Glucose C turnover in cell compartments and microbial groups in soil

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Abstract

Microorganisms regulate the carbon (C) cycle in soil, controlling the utilization and recycling of organic substances. To reveal the contribution of particular microbial groups to C utilization and C turnover within the microbial cells, fate of $^{13}$C-labeled glucose was studied under field conditions. The $^{13}$C was traced in cytosolic substances, amino sugars and phospholipid fatty acids (PLFA) at intervals of 3, 10 and 50 days after glucose addition.

$^{13}$C enrichment into PLFA (~1.5% of PLFA C at day 3) was one order of magnitude greater than into the cytosol, showing the importance of cell membranes for initial C utilization. $^{13}$C enrichment of amino sugars in living microorganisms at day 3 accounted for 0.57% of total pool, resulting that the renewal of C in cell wall components is two times slower than that of cell membranes. Turnover time of C in the cytosol (150 days) was three times longer than in PLFAs (47 days). Consequently, despite cytosol is a pool with fast process rates, intensive recycling of cytosol components, within the living cells, leads to a longer C turnover time. Amino sugars originate mainly from microbial residues, thus longer experimental periods are required for estimation of C turnover times in that pool.

Both PLFA and amino sugar profiles indicated that glucose C was preferentially used by bacteria. The $^{13}$C incorporated into bacterial cell membrane components decreased with time, but it remained constant or even increased for filamentous microorganisms. Hence, over a short period, bacteria contribute more to the utilization of low molecular weight organic substances, whereas filamentous microorganisms are responsible for further C transformations. Thus, tracing $^{13}$C in cellular compounds with contrasting turnover rates elucidated the role of microbial groups and their cellular compartments in C utilization and recycling in soil. The results also reflect that microbial C turnover is a phenomenon that is not restricted to the death or growth of new cells, but that even within living cells, highly polymeric cell compounds, including cell walls, are constantly replaced and renewed. This
information is especially important for assessing C fluxes in soil and the contribution of C from microbial residues to soil organic matter.

Keywords

Microbial biomarkers; phospholipid fatty acids; amino sugars; $^{13}$C labeling; glucose utilisation; soil microbial biomass.
1. Introduction

Over the last decade, numerous studies have demonstrated the role of soil microorganisms in regulating the fate and transformation of organic compounds. Soil microorganisms produce exoenzymes to carry out the primary degradation of plant as well as microbial polymers to monomers. Further transformations of monomers then take place within the microbial cells. Monomeric substances pass into the living microbial pool and are partly mineralised to CO$_2$, while part is assimilated into cell polymers and ultimately incorporated into soil organic matter (SOM) after cell death (Kindler et al., 2006). Understanding the fate of substances originated from plants and microbial residues into living biomass is therefore crucial for estimating the recycling of carbon (C) in soil and its stabilization as SOM.

Living microbial biomass (MB) is a highly active and heterogeneous pool (Malik et al., 2015), although it accounts for only 2-4% of the total SOM (Jenkinson and Ladd, 1981). Heterogeneity is evident at the level of single cells in the various cellular compartments with different properties, structures and biochemistry: from the highly heterogeneous cytosol (Malik et al., 2013), to well-structured cell membranes and cell walls. Due to their chemical composition and functions, compounds of cell membranes (phospholipid fatty acids (PLFAs)) and cell walls (amino sugars) have different turnover times within the cell as well as different stabilities within SOM.

Organic compounds that are taken up by microorganisms first enter the cytosol (Gottschalk, 1979), which has a high heterogeneity in composition (includes components of various chemical structure and molecular weight). However, due to the heterogeneity of this pool, the calculated C turnover time is a mean of turnover times of various components. The calculated turnover time of intact PLFAs in soil after microbial death is 2.8 days (Kindler et al., 2009), resulting PLFAs are mainly used to characterize the living microorganisms (Frostegard et al., 2011; Rethemeyer, 2004). However, no data concerning turnover time of
PLFA C in the living biomass are currently published. The formation of amino sugars from plant biomass is relatively rapid at 6.2–9.0 days (Bai et al., 2013), whereas their turnover times in soil vary between 6.5–81.0 yr⁻¹ (Glaser et al., 2006). Thus, PLFAs and amino sugars can be used to trace the fate of C within the living microorganisms and estimate their contribution to SOM (Schmidt et al., 2007).

Some cell compartments, such as the cytoplasm, are not specific for various microbial groups, whereas phospholipids are partly specific and consequently can be used to estimate microbial community structure. Thus, PLFAs of bacterial (i16:0, a16:0, i15:0, a15:0, 16:1ω7, 18:1ω7) and fungal communities (18:2ω6,9; 18:3ω6,9,12; 16:1ω5) are used to draw conclusions about the qualitative composition of living microbial communities, their contribution to utilisation of C by various origin (plant or microbial) and to understand trophic interactions within the soil (Ruess et al., 2005). In contrast, amino sugars (glucosamine, galactosamine, mannosamine and muramic acid) are usually used to assess the contributions of bacterial and fungal residues to SOM (Engelking et al., 2007; Glaser et al., 2004). Muramic acid is of bacterial origin, whereas glucosamine is derived from both fungal and bacterial cell walls (Glaser et al., 2004). Galactosamine is more abundant in fungal than in bacterial cell walls (Engelking et al., 2007; Glaser et al., 2004).

