints is hardly possible. Also, the interpretation of PLFA fingerprints has to consider that not only microbial community restructuring, but also modifications of fatty acids (e.g. due to changing environmental conditions) can occur within living cells. If isotope pulse

labeling is used to calculate PLFA or microbial turnover times, it has to be considered that ^{13}C from LMWOS is not incorporated homogenously into the PLFAs but mainly bound in functional or terminal groups of fatty acids.

This new view on PLFA formation and fatty acid transformations enables an improved interpretation of labeling experiments and microbial lipid transformations in soils. The final identification of the incorporation and transformations of fatty acids needs a combination of position-specific ^{13}C labeling with position-specific analysis in the metabolites.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft (DFG) for funding, Stefanie Boesel from the Martin-Luther University of Halle-Wittenberg for performing the bulk isotope measurements and Michael Zech and Björn Buggle for their helpful discussions about fatty acid transformations in soils.

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Supplementary Data

Supplementary Table A1: Fatty acids in the external standard

FA-type	Name	Common name	Abbreviation	Retention time (s)*
Saturated	Tetradecanoic acid	Myristic acid	14:0	150
	Pentadecanoic acid	-	15:0	355
	Hexadecanoic acid	Palmitic acid	16:0	630
	Heptadecanoic acid	Margaric acid	17:0	1010
	Octadecanioc acid	Stearic acid	18:0	1520
	Eicosanoic acid	Arachidic acid	20:0	2930
Branched chain	11-Methyltridecanoic acid	Anteisomyristic acid	a14:0	100
	12-Methyltridecanoic acid	Isomyristic acid	i14:0	90
	12-Methyltetradecanoic acid	12-Methylmyristic acid	a15:0	290
	13-Methyltetradecanoic acid	13-Methylmyristic acid	i15:0	270
	13-Methylpentadecanoic acid	Anteisopalmitic acid	a16:0	545
	14-Methylpentadecanoic acid	Isopalmitic acid	i16:0	525
	14-Methylhexadecanoic acid	14-Methylpalmitic acid	a17:0	890
	15-Methylhexadecan acid	15-Methylpalmitic acid	i17:0	860
Cyclo-propane	cis-9,10-Methylenhexadecanioc acid	cis-9,10-Methylpalmitic acid	cy17:0	940
	cis-9,10-Methylenoctadecanoic acid	Dihydrosterculic acid	cy19:0	2025
Methylated	10-Methylhexadecanoic acid	10-Methylpalmitic acid	10Me16:0	780
	10-Methyloctadecanoic acid	Tuberculostearic acid	10Me18:0	1745
Mono-unsaturated	9-Tetradecaeoic acid	Myristoleic acid	14:1w5c	130
	cis-11-Hexadecenoic acid	-	16:1w5c	595
	cis-9-Hexadecenoic acid	Palmitoleic acid	16:1w7c	570
	cis-Octadecenoic acid	Cis-Vaccenic acid	18:1w7c	1400
	cis-9-Octadecenoic acid	Oleic acid	18:1w9c	1375
	11-Eicosenoic acid	Eicosenoic acid	20:1w9c	2700
Poly-unsaturated	cis,cis-9,12-Octadecadienoic acid	Linoleic acid	18:2w6,9	1320
	6,9,12-Octadecatrienoic acid	g-linolenic acid	18:3w6,9,12	1355
	cis,cis,cis,cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4w6	2320

*for 45 m ± 0.5 m column lengths, deviations of ± 15 s possible

Supplementary Table A2: Fatty acids in the external standard

Rate [°C · m^{-1}]	Temperature [°C]	Hold time [min]
-	80	1
7	180	0
0.3	185	3
0.5	204	1.5
15	300	10
50	80	0

Supplementary Table A3: Result of factor analysis

Fatty acid	Factor 1	Factor 2	Factor 3	Factor 4	Microbial group
i16:0	**0.62**	-0.18	-0.32	0.49	Gram positives I
i17:0	**0.53**	0.54	-0.45	0.2	
a17:0	**0.56**	0.51	-0.47	23	
16:1w7c	**-0.61**	-0.07	0.41	0.56	Gram negatives II
cy19:0	**-0.6**	0.5	0.13	0.07	
10Me16:0	**0.82**	0.02	0.02	0	Actinoomycetes
10Me18:0	**0.54**	0.27	-0.17	0.46	
i15:0	-0.11	**-0.91**	0.29	-0.06	Gram positives II
a15:0	-0.16	**-0.92**	0.10	0.12	
14:1w5c	0.04	0.10	**0.86**	-0.14	Gram negatives I
18:1w7c	-0.59	-0.08	**0.62**	-0.28	
18:1w9c	-0.42	-0.14	**0.70**	-0.26	
18:2w6,9	0.58	0.12	**-0.53**	0.02	Fungi
20:1w9c	0.04	0.00	**-0.84**	0.08	
20:4w6	0.02	0.21	**-0.88**	0.15	Protozoa
16:1w5c	-0.31	0.40	0.20	**-0.51**	VA-Mykorrhiza
cy17:0	-0.01	0.06	0.02	**0.89**	N/A

Supplementary Table A4: Nested ANOVA for acetate and palmitate positions nested in the variable day, block as random variable and day. Degrees of freedom (df), F-values and significance level (p) are shown for the acetate and palmitate. If requirements for parametric tests (normal distribution + homogeneity of variances was not given, a Kruskal-Wallis ANOVA for the individual treatments was calculated (in this case H-Value is given instead of F value)

Ala concentration	factor	acetate			palmitate		
		df	F	p	df	F	p
Gram Neg I	position (nested in day)	4	1.18	n.s.	5	1.18	n.s.
	block = random factor	3	0.36	n.s.	3	0.36	n.s.
	day	1	12.31	**	1	12.32	**
Gram Neg II	position (nested in day)	4	3.65	*	5	0.18	n.s.
	block = random factor	3	0.06	n.s.	3	0.50	n.s.
	day	1	8.74	**	1	19.78	***
Gram Pos I	position (nested in day)	4	3.75	*	5	2.54	n.s.
	block = random factor	3	0.35	n.s.	3	0.45	n.s.
	day	1	9.08	**	1	1.71	n.s.
Gram Pos II	position (nested in day)	4	3.19	*			
	block = random factor	3	0.25	n.s.	7	H = 10.37	n.s.
	day	1	1.85	n.s.			
Actinomycetes	position (nested in day)	4	3.25	*	5	0.72	n.s.
	block = random factor	3	0.08	n.s.	3	0.36	n.s.
	day	1	0.88	n.s.	1	2.58	n.s.
Fungi	position (nested in day)	4	1.54	n.s.	5	0.50	n.s.
	block = random factor	3	2.79	n.s.	3	1.72	n.s.
	day	1	0.07	n.s.	1	8.69	**
VAM	position (nested in day)	4	2.63	n.s.			
	block = random factor	3	0.04	n.s.	7	H = 7.52	n.s..
	day	1	1.11	n.s.			
Protozoa	position (nested in day)	4	-	n.d.	5	0.45	n.s.
	block = random factor	3	-	n.d.	3	0.51	n.s.
	day	1	-	n.d.	1	4.84	*

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

Supplementary Table A5: Nested ANOVA for acetate and palmitate DI, with the independent variables position (being nested in the variable day), block (as random variable) and day. Degrees of freedom (df), F-values and significance level (p) are shown for the acetate and palmitate

Ala concentration	factor	acetate			palmitate		
		df	F	p	df	F	p
Gram Neg I	position (nested in day)	2	2.91	n.s.	4	4.94	n.s.
	block = random factor	3	0.54	n.s.	3	1.85	n.s.
	day	1	0.07	n.s.	1	1.56	*.
Gram Neg II	position (nested in day)	2	5.07	*	4	2.82	n.s.
	block = random factor	3	0.04	n.s.	3	2.75	n.s.
	day	1	0.00	n.s.	1	0.00	n.s.
Gram Pos I	position (nested in day)	2	6.18	*	4	4.04	*
	block = random factor	3	0.06	n.s.	3	1.26	n.s.
	day	1	0.01	n.s.	1	0.05	n.s.
Gram Pos II	position (nested in day)	2	8.38	*	4	0.41	n.s.
	block = random factor	3	0.44	n.s.	3	1.05	n.s.
	day	1	0.27	n.s.	1	1.37	n.s.
Actinomycetes	position (nested in day)	2	10.05	**	4	1.58	n.s.
	block = random factor	3	0.23	n.s.	3	0.46	n.s.
	day	1	0.30	n.s.	1	2.74	n.s.
Fungi	position (nested in day)	2	6.31	*	4	3.81	n.s.
	block = random factor	3	2.72	n.s.	3	5.55	*
	day	1	0.36	n.s.	1	0.30	n.s.
VAM	position (nested in day)	2	5.92	*	4	0.80	n.s.
	block = random factor	3	0.02	n.s.	3	1.23	n.s.
	day	1	0.00	n.s.	1	0.02	n.s.

* $p < 0.05$

2.9 Study 9: Organic nitrogen uptake by plants: Re-evaluation by position-specific labeling of amino acids

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Abstract

Current studies suggested that besides inorganic nitrogen (N), many plants are able to take up organic N in form of amino acids. However, reliable methods to quantify the uptake of intact amino acids are still missing and the relevance of organic N uptake is doubted. We used position-specific ^{14}C labeling to investigate the uptake of intact amino acids and their role in the N nutrition of plants.

