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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 decomposition. Combination of position-specific ^{13}C labeling with compound-specific ^{13}C-PLFA analysis is a novel tool to trace metabolic pathways. This combination was used to analyze short-term transformations (3 and 10 days after tracer application) of two key 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, microbial biomass (by CFE) and in cell membranes of microbial groups classified by ^{13}C-PLFA.

The ^{13}C incorporation in the Gram negative bacteria was higher by one order of magnitude compared to all other microbial groups. All of the ^{13}C recovered in soil on day 3 was allocated in microbial biomass. On day 10 however, a part of the ^{13}C was recovered in non-extractable microbial cell components or microbial excretions. As sugars are not absorbed by mineral particles due to a lack of charged functional groups, their quick mineralization from soil solution is generally expected. However, microorganisms transformed sugars to metabolites with a slower turnover. The ^{13}C incorporation from the individual glucose positions into soil and microbial biomass 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 ^{13}C incorporation from individual glucose positions into PLFAs showed intensive recycling of the added ^{13}C via gluconeogenesis and a mixing of both glucose utilizing pathways. The pattern of position-specific incorporation of ribose C also shows initial utilization in the pentose phosphate pathway but is overprinted on day 10, again due to intensive recycling and mixing. This shows that glucose and ribose – as ubiquitous substrates – are used in various metabolic pathways and their C is intensively recycled in microbial biomass.

Analyzing the fate of individual C atoms by position-specific labeling deeply improves our understanding of the pathways of microbial utilization of sugars (and other compounds) by microbial groups and so, of soil C fluxes.

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 (1–10 h mean residence time) and a quantitatively relevant gross flux of C passes through this pool (30% of total CO_2 efflux) (van Hees et al., 2005). 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

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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., 2012; 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 to 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 substrates 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 metabolic pathways – has recently reached an increasing consideration in soil science (Fischer and Kuzyakov, 2010; Dijkstra et al., 2011a; Apostel et al., 2013; Dippold and Kuzyakov, 2013). 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 et al., 1995; Zelles, 1999), 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-1and 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. Materials and methods

2.1. Field experiment

2.1.1. 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_{H2O} of 6.49, a TOC and TN content of 1.77% and 0.19%, respectively. CEC was 13 $cmol_C\ kg^{-1}$.

2.1.2. Experiment design

Field experiment is described in detail in Apostel et al. (2013). Briefly, the 12 × 12 m field was divided into four quadrants, for four replications. PVC-tubes (diameter: 10 cm, height: 13 cm) were installed 10 cm deep in the soil. 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 within the block.

2.1.3. Sampling and sample preparation

Sampling occurred 3 and 10 days after labeling. Complete columns from one set (background, uniformly and position-specifically labeled) were dug out. Soil volume and density were determined; a subset was sieved to 2 mm for further analysis and

Table 1
Locations of ^{13}C in position-specific 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})	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
C amount (μmol g $soil^{-1}$)	2.72	2.77	2.76	2.73	2.93	2.70	2.70	2.71
Atom%^{13}C	12.36	12.36	12.43	12.34	12.71	12.83	12.82	13.46

stored at 5 °C for less than one week until chloroform-fumigation extraction and at −20 °C for PLFA-extraction.

2.2. Analytical methods

2.2.1. Bulk soil measurements

Bulk soil C content and δ^{13}C-analysis were measured 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 the mixing model Eqs. (1) and (2), where the C content of the background in Eq. (1) was substituted according to Eq. (2).

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

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

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%)

2.2.2. Chloroform fumigation extraction

Microbial biomass and its δ^{13}C values were determined by Chloroform fumigation extraction according to Apostel et al. (2013).

The C content was measured on the TOC analyzer multi C/N® 2000 (Analytik Jena, Jena, Germany). δ^{13}C was 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 Eqs. (1) and (2) and microbial biomass C and for ^{13}C incorporation, an extraction factor of 0.45 was applied according to Wu et al. (1990).

2.2.3. PLFA-analysis

Phospholipid analysis was performed analogue to Apostel et al. (2013): Extraction and purification is a modified method by Frostegård et al. (1991). For gas chromatography (GC) measurements, the fatty acids were saponified, then derivatized into fatty acid methyl esters (FAME) according to Knapp (1979). External standards consisting of the 27 fatty acids given in Supplementary Table 1 and an internal standard were 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. Quantification occurred via a linear regression calculated from the external standards.