Bacteria and fungi have various chemical composition, which strongly contributes to their turnover rates in soil: for bacteria it consists 2.3-33 days, whereas for fungi it accounts for 130-150 days (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). Despite turnover of microorganisms directly effect the C turnover rates in intercellular compounds (cell membrane and cell wall biomarkers), this relationship has rarely been investigated so far. However, the comparison of C turnover for cell membrane and cell wall components can be used to characterize the contribution of various microbial groups to medium-term C utilisation and to the stabilization of microbially derived C in SOM.
Combination of PLFAs and amino-sugar biomarkers analyses, as well as cytosolic C measurement with isotope tracing techniques (based on $^{13}$C natural abundance or $^{13}$C/$^{14}$C labelling) have been used in various studies to characterize organic C utilisation by the microbial community (Bai et al., 2013; Brant et al., 2006). However, to date no systematic studies have compared these contrasting cell compartments in a single soil within a C turnover experiment. Therefore, this study aimed to examine C allocation to various cell compartments following $^{13}$C labelling with a ubiquitous monomer, glucose. Glucose has a higher concentrations in the soil solution compared to other low molecular weight organics (Fischer et al., 2007), due to its diverse origin: from cellulose decomposition, presence in rhizodeposition (Derrien et al., 2004; Gunina and Kuzyakov, 2015), and synthesis by microorganisms. It is also used by most of the microbial groups, and, thus, is the most suitable substance for such a study.

We analyzed glucose derived $^{13}$C partitioning into the cytosol, cell membranes and cell walls, to evaluate the turnover time of C in each pool, and to assess the contribution of bacterial and fungal biomass to SOM. We hypothesized that: 1) turnover times of C in pools follow the order cytosol<PLFA<amino sugars, because substances taken up by cells first are transported by membrane proteins into cytosol, from where they get distributed to other cellular pools and 2) incorporation of $^{13}$C glucose should be faster and higher for bacterial than for fungal biomarkers, because bacterial biomass has a faster cell turnover than fungal biomass.
2. Material and Methods

2.1. Field site and experimental design

The $^{13}$C labeling field experiment was established at an agricultural field trial in Hohenpötz, Germany (49°54'N, 11°08'E, at 500 m a.s.l.). Triticale, wheat and barley were cultivated by a rotation at the chosen site. The soil type was a loamy haplic Luvisol (IUSS Working group WRB, 2014) and had the following chemical properties in the uppermost 10 cm: total organic C content 1.5%, C/N 10.7, pH 6.6, clay content 22%, CEC 13 cmol$_c$ kg$^{-1}$. The annual precipitation is 870 mm and mean annual temperature is +7 °C.

In summer 2010, following harvest of the triticale, columns (diameter 10 cm and height 13 cm) were installed to a depth of 10 cm. Each column contained 1.5 kg of soil and bulk density was 1.36 g cm$^{-3}$. The 50 mL of uniformly labelled $^{13}$C glucose (99 atom % $^{13}$C) was injected into the columns via a syringe at five points inside the column to spread the tracer homogeneously. Syringe was equipped with a special pipe having length 13 cm and perforated along the whole length, while the end of the pipe was sealed to prevent glucose injection below of the column. Each column received 93.4 µmol $^{13}$C of tracer (0.06 µmol $^{13}$C g$_{-}$soil) and similar amounts of non-labeled glucose were applied to the control columns, to make the experimental conditions equal. The concentration was chosen to trace the natural pool of glucose in soil solution (Fischer et al., 2007), rather than stimulate the activity or growth of microorganisms.

The experiment was done in four field replicates, which were organized in a randomized block design. Labelled and control columns were present within each block. For the first 10 days of the experiment the rainfall was excluded by protective shelter, which was then removed and the experiment was run for 50 days in total. The rainfall was excluded to prevent the added glucose to be leached out from the soil profile, due to processes of microbial uptake go slower in the field conditions, than in the controlled laboratory. After 3,
10 and 50 days, separate soil columns (four columns where $^{13}$C was applied and four control columns) were destructively sampled. The columns had no vegetation by the collecting time, as well as when the $^{13}$C glucose was applied.

The soil was removed from the column, weighed and the water content was determined in a subsample. Soil moisture was determined by drying samples for 24 h at 105 °C and was essentially constant during the experiment, ranging between 21–25% (25.7±1.2 (3 days), 23.3±1.3 (10 days), 21.4±0.7 (50 days)). Each soil sample was sieved to <2 mm and divided into three parts. One part was stored frozen (-20°C) for PLFA analysis, another was cooled (+5°C) (during one week) before the microbial biomass analysis, and the rest was freeze-dried and used for amino-sugar analysis and for measurement of the total amount of glucose derived $^{13}$C remaining in the soil.

2.2. Bulk soil $\delta^{13}$C analysis

The soil for the $\delta^{13}$C analysis was 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 glucose derived $^{13}$C remaining in the soil was calculated based on a mixing model (Equations 1 and 2), where the amount of C in the background sample in Eq. 1 was substituted according to Eq. 2.

$$[C]_{soil} \cdot at\%_{soil} = [C]_{BG} \cdot at\%_{BG} + [C]_{glc} \cdot at\%_{glc} \quad \text{Eq. (1)}$$

$$[C]_{soil} = [C]_{BG} + [C]_{glc} \quad \text{Eq. (2)}$$

with:

$$[C]_{soil/BG/glc} \quad \text{C amount of enriched soil sample / background soil sample /}$$
glucose derived C in soil \((\text{mol} \cdot \text{g}_{\text{soil}}^{-1})\)

\[ \text{at}\%_{\text{soil/BG/gl}} \]

\(^{13}\text{C}\) in enriched soil sample / background soil sample /

applied glucose \((\text{at}\%)\)