Position specifically ^{14}C and ^{15}N labeled alanine, injected into the rhizosphere soil, enabled to trace the uptake of C from individual molecule positions by *Zea maize, Lupinus albus* and *Cichorium intybus*. As a control, uniformly ^{14}C labeled alanine, acetate and inorganic $^{15}NH_4^+$ and $^{15}NO_3^-$ were applied.

The same uptake of uniformly ^{14}C labeled alanine and acetate showed that low molecular weight organic substances are taken up by roots may occur by passive mechanisms, without differences for N containing and N free organics. Differences in plant uptake of ^{14}C from individual positions in alanine molecule confirmed that soil microorganisms split alanine within 6 h into transformation fragments (including mineral NH_4^+), which were then taken up by plants. Only 0.04 to 0.25% of the alanine added directly into the rhizosphere were taken up as intact molecule with the highest uptake observed for lupine – the plant adapted to organic N transport from *Rhizobia*.

Microbial utilization strongly dominated the fate of low molecular weight organic substances in soils and the majority of amino acid ^{14}C uptake by plants was explained by passive uptake of microbial transformation products. Position-specific labeling is an innovative tool that enables to separate easily the intact uptake from uptake of molecule fragments. Thus, it improves the quantification of intact uptake by avoiding the up to 3-fold overestimation of uniform labeling approaches.

Keywords: Alanine; Position-specific, dual isotope labelling; Organic N uptake; Chicory; Lupine; Maize; Isotopic approaches; Nitrogen cycle

2.9.1 Introduction

Over the past century, many studies have emphasized the role of dissolved inorganic nitrogen (DIN) in ecosystems (Matson et al., 1997; Vitousek et al., 1997; Vitousek et al., 1979). Ammonium (NH_4^+) and nitrate (NO_3^-) are the main representatives of mineral nitrogen. Ammonium is a reduced form of DIN and can be directly utilized by plants after uptake whereas nitrate needs to be reduced first. Nitrate reduction demands energy from plants (Doubnerova and Ryslava, 2011; Liu et al., 2011; Tischner, 2000) leading to additional CO_2 fluxes through the plant-soil system (Gavrichkova and Kuzyakov, 2008, , 2010). Both DIN species can be lost from ecosystems: nitrate by leaching into the ground water, denitrification to N_2O and N_2, or reduction to ammonium and ammonium can be lost by volatilization or irreversible fixation by soil minerals.

In ecosystems with low availability of DIN due to slow mineralization, like boreal or arctic ecosystems (Nasholm et al., 1998; Vitousek et al., 1979), plants may also rely on other N forms such as dissolved organic nitrogen (DON). This is not only a short-circuit in the traditionally assumed N nutrition pathways (the mineralization to NH_4^+ and NO_3^- is omitted), but also reduces potential N losses from ecosystems, e.g. by leaching.

In the past twenty years, there has been remarkable interest in DON as a plant N source (Chapin et al., 1993; Jones et al., 2005a; Nasholm et al., 1998; Paungfoo-Lonhienne et al., 2012; Schimel and Chapin, 1996). Organic N can be found in many compounds in soil from macromolecules like proteins (Jones et al., 2005d) or humic substances (Szajdak et al., 2003) to low molecular weight organic substances (LMWOS) like amino acids (Doerr et al., 2012; Jones et al., 2005c; Lipson et al., 1999; Streeter et al., 2000), amino sugars (Roberts et al., 2007; Roberts and Jones, 2012) and nucleic acids (Kuzyakov, 1996).

Many amino acids have very fast cycling rates and the half-life of amino acid C in soils is in the range of few hours (Jones et al., 2009; Kuzyakov, 1996). This fast cycling is connected with fast and almost complete uptake by microorganisms (Fischer et al., 2007). Another study demonstrated that LMWOS at average soil concentrations in soil solution (below 10 µmol l^{-1}) were taken up by microorganisms at a rate of 82% after 3 min (Fischer et al., 2010b), and the half-life of amino acids in soil solution ranges between 4-8 min (Jones et al., 2004). Due to this fast utilization, soil microorganisms are stronger competitors for amino acids than plants (Biernath et al., 2008; Hodge et al., 2000; Jones et al., 2005a; Kuzyakov and Xu, 2013b), whereas in the long-term this N is released by the microorganisms and is available for plants. In contrast, Chapin et al. (1993) showed in the early 90ies the preferential use of organic N by an arctic sedge which started the discussion about the relevance of amino acids as plant N source.

Further studies showed that boreal forest vegetation actively take up amino acids, probably due to a lack of other N sources (Delgado-Baquerizo et al., 2011; Nasholm et al., 1998). The parallel uptake of DIN and DON is dependent on their availability (Kranabetter et al., 2007). Therefore DON is discussed to be less relevant for agricultural crops (Jones et al., 2005a). In order to evaluate the relevance of DON, a comparison between LMWOS with inorganic N uptake was recommended (Glass et al., 2002; Jones et al., 2005a; Streeter et al., 2000) especially for agroecosystems, where the role of DON is still controversial.

Isotope labeling of LMWOS with ^{15}N coupled with ^{13}C or ^{14}C is a common tool to investigate uptake and allocation in plants as well as mineralization or microbial incorporation (Thede, 2010; van Hees et al., 2005). The uptake of amino acids by plants was mainly investigated by dual-labeling with ^{15}N and ^{13}C (Nasholm et al., 1998; Streeter et al., 2000). It is tacitly assumed in this approach that the uptake of ^{13}C corresponds to the uptake of the intact amino acid. However, dual isotope labeling has a methodological shortcoming leading to an overestimation of intact uptake: microorganisms produce labeled fragments from the added amino acids, and these fragments and mineralized N can be taken up in parallel (Rasmussen et al., 2010). This would contribute to the quantified intact uptake by the dual isotope labeling approach (Sauheitl et al., 2009a). The first evaluation of this overestimation was performed by the application of dual uniformly labeled amino acids and compound-specific ^{13}C and ^{15}N analysis during their root uptake. It was shown that due to uptake of labeled metabolites, bulk measurements caused an up to six-fold overestimation of the intact uptake (Sauheitl et al., 2009a). However, compound-specific ^{13}C and ^{15}N analysis has the disadvantage of being a time-consuming and expensive technique (Sauheitl et al., 2009a).

To prove the uncertainties of the original $^{13}C/^{15}N$ approach, Rasmussen et al. (2010) proposed position-specific labeling as a potential tool to overcome the problem of molecule splitting. Thus, uptake as a whole molecule could be distinguished from uptake as partially degraded amino acid fragments i.e. decarboxylated fragments (Dippold and Kuzyakov, 2013). Some recent studies (Dijkstra et al., 2011a; Fischer and Kuzyakov, 2010) clearly showed that position-specific ^{13}C and ^{14}C labeling enables tracing the fate of individual functional groups in various soil pools. If the uptake of amino acid C occurs as a broad spectra of various transformation products (and not as intact amino acids), this would strongly reduce the importance of N nutrition by amino acid – from a quantitative as well as a regulative view concerning N deficiency.

Here, we used the same technique of position-specific ^{14}C labeling to quantify the intact uptake of amino acid. We hypothesized that 1) the original $^{13}C/^{15}N$ approach over-

estimates the intact uptake of amino acids, and 2) organic N uptake is traceable in temperate ecosystems but is of minor relevance for the N nutrition of agricultural plants.

In order to consider the physiological differences of plant functional types (Weigelt et al., 2005), we performed our experiment with three species: maize, chicory and lupine. These species differ in their N uptake and transformation, their physiology and morphology, especially in the root system: 1) the grass maize (*Zea mays L.*) has a fibrous root system and reduces NO_3^- in roots and shoots (He et al., 2011), 2) the herb chicory (*Cichorium intybus L.*) reduces NO_3^- in roots (Goupil et al., 1998) and has a taproot system, where it can store N-containing compounds for the next year (Ameziane et al., 1997) and 3) the legume lupine (*Lupinus albus L.*) reduces NO_3^- in roots (Gavrichkova and Kuzyakov, 2008) and has the ability to reduce atmospheric N_2 in root nodules through symbiosis with *Rhizobia*.

As organic N source, we used alanine as one of the most abundant amino acids (Fischer et al., 2010a) and ammonia and nitrate as inorganic N sources. To evaluate the preference of amino acid uptake compared to N-free LMWOS, we included additional treatments with acetate, which has a structural resemblance to alanine. If uptake of N-LMWOS (alanine) occurs mainly by unselective mechanisms, it should be in a similar range to N-free LMWOS (acetate).

The aims of this study were: 1) to determine the fate of amino acids in soil with a special focus on the plant uptake of an initial substance versus the uptake of its transformation products, 2) to assess the ecological and physiological role of intact uptake of amino acids by different plant species and 3) to evaluate the relevance of three N sources (alanine, ammonium and nitrate) for N nutrition of agricultural plants.

2.9.2 Material and Methods

2.9.2.1 Experiment preparation

Soil sampling

Soil samples were collected from an agricultural field site close to Hohenpölz (Bavaria, Germany at 49.907 N, 11.152 E, 501 m.a.s.l.) that had been long-term cultivated with cereals (barley, wheat, triticale). The soil is a loamy haplic Luvisol (FAO, 2006). Soil was collected from 0-10 cm, sieved to 2 mm and roots were removed. The physico-chemical characteristics of the soil are described in Table 1.