δ^{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 δ^{13}C-values. The δ^{13}C of the second reference gas peak was defined as −40‰ and all other δ^{13}C values were calculated by comparison.

The chromatograms were evaluated with ISODAT NT 2.0.

Linear regressions were calculated from CO_2 reference gas (99.995% pure) peaks to correct for any drift during measurements.

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 δ^{13}C-values to their area were calculated. If both regressions were significant, that with the higher significance was applied. As the δ^{13}C-value for the derivatizing agents was unknown, the correction was performed according to Glaser and Amelung (2002) (Eq. (3)).

$$C_{FS}(at\%) = \frac{N(C)_{FAME}}{N(C)_{FS}} \cdot \left(C_{FAME-DK}(at\%) - \left(m_{lin/ln} \cdot A_{FAME} + t_{lin/ln} \right) \right) + C_{EA-FS}(at\%) \quad (3)$$

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. 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):

$$DI_i = \frac{n \cdot C_i}{\sum_1^n C_i} \text{ or } = \frac{n \cdot C_i}{C_u} \quad (4)$$

with:

n: number of C atoms in molecule
C_i: relative incorporation of tracer C [$mol \cdot mol^{-1}$]
C_u: relative incorporation of uniformly labeled tracer C [$mol \cdot mol^{-1}$]

The DI compares the incorporation of C from each position with the mean C incorporation from all positions of a molecule in any desired pool. 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.

2.4. 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 replication analyses of δ^{13}C-values. According to a factor analysis of PLFA amounts of the entire dataset, the PLFA were classified into corresponding microbial groups. 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 et al., 1995; Zelles, 1999). All data was 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 (03.04.2013).

3. Results

3.1. Pool sizes and 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 than glucose.

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, trends 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. These trends were 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-1was incorporated significantly less than ^{13}C-5. 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 remained on day 10. In microbial biomass, we observed equal recoveries of glucose ^{13}C-1 and ^{13}C-4, which were lower than the equally high recoveries of glucose ^{13}C-2 and ^{13}C-6 on day 3. There was also more than twice as much ribose ^{13}C-5 than ^{13}C-1 extracted in microbial biomass 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 tracer recovery

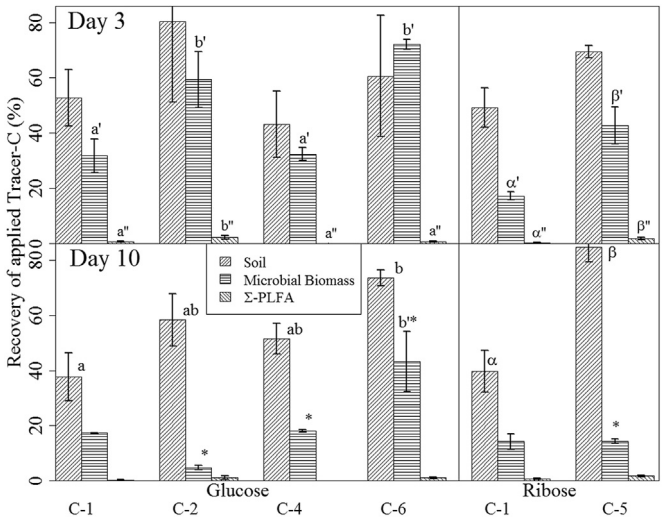

Fig. 1. Recovery of position-specific^{13}C labeled glucose and ribose in soil, microbial biomass and Σ-PLFA, three (top) and ten days (bottom) after application. Letters indicate significant differences ($p < 0.05$) between recovery in bulk soil (glucose: a, ribose α), microbial biomass (glucose: a′, 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.

decrease, but also the pattern of incorporation of individual positions changed. 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 soil, these positions were considered to be stabilized in the soil as microbial residues.

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 1). 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.