2.3. Cytosolic C pool

The cytosolic pool was determined by the fumigation–extraction technique from fresh soil shortly after sampling, according to Wu et al. (1990) with slight changes. Briefly, 15 g fresh soil was placed into glass vials, which were exposed to chloroform during 5 days. After removing the rest of chloroform from the soil, the cytosolic C was extracted from the soil with 45 mL 0.05 M \(\text{K}_2\text{SO}_4\). As fumigation–extraction technique allows to obtain not only soluble components, but also cell organelles and cell particles, we named pool of C in fumigated extracts as cytosol only for simplification of terminology. Organic C was measured with a high-temperature combustion TOC-analyser (Analyser multi N/C 2100, Analytik Jena, Germany). The cytosolic pool was calculated as the difference between organic C in fumigated and unfumigated samples without correcting for extraction efficiency. After organic C concentrations were measured, the \(\text{K}_2\text{SO}_4\) extracts were freeze-dried and the \(\delta^{13}\text{C}\) values of a 30–35 \(\mu\)g subsample were determined using EA-IRMS (instrumentation identical to soil \(\delta^{13}\text{C}\) determination). The recovery of glucose derived \(^{13}\text{C}\) in fumigated and unfumigated samples was calculated according to the above-mentioned mixing model (Eq. 1 and 2). The \(^{13}\text{C}\) in the microbial cytosol was calculated from the difference in these recoveries.
2.4. Phospholipid fatty acid analysis

The PLFA analysis was performed using the liquid–liquid extraction method of Frostegard et al. (1991) with some modifications (Gunina et al., 2014). Briefly, 6 g soil were extracted with a 25-mL one-phase mixture of chloroform, methanol and 0.15 M aqueous citric acid (1:2:0.8 v/v/v) with two extraction steps. The 19:0-phospholipid (dinnenadecanoylglycerol-phosphatidylcholine, Larodan Lipids, Malmö, Sweden) was used as internal standard one (IS1) and was added directly to soil before extraction (25 µL with 1 µg µL⁻¹). Additional chloroform and citric acid was added to the extract achieve a separation of two liquid phases, in which the lipid fraction was separated from other organics. Phospholipids were separated from neutral- and glycolipids by soild-phase extraction using a silica column. Alkaline saponification of the purified phospholipids was performed with 0.5 mL 0.5 M NaOH dissolved in dried MeOH, followed by methylation with 0.75 mL BF₃ in methanol. The resulting fatty acid methyl esters (FAMEs) were purified by liquid–liquid extraction with hexane (three times). Before the final quality and quantity measurements, internal standard two (IS2) (13:0 FAME) (15 µL with 1 µg µL⁻¹) was added to the samples (Knapp, 1979).

All PLFA samples were analysed by gas chromatograph (GC) (Hewlett Packard 5890 GC coupled to a mass-selective detector 5971A) (Gunina et al., 2014). A 25 m HP-1 methylpolysiloxane column (internal diameter 0.25 mm, film thickness 0.25 µm) was used (Gunina et al., 2014). 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 single standard substances, (13:0, 14:0, i14:0, a14:0, 14:1ω5, 15:0, i15:0, a15:0; 16:0, a16:0, i16:0, 16:1ω5; 16:1ω7, 10Me16:0, 17:0, a17:0, i17:0, cy17:0, 18:0, 10Me18:0, 18:1ω7, 18:1ω9, 18:2ω6,9, 18:3ω6,9,12, cy19:0, 19:0, 20:0, 20:1ω9, 20:4ω6) at six concentrations. The recovery of extracted PLFA was calculated using IS1 and the PLFA
contents of samples were individually corrected for recovery. Based on the measured PLFAs contents, the PLFAs C was calculated for the each single compound.

The $^{13}$C/$^{12}$C isotope ratios of the single fatty acids were determined by an IRMS Delta PlusTM coupled to a gas chromatograph (GC; Trace GC 2000) via a GC-II/III-combustion interface (all units from Thermo-Fisher, Bremen, Germany) (Gunina et al., 2014). A 15 m HP-1 methylpolysiloxane column coupled with a 30 m HP-5 (5% Phenyl)-methylpolysiloxane column (both with an internal diameter of 0.25 mm and a film thickness of 0.25 µm) were used. The measured δ$^{13}$C values of the fatty acids were corrected for the effect of derivative C by analogy to Glaser and Amelung (2002) and were referenced to Pee Dee Belemnite by external standards. The enrichment of $^{13}$C in single fatty acids was calculated by analogy to bulk soil and cytosol according to Eq. 1 and 2, following a two-pool dilution model (Gearing et al., 1991).

2.5. Amino sugar analysis

Acid hydrolysis was performed to obtain amino sugars from soil and further ion removal was performed according to the method of Zhang and Amelung (1996) with optimization for δ$^{13}$C determination (Glaser and Gross, 2005). Methylglucamine (100 µL, 5 mg mL$^{-1}$) was used as IS1 and was added to the samples after hydrolysis. Following iron and salt removal, non-cationic compounds such as monosaccharides and carboxylic acids were removed from the extracts using a cation exchange column (AG 50W-X8 Resin, H$^+$ form, mesh size 100–200, Biorad, Munich, Germany) (Indorf et al., 2012). For final measurement, IS2 – fructose (50 µL, 1 mg mL$^{-1}$) – was added to each sample. The amino sugar content and $^{13}$C enrichment were determined by LC-O-IRMS (ICS-5000 SP ion chromatography system coupled by an LC IsoLink to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo-Fischer,
Bremen, Germany) (Dippold et al., 2014). Amino sugars were quantified using a calibration curve, which was constructed using four single standard substances (glucosamine, galactosamine, mannosamine and muramic acid) as external standards at four different concentrations (Dippold et al., 2014).