Plant and material preparation

After sieving, soil was immediately filled into transfer pipettes made of low density polyethylene 30 cm in length and 1 cm diameter, which were used as rhizotubes (Biernath et al., 2008; Kuzyakov and Jones, 2006).

We used maize (*Zea mays* L), lupine (*Lupinus albus* L) and chicory (*Cichorium intybus* L). Plant seeds were pre-germinated at constant temperature (30 ºC) and watered for 36 hours (Gavrichkova and Kuzyakov, 2008). Then, one sprout of each plant was inserted into the rhizotubes. The rhizotubes were submerged in a plastic container half-filled with cold water to maintain the soil temperature around 12ºC. Thus, microbial activity e.g. mineralization rates should resemble field conditions (Jones, 1999). The pipette was connected with an air inlet (tube) at the bottom and directly under the soil surface (Biernath et al., 2008) to avoid water saturation of the soil and provide the soil and roots with air.

Table 1 The physicochemical properties of the Ap-horizon of the haplic Luvisol.

Soil parameters	**Values**
pH KCl	4.88 ± 0.12
pH H_2O	6.49 ± 0.11
Total Organic Carbon	1.77 ± 0.07%
Total Nitrogen	0.19 ± 0.01%
Cation-Exchange Capacity	13.6 $cmol_c\ kg^{-1}$ soil
Microbial biomass C	42.5 ± 1.1 µmol C g^{-1} soil
Microbial C/N ratio	9.9 ± 0.3

Chemicals and radiochemicals

The radiochemical stock solution had concentrations of 50 µM for alanine and acetate, both with 10^6 DPM ml^{-1} ^{14}C activity. Position-specific labeled alanine ([1-^{14}C], [2-^{14}C], [3-^{14}C]alanine, American Radiolabeled Chemical Inc., St Louis, USA), as well as uniformly labeled [U-^{14}C]acetate (Biotrend Köln, Germany) and [U-^{14}C]alanine (American Radiolabeled Chemical Inc., St Louis, USA) were used.

Nitrogen labeling was performed with a 99 atom-% ^{15}N enriched tracer of either alanine $CH_3CH(^{15}NH_2)COOH$ as the organic N-source or ammonium sulfate $(^{15}NH_4)_2SO_4$ or potassium nitrate $K^{15}NO_3$ as inorganic N forms (Biotrend Köln, Germany). Amount of applied C and N was identical in each treatment and lower than average concentrations of alanine, acetate, NH_4^+ or NO_3^- in agricultural soils.

2.9.2.2 Experimental setup

Treatment and labeling

Each plant (chicory, lupine, maize) grew until their root system reached the rhizotube bottom at 25 cm (chicory 5 weeks, lupine 4 weeks and maize 3 weeks after germination). Each treatment (the three plants, the four ^{14}C-LMWOS-treatments (acetate, alanine C-1, alanine C-2, alanine C-3 and alanine uniformly labeled) and the three ^{15}N nitrogen sources (alanine, ammonium and nitrate)) were performed in four replicates. For each plant the respective backgrounds with addition of the same amount but unlabeled substances were performed in four replicates.

Each tube was sealed with silicon (NG 3170 Thauer & Co., Dresden) on the top to avoid C fixation of microbially respired $^{14}CO_2$ by the plants. The seals were applied 24 h before tracer application in order to avoid air leakage (Tian et al., 2013). Pipes were installed at the bottom of the top of the rhizotube, which were connected to a gas volume separated from the aboveground biomass, to enable a gas exchange between soil and atmosphere.

Finally, 100 µl of tracer solutions were injected at three locations along the tube (at 5, 10 and 15 cm depth) to spread the tracer over the entire rhizosphere.

Harvesting plant biomass

To identify the uptake of intact amino acids, we harvested 6 h after labeling because the half-life of amino acids C in soil ranges from 1 to 12 h (Jones et al., 2005b). Aboveground biomass was cut at the top surface of the soil and immediately submerged in liquid nitrogen for 30 sec. The entire rhizotube was also frozen in liquid N_2. Afterwards, all samples were stored at -20 °C and before further analysis, roots and soil were separated manually and roots were washed according to Sauheitl et al. (2009b) to remove sorbed LMWOS and ions.

2.9.2.3 Laboratory analysis

Chemical and radiochemical analysis

Soil and plant samples were freeze-dried and ball-milled for ^{14}C and ^{15}N analysis. To quantify ^{14}C incorporation, we combusted 500 mg of soil and 20 mg of roots/shoots at 600 ºC for 10 min under a constant O_2 stream using an HT 1300 solid combustion module of a N/C analyzer 2100 (Analytik Jena AG, Jena Germany). The $^{14}CO_2$ was trapped in 10 ml of 1 M NaOH (two times 5 ml). For scintillation analysis, 3 ml of this NaOH with soil-, root- and shoot-derived $^{14}CO_2$ and 6 ml of scintillation cocktail (Ecoplus, Roth Company, Germany) were mixed and measured on an LS 6500 scintillation counter (LS 6500,

Beckman-Coulter, Krefeld, Germany). All samples were measured after 24 hours of dark storage in order to remove chemiluminescence.

In parallel, ^{15}N was measured in 5-6 mg of soil and 0.25 mg of root/shoot samples using a Euro EA Elemental Analyser (Eurovector, Milan, Italy) which was coupled via a ConFlo III interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage isotope ratio mass spectrometry (IRMS) (Thermo Fischer, Bremen, Germany) (Glaser, 2005).

Calculations of ^{14}C and ^{15}N uptake

The percentage of incorporated ^{14}C from the applied ^{14}C in the pools (C_{inc_pools}) was calculated by the ratio of the ^{14}C activity in each pool (soil, root, shoots or total biomass) divided by the applied ^{14}C activity per rhizotube. Decomposition of alanine and acetate to CO_2 was calculated as the difference between ^{14}C added and ^{14}C recovered in soil and plant biomass. Please note that additional unconsidered losses in ^{14}C would lead to an overestimation of the calculated, mineralized CO_2.

All $\delta^{15}N$ values were converted into ^{15}N atom% (r_{pool}), considering the isotope composition of international reference standards (Fry, 2006). The content of N (%) and the dry weight of the pools (DW_p) were used to calculate the total N ($[N]_{pool}$) content per sample. Thereafter, ^{15}N uptake (alanine, nitrate and ammonium) applied to the different pools was calculated following a mixing model in equation (1) (Amelung et al., 1999):

$$[N]_{inc.-N} = [N]_{pool} \cdot * \frac{r_{pool} - r_{pool-BG}}{r_{appl-N} - r_{pool-BG}} \quad (1)$$

N_{pool} represents the amount of N of an enriched pool and $[N]_{inc\text{-}N,}$ is the amount of newly incorporated tracer-N. The variables r_{pool} and $r_{pool\text{-}BG}$ are the measured at% ^{15}N values of the labeled pool, its background (BG), and $r_{app\text{-}N}$ is the enrichment of the purchased tracer, respectively.

The calculation of the percentage of relative N incorporation per pool [$N_{inc\ pool}$ (%)] is described in equation 2:

$$\mathrm{N}_{\text{ inc pool }}(\%) = \frac{[N]_{inc.-N}}{\sum_{pools}[N]_{appl-N}} \times 100 \quad (2)$$

with $\sum_{pools}[N]_{appl-N}$ being the sum of applied nitrogen measured in all of the pools.

2.9.2.4 Calculation of intact uptake of labeled substances

The calculation of intact uptake of alanine from the ^{14}C to ^{15}N ratio of the plant biomass ($R_{c/n}$) in equation 3 is based on the assumption of dual-isotope labeling that intact

uptake is characterized by the parallel uptake of ^{15}N and ^{14}C. This parallel uptake can either be calculated by a linear regression between C excess and N excess (Nasholm et al., 1998) or by the ratio of C to N incorporation:

$$R_{c/n} = \frac{\overline{^{14}C_{inc\_biomass}}}{\overline{^{15}N_{inc\_biomass}}} \quad (3)$$

$R_{C/N}$ was multiplied by 100 to obtain percentage values $R_{c/n}$ (%). The standard error of the mean of this ratio was calculated by Gaussian error propagation. Based on position-specific ^{14}C labeling, this ratio can be calculated for each C position of alanine. The label signal of all three positions must be equal in the case of intact uptake. However, fragment uptake would cause position-specific differences in this ratio. Thus, only the lowest value of the three C positions represents the real intact uptake of added alanine; all higher values represent uptake of fragments.

There are still factors which may cause over- or underestimation of this approach: First plant metabolism can cause an underestimation because of preferential respiration of specific positions similarly to microorganisms. Second, overestimation of intact uptake can occur if all three C atoms are transferred to the plant in different fragments.

In order to have a better understanding of the quantitative relevance of organic N uptake compared to DIN, the percentage of intact uptake of alanine from the total N taken up (ammonia, nitrate and alanine) was calculated. In addition, we calculated N uptake from mineralized alanine with equation 4:

$$DIN_{inc\_biomass} = \mathrm{N(ala)}_{\mathrm{inc\_biomass}}\ (\%) - \mathrm{R}_{\mathrm{c/n}}\ (\%) \quad (4)$$

Where $DIN_{inc\_biomass}$ (%) is the percentage of alanine-N being mineralized and afterwards taken up by the plant as DIN; $N(ala)_{inc\_biomass}$ is the total ^{15}N incorporation from ^{15}N-alanine into the plant biomass (in % of applied ^{15}N) and $R_{c/n}$ (%) is the alanine-^{15}N taken up as intact alanine into each plant.