3.3. Tracer uptake of functional microbial groups

The PCA of the PLFAs C-contents from both sampling times and the comparison of the statistical grouping with literature (Zelles et al., 1995; Zelles, 1999) led to a classification of PLFAs into 7 microbial groups (Supplementary Table 1). 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 into their PLFAs 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

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 (mg C g^{-1} soil)	Day 3	14.533 ± 2.616	0.493 ± 0.052	0.042 ± 0.003
	Day 10	16.332 ± 1.695	0.455 ± 0.111	0.050 ± 5.95
Glucose^{13}C recovery (ng glucose-^{13}C g^{-1} soil)	Day 3	1113.53 ± 34.73	576.96 ± 36.19	16.81 ± 0.54
	Day 10	1198.14 ± 120.61	312.48 ± 75.29	17.33 ± 0.66
Ribose^{13}C recovery (ng ribose-^{13}C g^{-1} soil)	Day 3	546.34 ± 23.42	335.24 ± 47.14	11.02 ± 0.16
	Day 10	649.71 ± 58.18	307.04 ± 22.52	13.61 ± 0.10

microorganism groups. There was little to no incorporation of glucose ^{13}C-4 and ribose ^{13}C-1 in all microorganisms' PLFAs. 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.

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.

4. Discussion

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%). It has to be considered that tracer decomposition was not quantified by direct $^{13}CO_2$ measurement but by the loss of ^{13}C from bulk soil and this indirect determination contains higher uncertainties than a direct measurement of mineralization. Thereafter, the ^{13}C incorporation in microbial biomass further decreased, but the total residue in soil did not decrease significantly (Fig. 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 less 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). Transformation of the applied ^{13}C towards polymeric, non-dissolvable compounds will increase the potential for stabilization in soils (Miltner et al., 2007, 2009).

The average amount of non-mineralized glucose and ribose remaining in soil on day 3 were in a comparable range, indicating that both substances are partitioned similarly between anabolism and 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 behavior of ribose and glucose ^{13}C in bulk soil confirms the similar microbial uptake and the utilization of both substances. Whereas the majority of glucose ^{13}C was found 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 faster into non-extractable pools of the microbial biomass. One possible, direct biochemical utilization of ribose C is 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, differences in the ratio of extractable, microbial ^{13}C to total soil ^{13}C between glucose and ribose already indicate that C from both substances is transferred into different compound classes by 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 labeling with acetate (data not presented), which showed that the incorporation of ^{13}C from LMWOS into PLFA after pulse labeling was not homogenous, but mainly occurred in terminal or specific functional groups of the PLFA, which have a higher turnover then the entire fatty acid molecules.

4.2. Microbial utilization of individual positions of glucose and ribose molecules

We tried to use the specific incorporation of ^{13}C from individual positions of the molecules to assess 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 process is equal to the decarboxylation described by Apostel et al. (2013) and Dippold and Kuzyaov (2013) for the highest oxidized position of alanine (which becomes deaminated to pyruvate) and pathways suggested by Dijkstra et al. (2011a) after position-specific pyruvate labeling. As a consequence, the labeled C-4 position is expected to show a lower incorporation into microbial products and no incorporation into PLFAs formed from acetyl-CoA – precursors (Caspi et al., 2008). Both phenomena can be observed in this study (see Fig. 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) (Fig. 5). However, we also observed a significantly lower incorporation of C-1 than C-6 into extractable microbial biomass products after 3 days. This indicates that, in addition to glycolysis, a portion of glucose molecules was transformed by other pathways, presumably 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 (Fig. 4) (Scandellari et al., 2009). The parallel existence of glycolysis and 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, suggesting a wide spectrum of various metabolic states of individual cells.

The loss of pathway-specific, positional 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 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). Therefore, position-specific labeling will loose its potential for metabolic tracing with continued transformation.

A similar loss of pathway-specific fingerprints could be observed for the microbial products formed from ribose: the clear preference for C-5 incorporation in the extractable microbial products at day 3 cannot be detected anymore at day 10. 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 is characteristic for 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. Conversion of C-1 and C-2 of ribose leads to a preferential oxidation of C-1 if this C is allocated to glycolysis afterward 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 afterward 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, 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 shown 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 the DI to approach 1 with continued microbial turnover.

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; Frostegård et al., 2011). Coupling ^{13}C biomarker analysis with uniform ^{13}C labeling enables tracing C utilization and partitioning within microbial cells and microbial communities. However, only position-specific labeling allows the identification of the transformation steps of the added substrate and the reconstruction of metabolic pathways (Dijkstra et al., 2011a; Dippold and Kuzyakov, 2013).