2.6. Calculations and statistical analysis

Factor analysis with the principal component extraction method of mass % of individual PLFAs was done. The final assignment of fatty acids to distinct microbial groups was made by combination the results of factor loadings table with databases about presence of particular fatty acids in microbial groups (Zelles, 1997). Fatty acids which were loaded into the same factor with the same sign (+ or -) and belonged to one group (base of the table provided in Zelles (1997)) were related to one specific microbial group and their PLFA contents were summed. This method enables quality separation of microbial groups within the soils (Apostel et al., 2013; Gunina et al., 2014). The results of the factor analysis are presented in Supplementary Table 1.

Recovery of glucose derived $^{13}$C ($^{13}$C$_{rec}$) (means $^{13}$C recovery represented as % of total applied $^{13}$C) and enrichment ($^{13}$C$_{enrichm}$) (means $^{13}$C recovery represented as % of total C pool) of the cytosol, PLFAs and amino sugars was calculated according to Eq. 3 and 4, respectively. The C turnover time in the cell pools was calculated as $1/k$; the value of $k$ was obtained from Eq. 5.

$$^{13}C_{incorp} = \frac{C_{Glc}}{C_{Applied}} \times 100\%$$  \hspace{1cm} \text{Eq. (3)}

$$^{13}C_{enrichm} = \frac{C_{Glc}}{C_{Total}} \times 100\%$$  \hspace{1cm} \text{Eq. (4)}

with
C\textsubscript{Glc} amount of glucose derived C incorporated into a distinct cell compartment calculated by equation (1) and (2) (µmol \textsuperscript{13}C per column)

\textsuperscript{13}C\textsubscript{Applied} amount of applied glucose \textsuperscript{13}C (µmol \textsuperscript{13}C per column)

Total \textsubscript{C Pool} amount of pool C (µmol C per column)

\begin{equation}
C_{\text{enrichm}(t)} = C_{\text{enrichm}(0)} \cdot \exp^{-kt}
\end{equation}

Eq. (5)

with

C\textsubscript{enrichm (t)} \textsuperscript{13}C enrichment of the compartment, obtained from Eq. 4 at time t (\%)

C\textsubscript{enrichm (0)} \textsuperscript{13}C enrichment of the compartment obtained from Eq. 4 at time 0 (\%)

k decomposition rate constant (% day\textsuperscript{-1})

t time (days)

One-way ANOVA was used to estimate the significance of differences in total \textsuperscript{13}C recovery and enrichment of cytosol, PLFAs and amino sugars. The data always represent the mean of four replications ± standard error. To calculate the turnover time of C in the cytosol, PLFA and amino sugar pools, a single exponential model was used (Eq. 5) (Kuzyakov, 2011; Parton et al., 1987).
3. Results

3.1. Glucose utilisation and its partitioning within microbial biomass pools

Amino sugars were the largest pool, due to their accumulation in SOM, whereas pools that mainly characterize living MB showed smaller C contents (Table 1). The cytosolic pool (C content 210±7.10 for day 3; 195±14.8 for day 10; 198±19.9 mg C kg\(^{-1}\) soil for day 50) as well as nearly all PLFA groups (Suppl. Table 2) remained constant during the experiment.

[Table 1]

The highest recovery of \(^{13}\)C was found for cytosol pool (15–25% of applied \(^{13}\)C), whereas the lowest amount was recovered in amino sugars (0.8–1.6% of applied \(^{13}\)C) (Fig. 1). The recovery of glucose derived \(^{13}\)C in the cytosolic pool decreased over time, with the largest decrease from day 3 to day 10, and then remained constant for the following month (Fig. 1). The \(^{13}\)C recovery into PLFA was generally very low and was in the same range as recovery into amino sugars (Fig. 1). The \(^{13}\)C recovery in PLFA showed no clear trend between the sampling points (high standard error) (Fig. 1). In contrast, \(^{13}\)C recovery in amino sugars increased two fold on the 50\(^{th}\) day experiment (p<0.05).

[Fig. 1]

3.2. Turnover time of C in microbial biomass pools

To evaluate C turnover in the cytosol, PLFAs and amino sugars, we calculated the enrichment (% of incorporated \(^{13}\)C relatively to pool C) of each pool by glucose derived \(^{13}\)C. The pool enrichment was the highest for PLFAs and the lowest for amino sugars (Fig. 2).
Based on the decrease of $^{13}$C enrichment over time (Fig. 2), the C turnover in the cytosol and PLFAs was calculated as 151 and 47 days, respectively. The C turnover time in the amino-sugar pool could not be calculated by this approach because the maximum enrichment had not yet been reached, and consequently a decomposition function could not be fitted.

[Fig. 2]

### 3.3 Phospholipid fatty acids

Fatty acids of bacterial origin dominated over those of fungal origin within the living microbial community characterized by PLFA composition (Table 1). The PLFA content of most groups did not change significantly during the experiment, reflecting steady-state conditions for the microbial community (see Suppl. Table 2).

Glucose derived $^{13}$C was incorporated in higher portions into bacterial than into fungal PLFAs (Fig. 3, top). Remarkably, the $^{13}$C enrichment decreased over time for all bacterial PLFAs, whereas it increased or remained constant for 16:1ω5, fungi and filamentous, bacterial actinomycetes (Fig. 3, bottom), indicating differences in C turnover in single-celled organisms compared to filamentous organisms.