In the same way, the C incorporated from alanine fragments was calculated:

$$C_{frag}\ (\%) = \left(^{14}C_{biomass}\right) - \mathrm{R}_{\mathrm{c/n}}\ (\%) \quad (5)$$

Where C_{frag} is the percentage of an individual C position taken up as a fragment and $^{14}C_{biomass}$ is the total percentage of ^{14}C incorporation from each position into the plant biomass.

Assuming fragment uptake as main uncertainty of uniform-labeling approached, overestimation of intact uptake compared to position-specific labeling was calculated: the mean of the fragmented uptake of the three position summed up with the percentage of intact alanine uptake (this resembles the result of uniformly labeling) was divided by the percentage of intact uptake.

2.9.2.5 Statistical analysis

Data were checked for normal distribution with Kolmogorov-Smirnov Test and checked for outliers with Nalimov Test. Factorial ANOVA (with factors plant compartment and substance or position) with the HSD post-hoc test for unequal N treatments were used for the data analysis. Calculations were performed by STATISTICA 8.0 (StatSoft Inc, Tulsa USA). Figures and tables were plotted using mean ± standard error of mean (SEM).

2.9.3 Results

2.9.3.1 ^{15}N uptake in plants from organic and inorganic N sources

Total N uptake was higher for maize than for chicory and lupine. Nitrate was the preferred N source for all plant species. After 6 hours, 34% of the $^{15}N\text{-}NO_3^-$ injected into the rhizosphere was incorporated into shoots and up to 15% in roots. On the other hand, less than 7% of the ammonium was taken up at the same time and alanine N uptake reached a maximum of 7% in roots and 4% in shoots.

Significantly higher uptake of nitrate was found in maize than in lupine and chicory ($p<0.001$), while alanine and ammonia were taken up in similar amounts by all three plant species (Fig. 1). Maize fed its high N demand mainly by uptake of nitrate in comparison with reduced N sources.

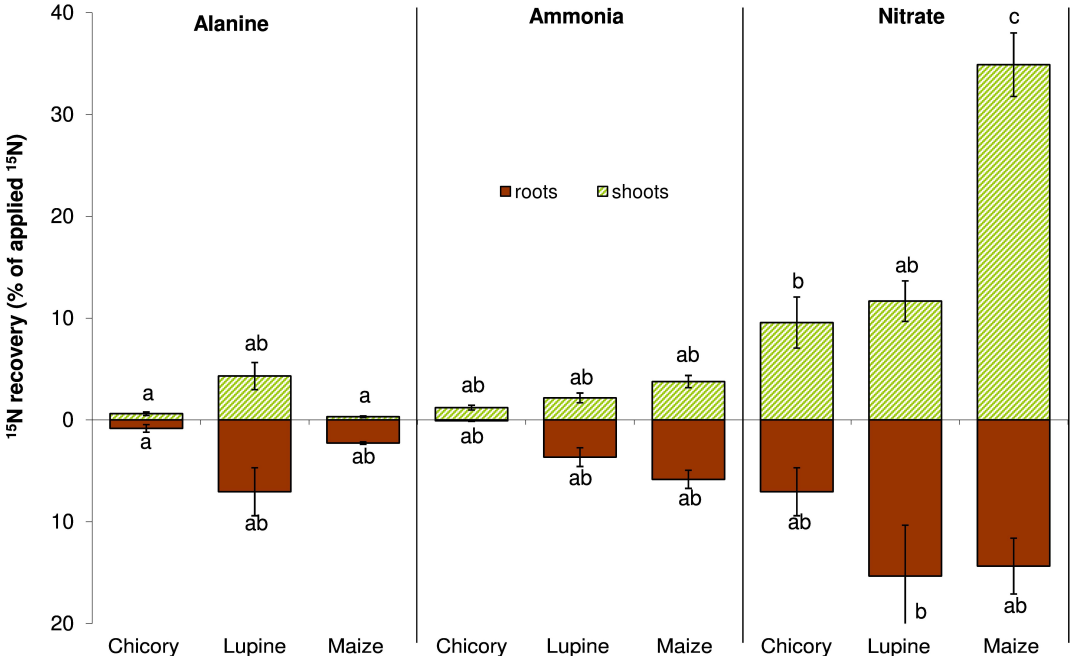

Fig. 1 Percentage of ^{15}N incorporation in roots and shoots as alanine-N, ammonia and nitrate in chicory, lupine and maize. Letters indicate significant differences ($p<0.001$) between alanine, ammonia and nitrate within the plants

Compared with inorganic N sources, alanine-derived N was preferentially incorporated into roots and to a lower amount allocated to the shoots. There was no species effect on the root/shoot ^{15}N ratio (Table 2) with the exception of preferential nitrate transport into the shoots of maize ($p<0.001$).

Table 2 Shoot/root ratio of ^{15}N from individual N sources

Shoot/root ^{15}N ratio	Chicory	Lupine	Maize
Alanine-^{15}N	0.71 ± 0.24	0.61 ± 0.23	0.13 ± 0.06
Ammonium-^{15}N	14.65 ± 11.81	0.59 ± 0.16	0.64 ± 0.12
Nitrate-^{15}N	1.35 ± 0.37	0.76 ± 0.15	2.43 ± 0.35

2.9.3.2 Plant uptake of uniformly ^{14}C labeled alanine and acetate

After six hours, 0.02 to 0.63% of the added ^{14}C activity were recovered in the shoots and 0.06 to 1.51% in roots with significant differences among the three plant species ($p<0.001$) (Fig. 2).

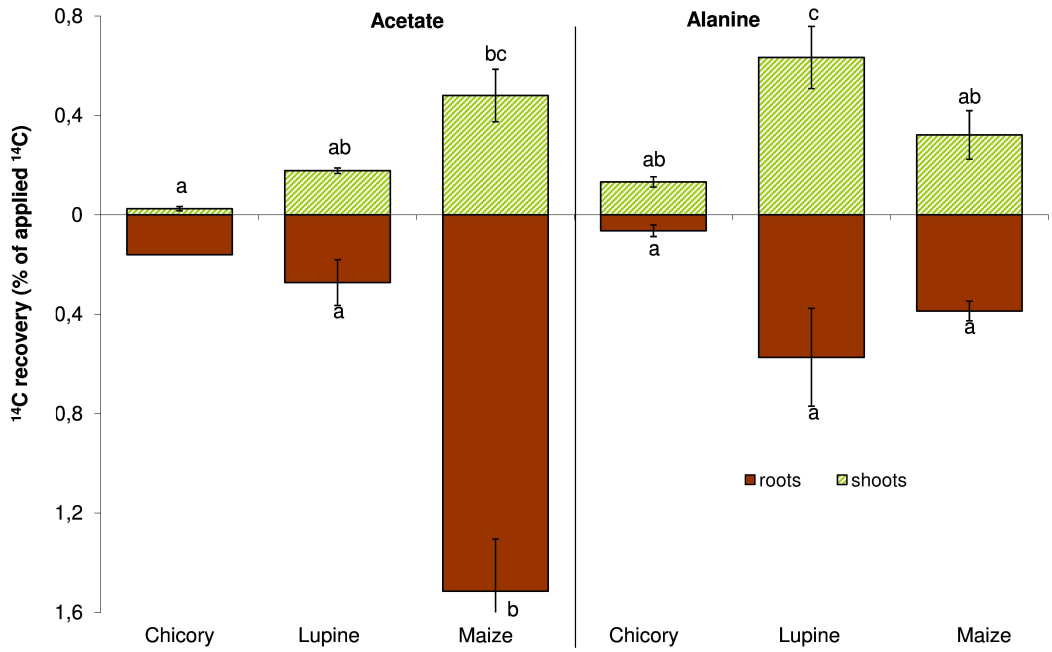

Fig. 2 Percentage of ^{14}C incorporation in roots and shoots after uniform ^{14}C labeling with acetate and alanine. Letters indicate the significant differences ($p<0.001$) of acetate and alanine C between plants

Maize took up more acetate than alanine. There was no clear preference of acetate uptake for lupine or chicory. Higher uptake of uniformly labeled acetate was found in

roots than shoots, reflecting its poor transport to the aboveground plant compartments. Plant uptake of uniformly labeled alanine was higher in lupine and lower in chicory with similar incorporation in roots and shoots by maize (Fig. 2). The LMWOS-C remaining in soil ranged from 42 to 65%, and potential mineralization to CO_2 (calculated as difference between applied and recovered ^{14}C) occurred in 35 to 57% of the applied tracer without significant differences between plants (Figure Supplementary).

2.9.3.3 ^{14}C plant uptake of position-specific labeled alanine

We compared the mean uptake of position-specific labeled isotopomers (mean of positions in Fig.3) with the results of uniform ^{14}C labeling (Fig. 2) to evaluate result quality and found no significant differences of the mean of the three positions to uniformly labeled alanine for any of the investigated pools.

Alanine C-3 was preferentially incorporated in maize and lupine shoots, whereas lupine roots preferred uptake of C-1 (Fig. 3). There was very low ^{14}C incorporation in chicory. In general, we observed higher incorporation of C-3 ($p<0.001$) than of the other positions, but with significant differences among the plant species ($p<0.001$) (Fig. 3).