Uptake of ^{13}C and incorporation in PLFAs (Fig. 2) was highest in Gram negatives. This is in accordance with previous studies using further LMWOS (Apostel et al., 2013; Gunina et al., 2014) and DNA studies on glucose ^{13}C uptake (Padmanabhan et al. 2003). It has to be taken into account that uptake into PLFA is not a quantitative measure of uptake into the microbial group represented by the biomarker PLFA, as intracellular C partitioning between the various cellular compounds as well as the surface (cell membrane) to volume ratio may differ for individual microbial groups. The last point is especially important when comparing eukaryotic to prokaryotic organisms. However, Gram negatives are already known to dominate in the rhizosphere and the uptake of low molecular weight rhizodeposits. Considering the whole microbial community in soil, Gram negatives (Treonis et al., 2004; Tian et al., 2013) are often

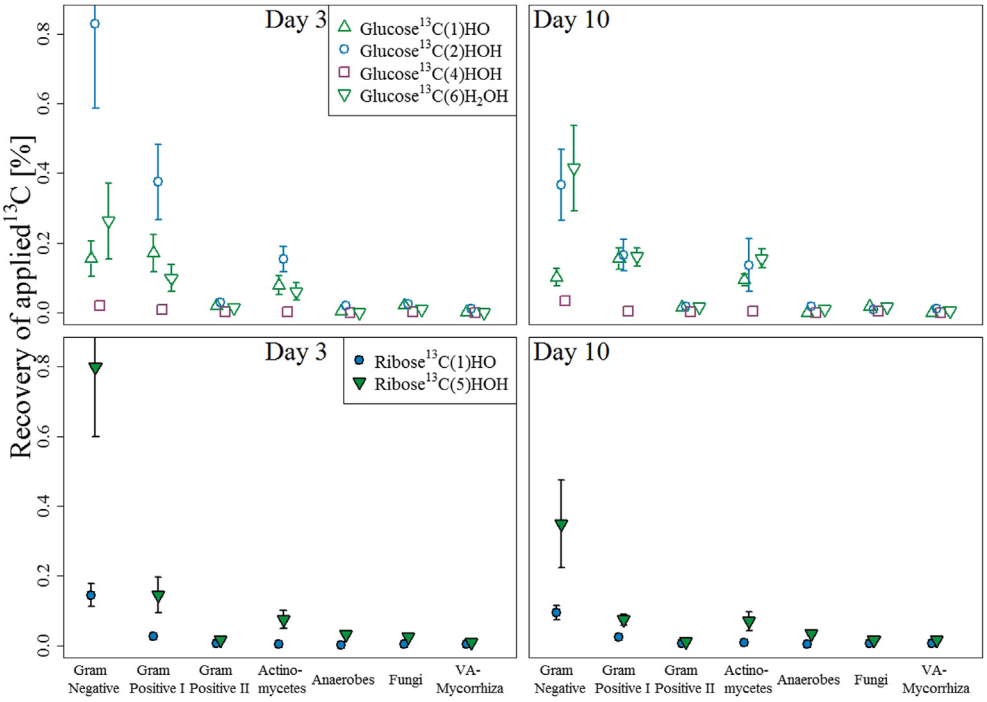

Fig. 2. Recovery of applied ^{13}C from molecule positions of glucose (top) and ribose (bottom) in microbial groups after three and ten days. Letters indicate significant differences ($p < 0.05$) between the individual C positions of glucose and ribose. Error bars show standard error of the mean from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

considered r strategists. In contrast, Gram positives may gain C mainly from old SOM and have properties of K strategists (Kramer and Gleixner, 2006). Theses studies suggest that the Gram positives are less competitive for LMWOS (like easily available monosaccharides), which is in accordance to the lower ^{13}C incorporation in PLFAs by Gram positives observed 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 (Fig. 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 and especially the DI (Fig. 3) revealed a more specific picture: the pattern of ribose ^{13}C recovery in PLFAs is characterized 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) (Fig. 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 (i.e. convergence) 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 Fig. 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 (Fig. 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