[Fig. 3]

### 3.4 Amino sugars

The content of amino sugars followed the order: muramic acid < galactosamine < glucosamine (Table 1). The glucosamine/muramic acid ratio varied between 17 and 55, whereas the galactosamine/muramic acid ratio ranged between 12 and 19 (Table 1). This
provides evidence that bacterial residues were dominant in the composition of microbial residues in SOM.

The recovery of glucose derived $^{13}$C into amino sugars increased in the order: muramic acid = galactosamine < glucosamine (Fig. 4, top) reflecting partly their pool sizes. The $^{13}$C recovery showed no increase from day 3 to day 50 for any amino sugars. The ratios of glucosamine/muramic acid and galactosamine/muramic acid, calculated for the incorporated $^{13}$C, were about six. This is much lower than the ratio observed for the pools of amino sugars. The $^{13}$C enrichment did not increase from day 3 to day 50 for any of the amino sugars. The highest enrichment was observed for muramic acid and the lowest for galactosamine (Fig. 4, bottom). The $^{13}$C enrichment in amino sugars was 10–20 times lower than for PLFA.

4. Discussion

4.1. Glucose decomposition

The amount of glucose derived $^{13}$C remaining in soil after 50 days was in the range 80 % which was higher than reported by other studies. Glanville et al. (2012) observed that 50% of glucose C remained in SOM after 20 days; Wu et al. (1993) reported that 55% of glucose derived $^{14}$C remained after 50 days; Perelo and Munch (2005) reported the mineralisation of 50% of $^{13}$C glucose within 98 days. The amounts of applied C (Bremer and Kuikman, 1994; Schneckenberger et al., 2008), as well as differences in microbial activity (Bremer and Kuikman, 1994; Schimel and Weintraub, 2003) in the investigated soils, explain the variation between studies in the portion of remaining glucose C.
The highest mineralization of glucose derived $^{13}$C (20 %) was found within the first three days after tracer application (Fig. 1), whereas at day 50 mineralization was much slower. Glucose is decomposed in soil in two stages (Gunina and Kuzyakov, 2015): during the first one, part of glucose C is immediately mineralized to CO$_2$ and part is incorporated into the microbial compartments and second one, when C incorporated into MB is further transformed and is used for microbial biosynthesis and mineralization of glucose-C to CO$_2$ occurs much slower (Bremer and Kuikman, 1994). This first stage takes place in the first day after substrate addition and is 30 times faster than the 2$^{nd}$ stage (Gregorich et al., 1991). Due to the first sampling point in our experiment was 3 days after glucose addition, the obtained data on glucose mineralization can be mainly related to the second stage.

A significant portion of glucose derived C was stored in the non-specific pool in SOM (Fig. 1), e.g., as microbial storage compounds and other cellular building blocks, which can contribute to C accumulation in microbial residues (Wagner GH, 1968; Zelles et al., 1997; Lutzow et al., 2006). This part cannot be extracted by the methods applied in this study. The amino sugar method detects only the peptidoglycan and chitin proportions of the cell walls, whereas other constituents can not be determined by this method (Glaser et al., 2004). Chloroform fumigation only partially extracts the cytosolic cell compounds, as high molecular weight compounds, which interact with the soil matrix, cannot be extracted with low molarity salt solution.

### 4.2. Partitioning of $^{13}$C-derived glucose between cell compounds

To estimate the residual amount of C derived from applied $^{13}$C-labelled low molecular weight organic substances (LMWOS), the $^{13}$C in SOM or in the total MB pool is frequently determined. This approach, however, does not allow the portions of $^{13}$C incorporated into
stable and non-stable C pools to be estimated, because the $^{13}$C in SOM includes the sum of $^{13}$C in living biomass and $^{13}$C in microbial residues. Furthermore, the living MB contains cell compartments with a broad spectrum of C turnover times. The approach applied in the present study allows the partitioning of glucose derived C in living MB to be estimated, as well as the contribution of LMWOS-C to SOM composition.

4.3. Cytosol

We calculated the $^{13}$C enrichment of the cytosolic microbial C pool, extracted after chloroform fumigation. The estimated turnover time of C in this pool was about 151 days. This value lies close to the previously reported range of 87–113 days, for the same pool for soils incubated for 98 days with $^{13}$C glucose (Perelo and Munch, 2005), but were lower than MB C turnover time calculated using a conversion factor (2.22) - 82 days, for soils incubated for 60 days with $^{14}$C glucose (Kouno et al., 2002). The long C turnover time in cytosol is related to the high heterogeneity of this pool, which includes compounds with various molecular masses (Malik et al., 2013) and functions, having different turnover times. Thus, C turnover time in cytosol presents the mean value of turnover times of these compounds.