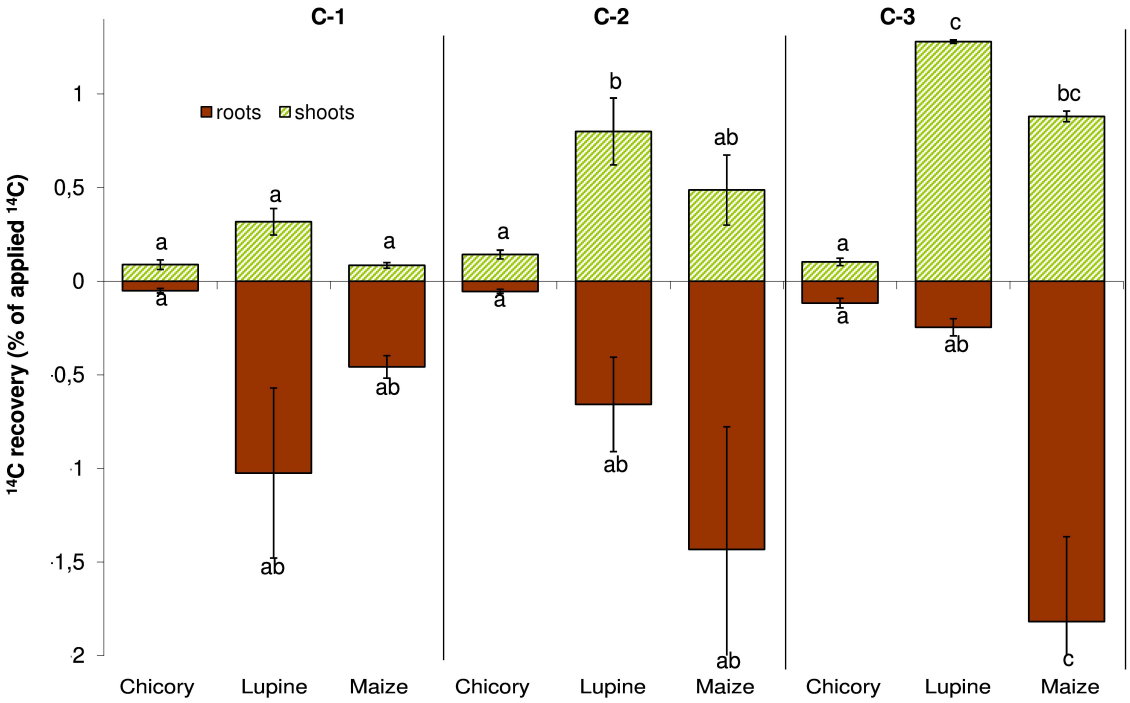

Fig. 3 Percentage of ^{14}C incorporation in roots and shoots after position-specific labeling with alanine. The alanine positions were C-1 (carboxyl group), C-2 (amino-bound group) and C-3 (methyl group). Letters indicate significant differences ($p<0.001$) between alanine C positions.

Plant species had no significant effect on the amount of mineralized ^{14}C (Figure. supplementary). Alanine showed significantly higher mineralization of C-1 (76%) than C-2 (45%) and C-3 (52%).

In general, we observed that after 6 h, the individual molecule positions of alanine had strongly differing fates concerning plant uptake as well as the proportions remaining in the soil.

2.9.3.4 Intact uptake of alanine assessed by position-specific labeling

The $^{14}C/^{15}N$ ratio in the plant biomass (shoots and roots) reflects the proportion of ^{14}C of each individual position, which was taken up together with ^{15}N. Based on position-specific ^{14}C labeling, this calculation can be performed for each C position of alanine (Fig. 4). This ratio showed the pattern C-3>C-2>C-1 for each plant. We considered that a molecule of alanine could only be taken up intact if all three positions were incorporated into the plant. Thus, the minimum of the $^{14}C/^{15}N$ ratio reflects the maximum intact uptake of alanine in plants, which was the case for the $^{14}C/^{15}N$ ratio of C-1 position. These values were in a similar range for the three investigated plant species: 7 to 14% of the alanine-N was taken up as intact alanine in the order maize<chicory<lupine (Table 3).

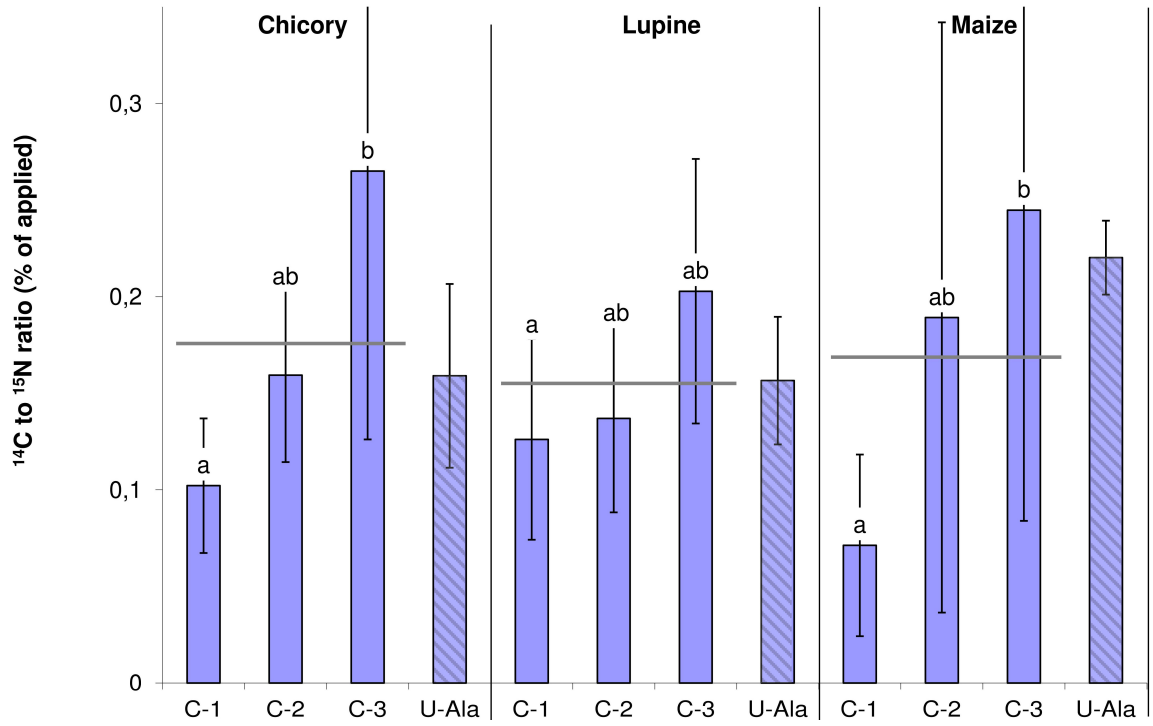

Fig. 4 Ratio of $^{14}C/^{15}N$ for individual alanine C positions incorporated in plant biomass. The alanine positions were C-1 (carboxyl group), C-2 (amino-bound group) and C-3 (methyl group). Letters indicate significant differences (p<0.001) between alanine C positions.

In order to compare the contribution of the three applied N sources, we estimated the tracer N nutrition budget. Comparing the role of alanine within the three investigated N sources, intact alanine uptake reached a maximum level of 0.25% of N. Lupine showed the highest N uptake in the form of intact alanine followed by chicory and maize (Table 3). The range of plant-specific relevance of intact alanine uptake (0.04-0.25%) reflected the plant-specific ability for N nutrition by organic sources.

Table 3 Intact uptake of alanine by chicory, lupine and maize and estimated contribution of intact alanine uptake to total N nutrition of these plants with respect to the other N sources.

	Chicory	**Lupine**	**Maize**
% ^{15}N uptake as intact alanine of total alanine-derived ^{15}N uptake	10.21 ± 3.48	13.70 ± 5.19	7.20 ± 4.70
% intact alanine of the three investigates N sources (alanine+ ammonium+ nitrate)	0.07 ± 0.04	0.25 ± 0.12	0.04 ± 0.02
Factor of overestimation of intact uptake based on uniform labeling	1.47 ± 0.25	1.14 ± 0.08	2.81 ± 1.89

The uptake of intact alanine reached a maximum of 13.7% of the total ^{15}N uptake from alanine (Table 3). The majority of the alanine molecules were metabolized within 6 h, when the initially organic-bound N was taken up as mineralized ammonium or even already oxidized to nitrate. This degradation of alanine as a percentage of the applied alanine is illustrated in Fig. 5. Intact alanine as well as mineralized alanine-derived N uptake was highest for lupine. Once fragmented, the uptake of C-1 was only half of that of C-3. This corresponds to the highest decomposition of C-1. This different fate of individual molecule positions demonstrates splitting of LMWOS which may have occurred in plant or soil.

However, less than 1% of the alanine C was recovered in plants at all, and the majority of the alanine (~99%) remains in soil or microbial biomass. From the alanine fragments, 1.1% to 9.2% of the mineralized N was taken up by plants, whereas C incorporation in plants ranged only from 0.01 to 1.58% (Fig. 5). Consequently, only a small portion of the applied ^{14}C but a relatively higher portion of the applied ^{15}N was taken up by plants and incorporated into their biomass after 6 h.