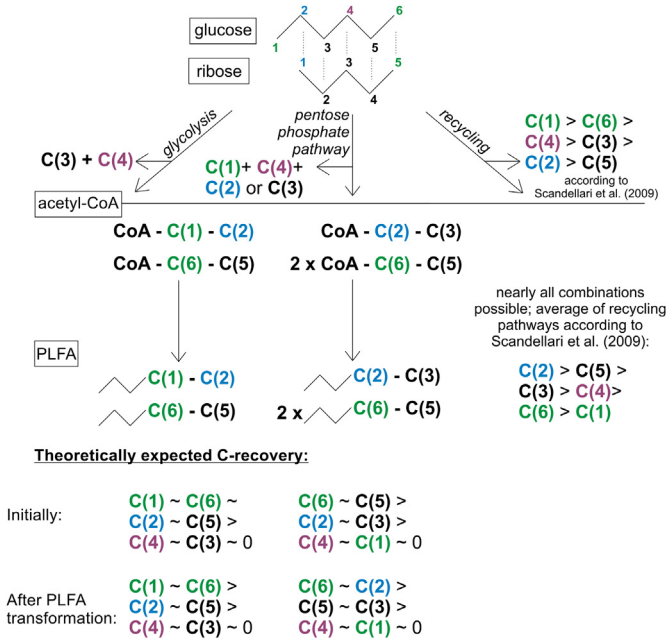

Fig. 4. Theoretical fate of individual glucose and ribose C positions of single pathways. Left: glycolysis; middle: pentose phosphate pathway and right: mixing of glycolysis, backflux via triose-triangle and pentose phosphate pathway: results for the right branch are experimental data (for fungi) taken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

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, reversal of the hexose backbone (which results in a molecule with C-6 at the position of C-1 and accordingly, C-5 as C-2, C-4 as C-3 and vice versa) by the triose

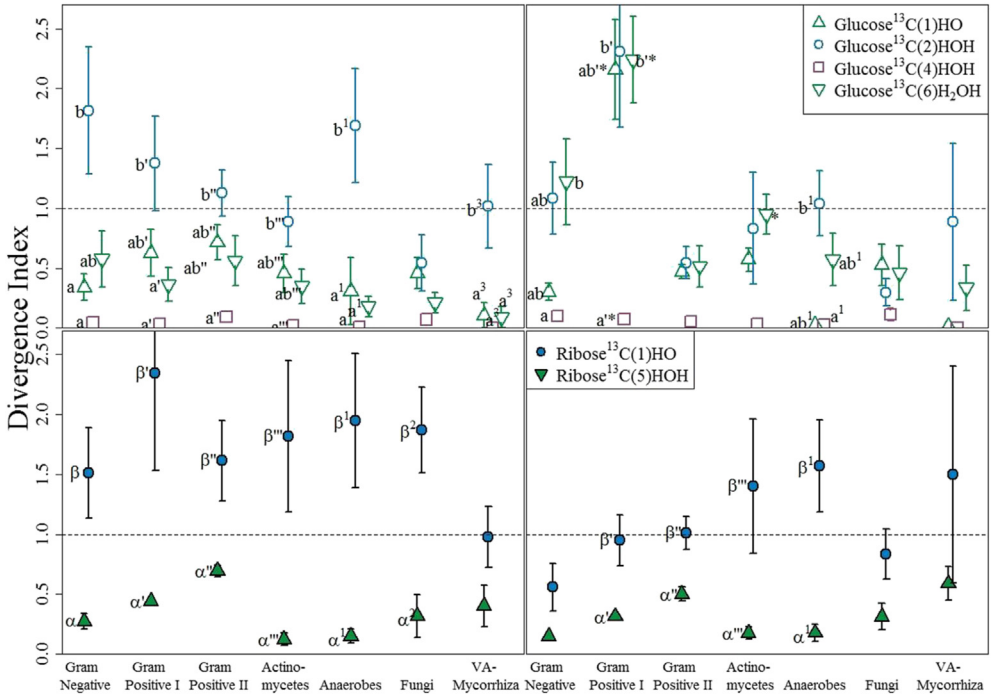

Fig. 3. Divergence Index (DI), reflecting discrimination between the C positions of glucose and ribose, three (top) and ten (bottom) days after application. Letters indicate significant differences ($p < 0.001$) between the relative incorporation of the C positions into the individual microbial groups.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