4.4. Phospholipid fatty acids

4.4.1. Phospholipid fatty acid content and turnover

Phospholipid fatty acid C comprised 0.27% of the soil organic carbon (SOC). The $^{13}$C recovery into PLFAs, in case of constant PLFAs content during the experiment, reflects microbial activity under steady-state conditions (growth and death of microorganisms occur with the same rates) and processes of the exchange and replacement of existing PLFAs within living cells.
Few studies have estimated the C turnover time in PLFAs or the turnover time of PLFAs themselves in soil as very few options exist to estimate these parameters under steady-state conditions. The turnover time of $^{13}$C-labelled PLFAs contained in dead microbial cells, was 2.7 days (Kindler et al., 2009). The PLFAs turnover times estimated in the field conditions using a C$_3$/C$_4$ vegetation change (Amelung et al., 2008; Glaser, 2005) or $^{14}$C dating (Rethemeyer et al., 2005) were between 1 and 80 years. However, these approaches estimate the turnover time of C bound in PLFA, which can be much older than the PLFA molecules due to repeated C recycling before incorporation. In contrast, $^{13}$C pulse labeling is an approach that enables direct estimation of the turnover of freshly added C by the initial incorporation peak. The approach used in the present study showed that the C turnover time in PLFA is about 47 days (Fig. 2). Accordingly, if the decomposition after cell death is about three days, the PLFA turnover time in living cells is about 44 days. This short turnover time of PLFAs is significantly lower than the C turnover time in the cytosol (Fig. 2). This is because the membrane is the interacting surface between the cell and the environment and thus, frequent and rapid adaptations of its structure are crucial for active microorganisms (Bossio et al., 1998, Kieft et al., 1997). In contrast, the extracted cytosolic pool includes C from both active and dormant microorganisms (Blagodatskaya and Kuzyakov, 2013), and the latter can dilute the $^{13}$C signal incorporated into the active pool with non-labelled C, yielding a lower turnover of this pool.

4.4.2. Contribution of microbial groups to glucose derived C utilisation

More glucose derived $^{13}$C was incorporated into bacterial PLFAs (Fig. 3, top), than filamentous microorganisms. This can be a consequence of low C loading rates (less than 4 mg C g$^{-1}$ soil, see (Reischke et al., 2014), under which conditions the added C is utilized
primarily by bacterial communities, whereas at higher concentrations of applied substrate, the
dominance of fungi in substrate utilisation is observed (Reischke et al., 2014).

The $^{13}$C recovery into gram-negative fatty acids was higher (taking both G- groups
together) compared to G+ bacterial PLFAs (Fig. 3, top), which might be due to: i) the
abundance of their fatty acids, which was higher (Table 1) or ii) glucose uptake activity,
which was higher for G- than G+ groups. In contrast, the $^{13}$C enrichment ($^{13}$C recovery
related to total C in particular biomarkers) for G- bacterial PLFAs was not higher than that
for G+ (Fig. 3, bottom). Thus, the high $^{13}$C recovery into G- bacterial biomarkers can mainly
corresponds to their high content in the soil, not to higher activity of microbial groups.
However, replacement of PLFAs C by glucose derived $^{13}$C is only a proxy of microbial
activity and can only partly estimate the real activity of microbial groups. This clearly
suggests that the analysis of isotope data after labeling in general requires the calculation and
combined interpretation of both the total tracer C recovery as well as the $^{13}$C enrichment in
the investigated pool.

In contrast to our results, a higher recovery of glucose derived $^{13}$C into G+ than G-
PLFAs was observed in other studies (Dungait et al., 2011; Ziegler et al., 2005). However, in
these studies, much higher amounts of C were applied to the soil (15 µg C g$^{-1}$ soil), which
stimulated the growth of G+ bacteria. In contrast, under steady-state conditions with low
glucose concentrations in soil, G- bacteria were the most competitive group for glucose
uptake (Fig. 3).

The $^{13}$C enrichment of bacterial PLFAs decreased from day 3 to day 50, whereas $^{13}$C
in fungal PLFAs increased (in the case of 16:1ω5) or stayed constant (Fig. 3, bottom). The
decline in $^{13}$C enrichment in bacterial fatty acids indicates a partial turnover of bacterial
lipid membranes, which is much faster than turnover in fungal membranes. This result is
consistent with the turnover time of bacterial biomass in soil (Baath, 1998), which is about 10
days, whereas fungal biomass turnover times range between 130–150 days (Rousk and Baath, 2007). Consequently, the increase in $^{13}$C enrichment in fungal PLFAs at late sampling points indicates that fungi consume the exudation products of bacteria or even dead bacterial biomass (Zhang et al., 2013; Ziegler et al., 2005).

4.5. Amino sugars

4.5.1. Amino sugar content and amino sugar C turnover in total and living microbial cell walls

Amino sugars represented the largest microbial pool investigated in this study (Table 1) and comprised 3.7% of SOC. Chitin and peptidoglycan, the direct sources of the amino sugars, comprise no more than 5% of cell biomass (Park and Uehara, 2008; Wallander et al., 2013). Therefore, the high amount of amino sugars relative to PLFA can only be explained by their high proportion in microbial residues/necromass (Glaser et al., 2004; Liang et al., 2008; Glaser et al., 2004). Irrespective of the large pool size of the amino sugars, their recovery and pool enrichment with glucose derived $^{13}$C was the lowest compared to other compartments in living cells and increased during the experiment. Consequently, amino sugars can have the slowest turnover in soils, presumably even within living cells, for two reasons: 1) cell walls are polymers that require a rather complex biosynthesis of the amino-sugar fibers, 2) cell-wall polymerization occurs extracellularly (Lengeler et al., 1999) and 3) microorganisms do not need to synthesize peptidoglycan unless they multiply. To calculate C turnover time in this pool, conducting of long-term experiments is necessary.