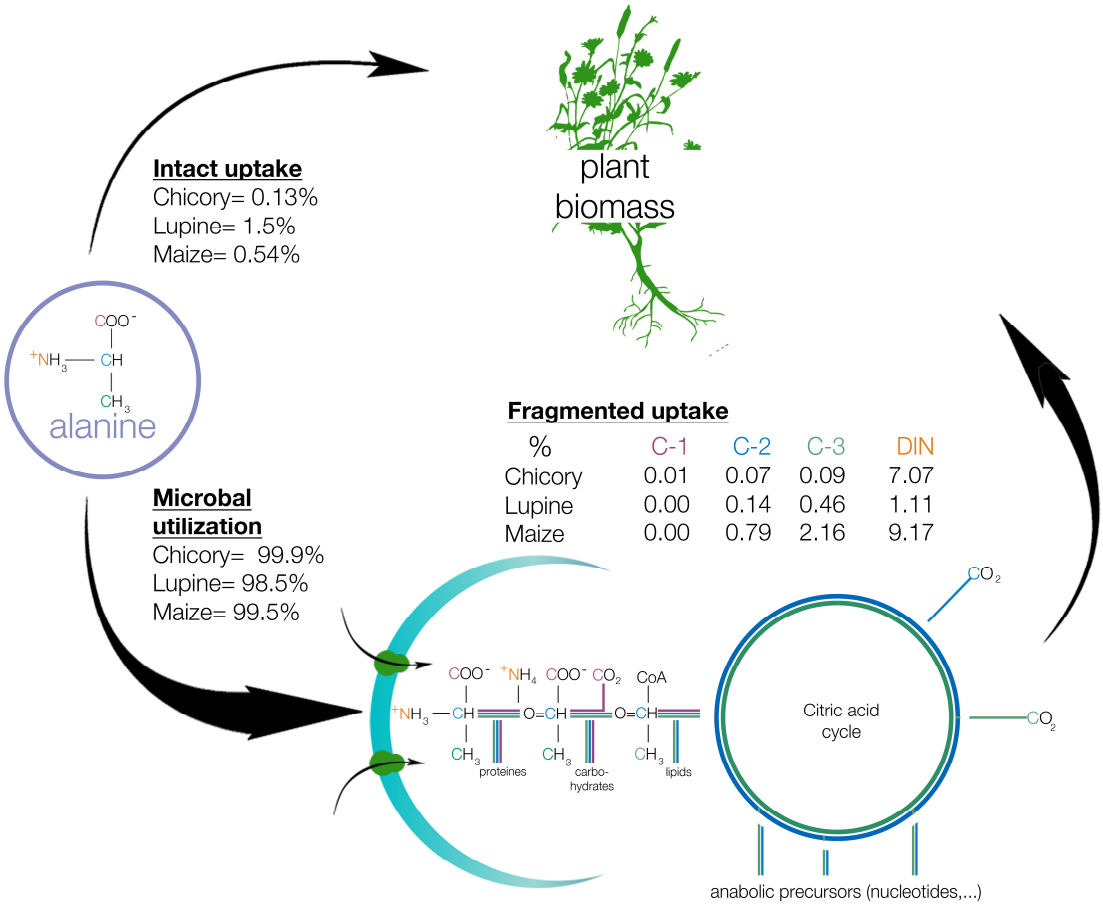

Fig. 5 Illustration of the fate of alanine tracer molecules, which are either taken up intact or degraded/mineralized to fragments and subsequently incorporated into plant biomass or microorganisms. Microbial metabolism of alanine by microorganisms is adapted from Dippold & Kuzyakov (in press)

2.9.4 Discussion

2.9.4.1 Plant uptake of N-containing and N-free organic substances

Our results showed no preferential uptake of ^{14}C from N-LMWOS alanine compared to ^{14}C from acetate – either taken up intact or as fragments - for any of the investigated plants. Biernath et al. (2008) found that maize had even higher uptake of acetate than alanine. This high uptake of acetate could mainly be attributed to passive uptake mechanisms (Rasmussen et al., 2010). As shown by Jones et al. (2005c) and Ge et al. (2009), a higher concentration of LMWOS and well-developed root systems increases plant competitiveness for LMWOS compared to microorganisms (Kuzyakov and Xu, 2013a; Xu et al., 2011). Stating a passive uptake means in this case the passive, unspecific transport of all LMWOS with the water flux towards the root surface, without any direct root-

specific regulation of amino acid transport. Specific conclusions about the contribution of various uptake systems at the plant surface cannot be stated from this study. If uptake is dominated by this passive flow of LMWOS towards the root surface, the bioavailability of alanine and acetate for plants would be the main driver for their uptake. Alanine can strongly interact with the soil matrix by its amino group, whereas acetate is less retained and consequently better available in the soil solution. In addition, microorganisms prefer alanine to acetate as a substrate (Fischer et al., 2010b; van Hees et al., 2002). We did not observe faster decomposition of acetate than alanine, but did not quantify incorporation into microbial biomass in this study. Alanine may have been preferentially incorporated by microorganisms, as previously observed by Fischer et al. (2010b), if the concentration of free alanine in the soil solution was lower than that of acetate.

The combination of high root development and higher availability of acetate explains the higher uptake of acetate by maize under the dominance of passive flux of LMWOS towards the roots. Thus, a potential explanation from these results is that LMWOS are taken up by plants passively, irrespective whether they contain N or not.

2.9.4.2 Fate of functional groups of alanine in soil

The loss of the carboxyl group by mineralization is higher than that of the methyl group in soil. Similar results were shown by Fischer & Kuzyakov (2010) for acetate, Nasholm et al. (2001) for glycine, Dijkstra et al. (2011a) for pyruvate, and Dippold & Kuzyakov (in press) for alanine. In all of these studies based on position-specific labeling, the mineralization of the carboxyl group was fastest compared to all other functional groups.

Comparing remaining alanine-^{14}C in soil after 6 h revealed the highest mineralization of C-1 with 68-70% followed by 30-45% and 34-52% for C-2 and C-3, respectively. This decomposition was even higher than that observed for 3 days in a field experiment being 89%, 49% and 29% for C-1, C-2 and C-3, respectively (Apostel et al., 2013). This higher mineralization reflects higher microbial activity under rhizosphere conditions compared to root-free soil (Blagodatskaya et al., 2009).

The C-1 position is rapidly oxidized by decarboxylation of the C-1 group of pyruvate, the most abundant microbial transformation product of alanine, within the microbial metabolism. This is an extremely fast process in soil (Dippold and Kuzyakov, in press) and kinetics of microbial uptake and metabolization are known to be faster than plant uptake (Jones et al., 2005a). In contrast, methyl groups represent reduced C and do not need to be further reduced for many anabolic pathways (Apostel et al., 2013; Dijkstra et al., 2011b). Therefore, C-3 was less mineralized, preferentially incorporated into microbial

metabolites, also into those metabolites released by microorganisms into soil solution.. In addition, Dippold and Kuzyakov (2013) found a partial extracellular oxidation of alanine following the order C-1>C-2>C-3, which may also contribute to a higher amount of C-3-fragments in soil. These fragments, if taken up by plants through passive mechanisms, cause the preferential incorporation of the C-3 position (Fig. 3 and Fig. 4). This preferential C-3 uptake as microbial metabolites seems to predominate the dark-fixation in the roots of microbially respired CO_2 (which would consequently have a C-1 enrichment).

In summary, microbial uptake and utilization were the main processes affecting the fate of individual C positions of LMWOS in soil. Preferential oxidation of C-1 and preferential incorporation of C-3 by microorganisms are likely to explain the preferential loss of C-1 and accumulation of C-3 in the entire plant-soil system.

2.9.4.3 Allocation and transformation of C and N within plants

The preference of crop plants for NO_3^- uptake has been reported in many studies (Ge et al., 2008; Hermans et al., 2006; Jones et al., 2005a) and was also confirmed in this experiment. When nitrate was used as the N source, maize reduced NO_3^- in shoots and in roots (Gavrichkova and Kuzyakov, 2008); lupine as well as chicory reduced NO_3^- mainly in roots (Gavrichkova and Kuzyakov, 2008; Goupil et al., 1998; Pate et al., 1981). Our results corroborate these findings, as the highest NO_3^- transport into shoots could be found in maize with a ^{15}N shoot/root ratio of 2.42 and lower ratios in chicory (1.35) and lupine (0.57) (Table 2). Low N allocation into shoots for chicory was also found by Ameziane et al. (1997): 8 days after labeling, chicory kept the majority of ^{15}N in its roots (shoot/root ratio 0.3). Reduced N sources like NH_4^+ or alanine showed no clear preference for allocation from root to shoot.

Svennerstam et al. (2007) found that incorporation of amino acids after intact uptake occurred as intact molecules, but could not prove this assumption as they neither labeled position-specific nor used compound-specific isotope analysis (CSIA) to measure plant amino acids. If intensive metabolization of amino acids in plants occurred, this would lead to a preferential decarboxylation and loss of C-1 and preferential incorporation of C-3, as observed in our study. Consequently, this approach would lead to an underestimation of intact uptake.

There are not many studies investigating the transformation of amino acids taken up by plants by means of CSIA. The intact incorporation without further transformation was first shown by Persson and Nasholm (2001) by GC-MS. Sauheitl et al. (2009a), who performed similar experiments with GC-C-IRMS, also found no indication for oxidation of incorporated amino acids within the plant metabolism. Both studies excluded transforma-

tion of the C backbone of amino acids into other amino acids, but not into other metabolic products. Total ^{14}C and ^{15}N uptake reflected that only minor portion of the amino acids is taken up and consequently could be metabolized by plants. However, there is a remaining uncertainty of the effect of plant metabolism, which may contribute to an underestimation of the calculated intact uptake if preferential C-1 oxidation occurred in plants.