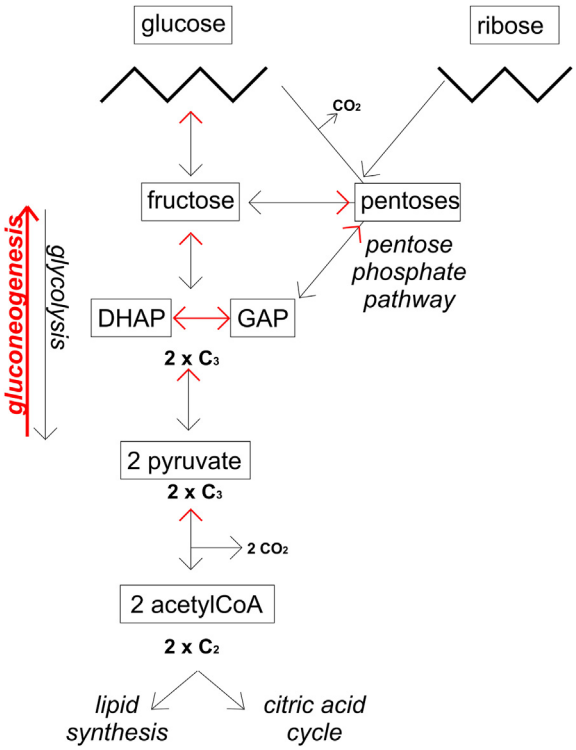

Fig. 5. Microbial transformation pathways of glucose and ribose. Black arrows indicate catabolic pathways, transferring C towards the oxidizing steps of pyruvate dehydrogenase reaction and citric acid cycle. Red arrows indicate anabolic pathways transferring C "up" for the construction of new microbial biomass compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

triangle (Scandellari et al., 2009) and gluconeogenesis (Fig. 4). Therefore, a significant part of glucose has to flow through these reversible pathways more than once in the prokaryotic groups (Fig. 5) to make this pattern apparent. 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, visible in the bacterial PLFAs, supports the concept of a slow-cycling, fungi-based and a fast-cycling, bacteria-based branch of the food web (Moore et al., 2005).

4.4. Metabolic tracing by position-specific labeling of monosaccharides

Monosaccharaides, 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 (Wegener et al., 2010; Dijkstra et al., 2011b, 2011c; Apostel et al., 2013; Dippold and Kuzyakov, 2013). Fatty acid synthesis pathways branch off from basic C metabolism after the pyruvate dehydrogenase step forming acetyl-CoA (Fig. 5). Therefore, pyruvate is a more direct precursor for metabolic tracing in lipids, which causes advantages for pathway reconstruction: A lower number of reversible steps, mixing the C positions, are located between educt and product. Consequently, this enables a 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). 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 frequently located in the citric acid cycle or fatty acid formation (Apostel et al., 2013).

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. 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, which would be impossible based on uniform labeling, only. However, for further studies with LMWOS labeling, it is advantageous to sample 1) earlier than 3 days after application of tracer and 2) with a higher temporal resolution. Even after 3 days, the results of this study suggest a complex network of C-recycling and reconstructing, which increases uncertainties and therefore the risk for misinterpretations of metabolic pathways.

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 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 identified by the 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. 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 ^{13}C-2 into PLFA was the highest, followed by the equal recovery of C-1 and C-6. This could only be explained by the parallel pathways or rapid succession of 1) glycolysis, 2) pentose phosphate pathway 3) reversal of the trioses and 4) gluconeogenesis, followed by a repetition of these processes with the newly formed glucose molecules. This shows that not only anabolic and catabolic pathways exist in parallel in soil microbial communities, but they are also used in fast succession 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. Already after 3 days, C-recycling creates an ambiguous picture, and necessary interpretations increase the possibility of misidentification of metabolic pathways. Thus, for monosaccharide metabolite tracing time intervals have to be shortened and frequency of sampling increased. To further reduce the ambiguity of the results, the "metabolic distance" between educt and product should be decreased, e.g. by using pyruvate or its metabolic precursors for tracing lipid synthesis or citric acid cycle activity.

The method of coupled position-specific ^{13}C labeling and compound-specific isotope analysis helps to identify the transformation steps of LMWOS. Determination C partitioning within microbial biomass pools will improve our understanding of C stabilization processes of microbial compounds in soil.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.09.005.

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