The majority of amino sugars extracted after acid hydrolysis represent microbial necromass, which does not incorporate any glucose derived $^{13}$C, but strongly dilutes the $^{13}$C incorporated into the walls of living cells. To estimate the $^{13}$C recovery into amino sugars of
living cells, we first calculated the amount of amino sugars in the living MB pool based on the fatty acids content. Assuming that PLFAs are present only in living biomass, and that the ratio of fatty acids to amino sugars in living biomass is about 0.23 (Lengeler et al., 1999), we estimated the amount of amino sugars in living MB to be 0.20 µmol g\(^{-1}\) soil fatty acids/0.23 = 0.87 µmol g\(^{-1}\) soil. The estimated percentage of amino sugars in living biomass from the total amino sugar pool was 0.87/7.70 (total AS (µmol g\(^{-1}\) soil))*100 = 11%. This estimate agrees with that of Amelung et al. (2001a) and Glaser et al. (2004), who reported that the amount of amino sugars in living biomass is one to two orders of magnitude lower than in the total amino-sugar pool. We calculated the \(^{13}\)C enrichment in amino sugars for the first sampling point, assuming that all replaced C is still contained within living MB after three days of glucose C utilisation. Total tracer recovery into amino sugars consisted of 0.00071 µmol glucose derived \(^{13}\)C in amino sugars g\(^{-1}\) soil/0.87 (µmol amino sugars g\(^{-1}\) soil)*7 (mean amount of C atoms in amino sugars)*100 = 0.57% of the C pool. Comparison of these data with the \(^{13}\)C enrichment into PLFAs and the cytosol allowed us to conclude that the replacement of the amino sugar C with glucose derived \(^{13}\)C in living biomass is two-fold slower than the replacement in PLFAs, and faster than in the cytosolic pool. This reflects that microbial C turnover is a phenomenon that is not restricted to the death or growth of new cells, but that even within living cells, highly polymeric cell compounds, including cell walls, are constantly replaced and renewed (Park and Uehara, 2008).

4.5.2. Contribution of bacterial and fungal cell walls to SOC

Glucosamine was the dominant amino sugar in the soil, whereas muramic acid was the least abundant (Table 1), which agrees with most literature data (Engelking et al., 2007; Glaser et al., 2004). To conclude about the proportions of bacterial and fungal residues in the SOM, the ratio of galactosamine/muramic acid (Glaser et al., 2004) was calculated (Table 1) and
showed bacteria to be the dominant within the soil microbial community. The bacterial origin of microbial residues in the soil is supported by: 1) the dominance of bacterial PLFA biomarkers 2) the environmental conditions of the site, namely, long-term agricultural use, which promotes the dominance of bacterial communities.

Three-fold more glucose derived $^{13}$C was incorporated into glucosamine than into galactosamine and muramic acid (Fig. 4, top). This correlates with the pool size and indicates that glucosamine is the most dominant amino sugar not only in total amino sugars, but also within the walls of living cells. The galactosamine/muramic acid ratio of the incorporated $^{13}$C was six, and consequently was significantly lower than the ratio calculated for the amount of amino sugars (Table 1). This indicates that bacteria are more active in glucose derived $^{13}$C utilisation than fungi, a conclusion also supported by the $^{13}$C-PLFA data (Fig. 3). Thus, even if the composition of amino sugars does not allow a clear conclusion concerning living microbial communities in soil, amino sugar analysis combined with $^{13}$C labeling reveals the activity of living microbial groups in terms of substrate utilisation.

The calculated $^{13}$C enrichment was the highest in muramic acid (Fig. 4, bottom). This is in agreement with the high $^{13}$C enrichment of bacterial PLFAs compared to 16:1ω5 and fungi (Fig. 3). Due to differences in cell-wall architecture, G+ bacteria contain more muramic acid (approximately four times) than G- bacteria (Lengeler et al., 1999), and thus make a higher contribution to the $^{13}$C enrichment of muramic acid.

The $^{13}$C enrichment of glucosamine was two-fold lower than muramic acid (Fig. 4, bottom). This confirms the hypothesis that glucosamine originates from bacterial as well as fungal cell walls and consequently has a mixed enrichment between that of the fungal galactosamine and the bacterial muramic acid.
5. Conclusions

Tracing the $^{13}$C labelled glucose through cytosol, PLFAs and amino sugars is a prerequisite for understanding the fate of organic substrates in soil. The highest $^{13}$C enrichment, and thus turnover of C, was found for the PLFA pool, corresponding to a turnover time of 47 days, whereas the turnover was slower in the cytosol (150 days), which contradict to the first hypothesis. Such results can be explained by 1) efficient C recycling in the cytosol, and 2) its heterogeneous composition, which contains compounds with different turnover rates. The $^{13}$C enrichment of amino sugars was still increasing at the end of the experiment, reflecting the slowest C turnover within the investigated pools and that most of this pool consisted of microbial residues and not living biomass. An approximate calculation of $^{13}$C enrichment of amino sugars in the living biomass gave values 0.57% of pool size, which was still lower than for PLFAs. Thus, C turnover in membrane components is faster than in cell wall components, even if only the portion of the amino sugar pool in living biomass is considered.

Bacterial PLFAs dominated in the microbial community composition, and much higher glucose derived $^{13}$C was incorporated to bacterial than to fungal PLFAs too. This agrees with prevailing role of bacteria in the utilisation of easily available organic substrates that are present at low concentrations in soil. A lower $^{13}$C enrichment of filamentous PLFAs compare to bacterial can evidence that i) C turnover in filamentous PLFAs is slower compare to bacterial and ii) filamentous organisms might utilize the products of bacterial metabolism and biomass.

The galactosamine/muramic acid ratio was between 12 and 19, indicating a predominance of bacterial vs. fungal residues in SOM. The ratio of galactosamine/muramic acid for incorporated $^{13}$C confirmed that bacteria were more active in glucose utilisation than fungi. The $^{13}$C enrichment was the highest for muramic acid and the lowest for
galactosamine, demonstrating that the turnover of bacterial cell wall components is more rapid than fungal.