Position-specific labeling in this experiment provided the first information about plant transformation of alanine by comparing the fate of individual molecule positions within the plant compartments. In lupine (Fig. 2), position C-1 was preferentially kept in the root and from position C-1 to C-3, an increasing allocation from root to shoot could be observed. Hence, either different fragments of alanine were allocated differently within the plant or intact alanine was partially split during 6 h by the plant metabolism. Ge et al. (2008) and Warren et al. (2012) found that amino acids can be transformed to other compounds to be transported to shoots. However, Warren et al (2012) and Sauheitl et al. (2009a) also indicate that transaminations are the most likely metabolic transformation within plants and that oxidation of the C skeleton is less likely.

The ^{14}C uptake by chicory was too low compared to variations between repetitions to detect a comparable position-specific trend. Maize showed increasing amounts of ^{14}C from alanine from C-1 to C-3 for shoots and roots. This could either result from a preferential oxidation of C-1 and C-2 after intact uptake or from a preferred uptake of C-3 fragments and their allocation into the shoots without transformation.

In summary, our results show that even if intact uptake occurs, plants tend to transform LMWOS rather quickly in their metabolism (Wegener et al., 2010) but mainly by transamination (Sauheitl et al., 2009a). The molecular nature of the newly formed metabolites can only be clarified by CSIA of the transformation products.

2.9.4.4 Intact uptake of alanine in plants

Physiological ability of intact alanine uptake by plants was shown by Svennerstam et al. (2007) who identified lysine histidine transporter 1 (LHT1) as a facilitator for amino acid uptake (lysine, glycine and alanine) by the roots of *Arabidopsis thaliana*. Many studies evaluated the relevance of N nutrition by intact amino acid uptake under natural soil conditions by using dual-isotope but uniformly labeled ^{13}C- and ^{15}N-tracers (Bardgett et al., 2003; Nasholm and Persson, 2001; Weigelt et al., 2003). Calculating the $^{14}C/^{15}N$ ratio of plant uptake (Fig. 4) is based on this approach (Nasholm et al., 1998) and reflects the intact alanine uptake. If we would average our alanine C positions, which correspond to the uniform labeling approach, we would detect intact uptake of around 15-18% of alanine-derived N without species-specific differences (Fig. 4). Calculating the uptake of

intact alanine based on the C-1 position, i.e. the position with the lowest uptake, gives values of intact uptake of 7-14% of alanine-derived N. This demonstrates a 1.2 to 3-fold overestimation, if the calculation is based on uniform labeling results compared to position-specific labeling.

In general, data for the highest intact uptake were found in boreal forests. This was partly explained by their nutrition via ecto-mycorrhization. However, many studies with grassland species and annual herbs also showed higher intact amino acid uptake than those observed in this study. This is a result of the methodological shortcomings of the uniform ^{13}C or ^{14}C labeling approaches. The use of position-specific labeling enables us to distinguish fragment uptake from whole molecule uptake and consequently demonstrates much lower uptake.

Rasmussen et al. (2010) expected the highest plant uptake of the C-1 position. They postulated that the high mineralization of C-1 leads to an increase in HCO_3^- from C-1 in the soil solution, which can be passively taken up by plants (Demidchik and Maathuis, 2007). Our results contradict this concept as we observed the highest incorporation rate with C-3. Thus, irrespective of the soil pH, a fast exchange of mineralized $H^{14}CO_3^-$ with atmospheric CO_2 leads to fast ^{14}C losses from mineralized molecule positions. The highest uptake of C-3 supports the idea of plant uptake of molecule fragments, i.e. microbial transformation products, by passive uptake mechanisms.

In contrast, position-specific C-2-labeling revealed that ~20% of the glycine-derived N was taken up as the intact amino acid by *Triticum aestivum* (Nasholm et al., 2001). However, C-2 of glycine as a methyl group resembles C-3 of alanine which had the highest uptake. This suggests that labeling of reduced C positions (Nasholm et al., 2001) is likely to cause an overestimation of the real intact amino acid uptake. In addition, intact uptake of glycine may be facilitated compared to alanine due to decreased competitiveness of soil microorganisms for glycine (Hocking and Jeffery, 2004). In addition, its smaller molecular weight facilitates passive uptake. The applied amino acid concentration can be another aspect to explain the higher range of intact uptake observed in many previous studies. For example, Nasholm et al. (2001) applied a 1 mM tracer solution, whereas we used a much lower concentration of 50 µM. An increased amino acid concentration improves plant competitiveness due to early saturation of microbial amino acid transporters (Kuzyakov and Xu, 2013a). Thus, amino acid uptake quantified at high concentrations may not resemble natural conditions as free amino acid concentrations rarely exceed 100 µM in soils (Jones and Willett, 2006) and bioavailable amino acid concentrations are even lower (Hobbie and Hobbie, 2012).

After glycine application to *Plantago lanceolata*, Sauheitl et al. (2009a) quantified intact uptake around 16.5% of glycine-derived N using ^{13}C- and ^{15}N-CSIA of amino acids.

This percentage is slightly above the values quantified here by position-specific ^{14}C labeling but up to 6fold lower than values gained by bulk isotope analysis. This confirms the overestimation of intact uptake gained by uniformly-labeling with bulk isotope analysis approaches.

We also found significant species-specific differences in the proportion of intact alanine-^{15}N to mineralized alanine-^{15}N uptake (Table 3). Lupine had the highest uptake of intact alanine followed by chicory and maize (Table 3). Maize is known to take up either amino acids or their degradation fragments (Adamczyk et al., 2012; Godlewski and Adamczyk, 2007). The highest position-specific differences measured in our study reveals that mainly microbially transformed C-3 fragments of alanine were taken up.

In contrast, lupine had a high total incorporation of alanine-derived N as well as high uptake of intact alanine. This can be attributed to its cluster roots (Hawkins et al., 2005) and to the very efficient amino acid transport systems, characteristic for legumes to facilitate transfer from the nodules of rhizobia (Day et al., 2001). Thus, plant ecophysiological characteristics can increase their chances to gain organic N.

In summary, the use of position-specific ^{13}C or ^{14}C labeling improved the quantification of intact uptake of amino acids by plants by revealing the contribution of fragment uptake. The highly efficient microbial competition for alanine decreases the intact uptake by plant roots.

2.9.4.5 Relevance of amino acids as a N source for agricultural plants

Within the three applied N sources, nitrate was preferred by the three plants irrespective of their ecophysiology. This preference of crops for nitrate has been shown in previous studies (Gavrichkova and Kuzyakov, 2008; Ge et al., 2009; Glass et al., 2002; Jamtgard et al., 2008) and is consistent with the soil properties in this study: The Luvisol, developed from loess, contains clay minerals (mainly illites) which can fix NH_4^+ and cause lower plant availability of cationic nutrients. Species specific preferences for N sources are in accordance with previous studies in grasslands (Weigelt et al., 2005): fast growing species – in our study maize – showed the highest uptake of nitrate.

The uptake of alanine-^{15}N was in the same range as ammonium-^{15}N. This indicates that presumably the majority of alanine-^{15}N was very fast mineralized to and taken up as ammonium which is confirmed by results of a previous study with tundra species (Schimel and Chapin, 1996).. Thus, ^{15}N uptake confirms the position-specific ^{14}C results (Fig. 3) that mainly partially metabolized or mineralized fragments are taken up. Also, other studies have demonstrated fast transformation of N-containing LMWOS: Jones et al. (2004) determined amino acid half-lives of 4 to 8 min in soil solution. Thus, within one

hour, applied amino acids are completely removed from soil solution and either incorporated into soil microorganisms, mineralized to ammonia or irreversibly fixed by the soil matrix.

Table 1 shows a small C:N-ratio of the microbial biomass (~9.9) and thus a low N demand of the microbial community. Hence, the main fate of microorganisms using N-LMWOS is the C skeleton. A similar strategy of microorganisms was observed for P-containing LMWOS in P-rich soils (Spohn and Kuzyakov, 2013). Thus, the majority of alanine-^{15}N will be mineralized, released as $^{15}NH_4^+$ and then be available for plant uptake. Therefore, the N mineralization activity of the soil microbial community is a crucial factor deciding whether the incorporation of amino acids N occurs intact or mineralized.

In summary, the majority of alanine-derived N was taken up by plants after mineralization and less than 1.5% of applied alanine as intact alanine. Thus, intact uptake of amino acids was the least relevant N source, contributing to less than 0.25% to the total N nutrition of the plant. The maximal relevance of amino acid-based N nutrition can be calculated assuming that all 20 proteinogenous amino acids have an uptake similar to alanine (although some of them have much lower concentrations in the soil than alanine). Thus, multiplying the alanine uptake with 20 gives an estimate of the total amino acid uptake. Comparing this with the ammonium and nitrate uptake measured in this study revealed that a maximum of 5% of plant N nutrition can be expected from all amino acids.

2.9.5 Conclusions and Outlook

This study emphasizes that position-specific labeling is a novel and unique technique to gain detailed insight into the importance of organic N sources and the uptake of LMWOS by roots from soil. The precision of previous estimates of intact uptake can be strongly enhanced using this new labeling approach without performing time- and cost-consuming measurements like compound-specific isotope $^{13}C/^{15}N$ analyses.