Consequently, the combination of $^{13}$C labeling with the subsequent analysis of several microbial cell compartments and biomarkers is a unique approach to understanding C partitioning within microbial cells and the microbial communities in soil. This knowledge is not only crucial for assessing C fluxes and recycling in soil, but is also of special importance concerning the contribution of C from microbial residues to SOM.

**Author contribution**

Y. Kuzyakov and B. Glaser designed the experiments and M. Dippold and A. Gunina carried them out. A. Gunina prepared the manuscript with contributions from all co-authors.

**Data availability**

Underlying research data can be accessed by a request from the first author of paper.

**Acknowledgements**

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References


Zhang, H., Ding, W., Yu, H., and He, X.: Carbon uptake by a microbial community during 30-day treatment with C-13-glucose of a sandy loam soil fertilized for 20 years with NPK or compost as determined by a GC-C-IRMS analysis of phospholipid fatty acids, Soil Biology & Biochemistry, 57, 228–236, doi:10.1016/j.soilbio.2012.08.024, 2013.

Table 1 Amount of microbial biomass compartments, their C content, PLFA content of microbial groups and composition of microbial residues in investigated soil. G-1 and G-2 are gram-negative group one and two, respectively; G+1 and G+2 are gram positive group one and two, respectively; Ac – actinomycetes; 16:1ω5 - saprotrophic fungi. Data present mean of three time points (with four replications for each time point) ± SE

<table>
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<th>Compartment</th>
<th>mg component C kg⁻¹ soil</th>
<th>mg kg⁻¹ soil</th>
<th>Ratio</th>
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<tr>
<td>Cytosol</td>
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<td>Phospholipid fatty acids</td>
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<td></td>
<td></td>
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<tr>
<td>G-1</td>
<td>8.9±3.6</td>
<td>11.6 ± 4.6</td>
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<tr>
<td>G-2</td>
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<td>7.4 ± 1.1</td>
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<tr>
<td>G+1</td>
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<td>7.9 ± 1.6</td>
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<td>G+2</td>
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<td>1.0 ± 0.4</td>
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<tr>
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<td>Fungi</td>
<td>1.0±0.2</td>
<td>1.3 ± 0.2</td>
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<td>Amino sugars</td>
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<td>Glucosamine</td>
<td>560.7 ± 68.2</td>
<td>1393.8 ± 170.0</td>
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<tr>
<td>Galactosamine</td>
<td>460.7±79.3</td>
<td>1146.5 ± 197.3</td>
<td></td>
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<tr>
<td>Muramic acid</td>
<td>90.9±11.3</td>
<td>226.3 ± 28.2</td>
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*Data are taken from Glaser et al. (2004).
**Table captions**

**Table 1** Amount of microbial biomass compartments, their C content, content of microbial groups and composition of microbial residues in investigated soil. G-1 and G-2 are gram-negative group one and two, respectively; G+1 and G+2 are gram positive group one and two, respectively; Ac – actinomycetes; 16:1ω5 - saprotrophic fungi.

**Figure captions**

**Fig. 01** Partitioning of glucose derived $^{13}$C in SOM presented as the $^{13}$C recovery (% of initially applied $^{13}$C found in soil) between the following pools: non-specified SOM (calculated as total $^{13}$C recovery subtract $^{13}$C recovery in cytosol, PLFAs and amino sugars), cytosolic, PLFAs and amino sugars. Small letters reflect differences between the sampling points for the distinct pool. Data present mean (n=4) and bars present standard errors (SE). The SE for the amino sugars are not fully shown.

**Fig. 02** $^{13}$C enrichment in the cytosolic, PLFA and amino-sugar cell pools as well as functions to calculate the C turnover times in these microbial cell pools. The left y-axis represents the PLFA pool, the first right y-axis, the cytosolic and the second y-axis, the amino-sugar pool. Data present mean (n=4) and bars present standard errors.

**Fig. 03** Recovery of glucose derived $^{13}$C (top) and $^{13}$C enrichment (bottom) of the microbial PLFAs. Note that the values for 16:1ω5 and fungi are scaled-up 10 times (secondary Y axis) compared to those of other groups (Y axis at the left). Data present mean (n=4) and bars present standard errors. Small letters reflect differences between the microbial groups for $^{13}$C
recovery and $^{13}$C enrichment from glucose; letters a-d are for day three, l-o are for day 10, x-z are for day 50.

**Fig. 04** Recovery of glucose derived $^{13}$C (top) and $^{13}$C enrichment (bottom) of amino sugars and muramic acid. Letters reflect significant differences in the recovery and $^{13}$C enrichment from glucose into amino sugars on a particular day; letters a-b are for day three, l-m are for day 10, x-y are for day 50. No significant differences were observed between the three sampling days. Data present mean (n=4) and bars present standard errors.

**Fig. 05** Dynamic relationship of microbial utilization of glucose and turnover of cytosol, cell membrane and cell wall components.
Figure 01.
Figure 02.

\[ ^{13}\text{C}_{\text{replacement}} = 69.9 \cdot e^{(-0.021t)} \quad \text{C}_{\text{turnover time}} = 47 \text{ d} \]

\[ ^{13}\text{C}_{\text{replacement}} = 18.1 \cdot e^{(-0.007t)} \quad \text{C}_{\text{turnover time}} = 152 \text{ d} \]

\[ ^{13}\text{C}_{\text{replacement}} = 0.007 \cdot e^{(0.010t)} \]
Figure 04.

Galactosamine  Glucosamine  Muramic acid

% of applied glucose $^{13}\text{C}$

$^{13}\text{C}$ recovery

% of C pool size

$^{13}\text{C}$ enrichment

Days after $^{13}\text{C}$-glucose application