The comparison of N-LMWOS versus N-free LMWOS uptake revealed no significant differences in the ^{14}C incorporation from these sources. This supported the concept of passive uptake as one of the main uptake mechanisms for LMWOS by plants.

Position-specific ^{14}C labeling revealed that a minor portion of amino acids was taken up intact, whereas the majority of alanine was degraded by soil microorganisms. Some uncertainties remain as plant metabolization like root dark fixation (leading to an overestimation of intact uptake) and plant respiration (leading to an underestimation of intact uptake) cannot be quantified by this approach, too.

Mineralized N as well as fragments of the C skeleton was partially available in the soil solution for root uptake. Lupine, as the representative of the legumes in this study,

confirmed the general trend for a greater preference of legumes for organic N sources compared to non-legumes which might be attributed to their ecophysiological capability for amino acid transfer between nodules and roots. Maize, a plant species with fast growth, high N demand and water uptake showed a higher contribution of passive uptake and thus uptake of microbial transformation products (^{14}C-fragment and DIN).

In summary, comparing the relevance of DIN and amino acids for each of the investigated plants, irrespective of their ecophysiological specifics, the role of intact amino acid uptake within N nutrition was rather low. Our study suggests N uptake from organic sources is of minor importance for N nutrition of agricultural plants. Nevertheless, the ecophysiological role cannot be fully understood as long as the uptake and allocation mechanisms (passive/active transport, metabolization within the plant) as well as their regulating factors are not identified. Therefore, investigations with a broad spectrum of position-specific labeled LMWOS coupled with CSIA of plant and microbial transformation products are needed.

Acknowledgements

This study was financed by Deutsche Forschungsgemeinschaft DFG. We thank Ilse Thaufelder and Stefanie Bösel, technical staff at the University of Bayreuth and Martin-Luther University of Halle, respectively, C. Cavedon for figure design and C. Werner for her thorough review.

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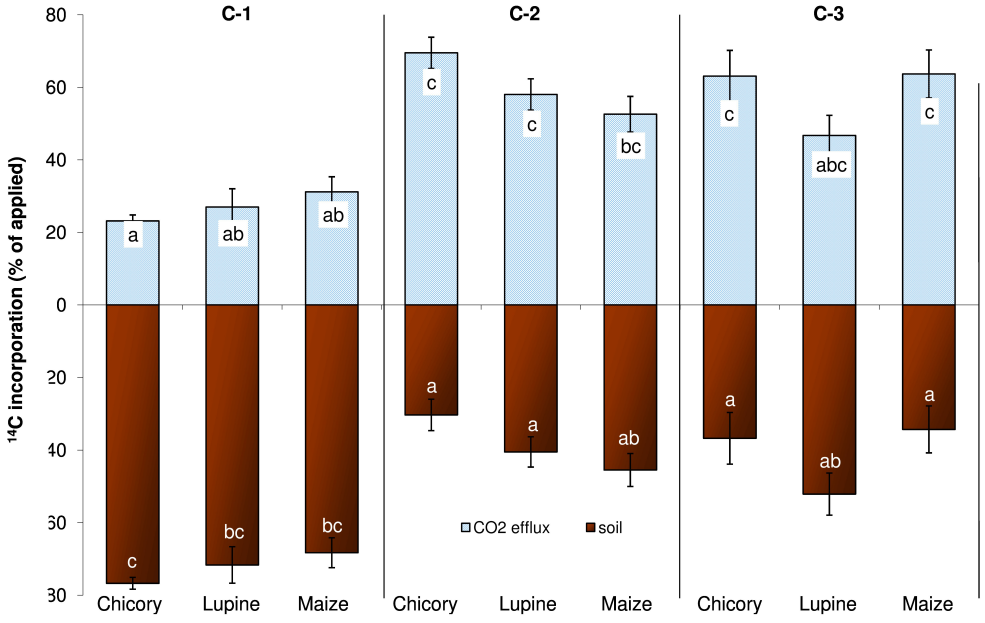
Supplementary Data

Supplementary Table: Mean of above- and belowground biomass (dry weight in g) ± standard error of the three plant species determined after biomass harvesting.

Biomass (dry weight in g)	belowground			aboveground		
Chicory	4.57	±	3.78	10.13	±	5.46
Lupine	12.78	±	4.75	8.24	±	4.43
Maize	15.55	±	6.77	20.47	±	2.46



Supplementary Figure: Percentage of ^{14}C incorporation in soil and CO_2 efflux after position-specific labeling with alanine. The alanine positions were C-1 (carboxyl group), C-2 (amino-bound group) and C-3 (methyl group). Letters indicate significant differences ($p<0.001$) between alanine C positions.

Additional peer-reviewed pubications

Tian, Jing, Dippold M, Pausch J, Blagodatskaya E, Fan M, Li X, Kuzyakov Y (2013): Microbial response to rhizodeposition depending on water regimes in paddy rice soils. Soil Biology and Biochemistry: http://dx.doi.org/10.1016/j.soilbio.2013.05.021

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Acknowledgements

Ich danke allen, die mir während meiner Doktorarbeit zur Seite standen ganz herzlich.

Mein besonderer Dank gilt meinem Doktorvater Prof. Dr. Yakov Kuzyakov: durch einem auf mein Interessengebiet zugeschnittenen DFG Antrag ermöglichte er mir die Promotion in diesem spannenden Forschungsfeld zwischen Biochemie und Bodenkunde. Er lies mir stets den nötigen Freiraum eigene Ideen in allen Ebenen wissenschaftlich Arbeitens zu verwirklichen, was mich außerordentlich motivierte. Auch für die intensive Einführung in die Wissenschaftsgemeinde im Rahmen zahlreicher Tagungsteilnahmen möchte ich mich bei ihm recht herzlich bedanken.

Ein außerordentlicher Dank geht an Prof. Dr. Bruno Glaser an dessen Institute ein maßgeblicher Anteil der Analytik erfolgte. Aus den zahlreichen Diskussionen und Gesprächen konnte ich sowohl wissenschaftlich als auch persönlich sehr profitieren. Im speziellen für seine Freundschaft und sein immer offenes Ohr möchte ich mich recht herzlich bedanken.

Des Weiteren gilt mein Dank Prof. Huwe, der während meiner Doktorarbeit Laborräume und Gerätschaften zur Fertigstellng meiner Disseration bereitstellte. Ebensogroßer Dank gilt Prof. Dr. Christiane Werner Pinto, die mich sehr herzlich in ihrer Arbeitsgruppe aufnahm und mir in vielen Gesprächen stets hilfreich zur Seite stand. Insbesondere möchte ich Prof. Dr. Wolfgang Zech danken, durch dessen Förderung ich für die Bodenkunde begeistert wurde und auf dessen Unterstützung ich immer zurückgreifen konnte.

Der DFG danke ich für die Finanzierung dieses Projekts (DFG KU 1184/19-1) zur Aufklärung positionsspezifischer Transformationen in Böden.

Ein besonderes Dankeschön geht an Stefanie Bösel, deren Geschick am Isotopenmassenspektrometer wichtigste Voraussetzung für das Zustandekommen des Umfangreichen Datensatzes war. Die aus den vielen gemeinsamen Stunden am IRMS entstandene Freudschaft hat mir im Rahmen meiner Dissertation sehr geholfen. Auch Ilse Thaufelder, die immer mit Rat und Tat zur Seite stand, hat meine Laborarbeit in Bayreuth sehr erleichtert und bereichert.

Spezieller Dank gilt meinen beiden Diplomarbeitsbetreuern Dr. Leopold Sauheitl und Jago Birk, denen ich das Wissen und die analytischen Fähigkeiten zur Durchführung dieser Doktorarbeit verdanke. Neben einer fortlaufend, konstruktiven Zusammenarbeit mit beiden standen sie mir auch als Freunde in jeder Lebenslage zur Seite. Ebenso in-

tensive Unterstützung habe ich durch PD Dr. Michael Zech erfahren, der sowohl fachlich in vielen Diskussionen zum Gelingen der Dissertation beigetragen hat als auch persönlich mein Leben sehr bereichert hat. Für die konstruktive Zusammenarbeit und ihre stets hilfreiche Freundschaft möchte ich mich außerdem bei Dr. Guido Wiesenberg und Dr. Björn Buggle bedanken.

Tiefe Dankbarkeit empfinde ich für meine beiden, treuen Freundinnen Janine Sommer und Katharina Leiber, deren Freundschaft ich mir während all der Jahre unabhängig von allen äußeren Faktoren immer sicher sein konnte. Neben der aktiven Unterstützung in Feld und Labor hat v.a. die Kraft, die ich aus diesen Freundschaften ziehen konnte, das Zustandekommen dieser Doktorarbeit ermöglicht.

Meinen herzlichen Dank möchte ich allen Korrekturlesern dieser Arbeit ausprechen, Leopold Sauheitl, Bruno Glaser, Michael Zech, Thomas Friedel, Carolin Apostel und meiner Schwester Christine.

Ein in Worte nicht zu fassender Dank geht geht jedoch an meine Familie – meiner Schwester Christine, meinem Bruder Tobias, meinem Vater Klemens, Gerlinde, und meiner Patin Anna, die allen nächtlichen Laborschichten, ständigen Dienstreisen und tagelangen Computersessions zum Trotz mich immer unterstützt haben. Ihre Liebe und Rückendeckung in jeder Lebenslage waren die Grundlage für das Zustandekommen dieser Arbeit.

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