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Turnover of microbial groups and cell components in soil: ^{13}C analysis of cellular biomarkers

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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 turnover within the microbial cells, the fate of ^{13}C-labelled glucose was studied under field conditions. Glucose-derived ^{13}C was traced in cytosol, amino sugars and phospholipid fatty acid (PLFA) pools at intervals of 3, 10 and 50 days after glucose addition into the soil.

^{13}C enrichment in PLFAs ($\sim$ 1.5 % of PLFA C at day 3) was an order of magnitude greater than in cytosol, showing the importance of cell membranes for initial C utilization. The ^{13}C enrichment in amino sugars of living microorganisms at day 3 accounted for 0.57 % of total C pool; as a result, we infer that the replacement of C in cell wall components is 3 times slower than that of cell membranes. The C turnover time in the cytosol (150 days) was 3 times longer than in PLFAs (47 days). Consequently, even though the cytosol pool has the fastest processing rates compared to other cellular compartments, intensive recycling of components here leads to a long C turnover time.

Both PLFA and amino-sugar profiles indicated that bacteria dominated in glucose utilization. ^{13}C enrichment decreased with time for bacterial cell membrane components, but it remained constant or even increased for filamentous microorganisms. ^{13}C enrichment of muramic acid was the 3.5 times greater than for galactosamine, showing a more rapid turnover of bacterial cell wall components compared to fungal. Thus, bacteria utilize a greater proportion 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 not restricted to the death or growth of new cells. Indeed, even within living cells, highly polymeric cell compounds are constantly replaced and renewed. This is especially important for assessing C fluxes in soil and the contribution of C from microbial residues to soil organic matter.

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 are taken up by the living microorganisms and are partly mineralized 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 C turnover times in 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 C in PLFA of 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 and 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 as well as to estimate the contribution of microbial residues to SOM (Schmidt et al., 2007).

Some cell compartments, such as the cytosol, 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 ($i16:0$, $a16:0$, $i15:0$, $a15:0$, $16:1\omega7$, $18:1\omega7$) and fungal communities ($18:2\omega6,9$; $18:3\omega6,9,12$; $16:1\omega5$) are used to draw conclusions about the qualitative composition of living microbial communities, their contribution to utilization 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 different chemical composition, which strongly contributes to their turnover rates in soil: for bacteria it constitutes 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 the fact that the turnover of microorganisms directly affects 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 utilization and to the stabilization of microbially derived C in SOM.

Combination of PLFAs and amino-sugar biomarker analyses, as well as cytosol C measurement with isotope tracing techniques (based on ^{13}C natural abundance or ^{13}C / ^{14}C labelling), has been used in various studies to characterize organic C utilization by 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 is thus the most suitable substance for such a study.

We analysed glucose-derived ^{13}C partitioning into the cytosol, cell membranes and cell walls in order 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) recovery 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 labelling field experiment was established at an agricultural field trial in Hohenpölz, 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. The syringe was equipped with a special pipe (13 cm long) with perforations along the whole length, while the end of the pipe was sealed to prevent glucose injection below the column. Each column received 93.4 µmol ^{13}C of tracer (0.06 µmol ^{13}C g^{-1}soil), and similar amounts of non-labelled 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 rainfall was excluded by a protective shelter, which was removed thereafter, with the experiment running for 50 days in total. The rainfall was excluded to prevent the added glucose from being leached out from the soil profile, due to processes of microbial uptake progressing 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, nor was there any when the ^{13}C glucose was applied.

The soil was removed from the column and 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 and 25 % (25.7 ± 1.2 (3 days), 23.3 ± 1.3 (10 days), and 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) (over 1 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 δ^{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 Fisher, Bremen, Germany) to a Delta V Advantage isotope ratio mass spectrometer (IRMS; Thermo Fisher, Bremen, Germany). The amount of glucose-derived ^{13}C remaining in the soil was calculated based on a mixing model (Eqs. 1 and 2), where the amount of C in the background sample in Eq. (1) was substituted according to Eq. (2).

$$[C]_{soil} \cdot atom\%_{soil} = [C]_{BG} \cdot atom\%_{BG} + [C]_{glc} \cdot atom\%_{glc} \quad (1)$$

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

where $[C]_{soil/BG/glc}$ is the C amount of enriched soil sample/background soil sample/glucose-derived C in soil (mol × g_{soil}^{-1}) and atom $\%_{soil/BG/glc}$ is ^{13}C in enriched soil sample/background soil sample/applied glucose (atom %).

2.3 Cytosol C pool

The cytosol 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 of fresh soil was placed into glass vials, which were exposed to chloroform over 5 days. After removing the rest of chloroform from the soil, the cytosol C was extracted with 45 mL of 0.05 M K_2SO_4. As the fumigation–extraction technique produces not only soluble components but also cell organelles and cell particles, we referred to the pool of C in fumigated extracts as "cytosol" only for simplification of terminology. Organic C was measured with a high-temperature combustion TOC analyser (multi N/C 2100 analyser, Analytik Jena, Germany). The cytosol 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 K_2SO_4 extracts were freeze-dried and the $\delta^{13}C$ values of a 30–35 µg subsample were determined using EA-IRMS (instrumentation identical to soil $\delta^{13}C$ determination). The recovery of glucose-derived ^{13}C in fumigated and unfumigated samples was calculated according to the above-mentioned mixing model (Eqs. 1 and 2). The ^{13}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 of soil was 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 (dinonadecanoylglycerol-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 were added to the extract to achieve a separation of two liquid phases, in which the lipid fraction was separated from other organics. Phospholipids were separated from neutral lipids and glycolipids by solid-phase extraction using a silica column. Alkaline saponification of the purified phospholipids was performed with 0.5 mL of 0.5 M NaOH dissolved in dried MeOH, followed

by methylation with 0.75 mL of BF_3 dissolved 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 PLFA contents, the PLFA 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 Plus™ 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) was used. The measured $\delta^{13}C$ 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 Eqs. (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 $\delta^{13}C$ 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 contents and ^{13}C enrichments were determined by LC-O-IRMS (ICS-5000 SP ion chromatography system coupled by an LC IsoLink to a Delta V Advantage IRMS (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 combining the results of the factor loadings table with databases of the 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 Supplement Table S1.

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 Eqs. (3) and (4), respectively. The C turnover times in the cell pools were calculated as $1/k$; the k values were obtained from Eq. (5).

$$^{13}C_{rec} = \frac{C_{Glc}}{^{13}C_{Applied}} \times 100\,\%, \quad (3)$$

$$^{13}C_{enrichm} = \frac{C_{Glc}}{^{Total}C_{Pool}} \times 100\,\%, \quad (4)$$

where C_{Glc} is the amount of glucose-derived C incorporated into a distinct cell compartment calculated by Eqs. (1) and (2) (µmol ^{13}C per column), $^{13}C_{Applied}$ is the amount of applied glucose ^{13}C (µmol ^{13}C per column), and $^{Total}C_{Pool}$ is the amount of pool C (µmol C per column).

$$C_{enrichm(t)} = C_{enrichm(0)} \cdot \exp^{-kt}, \quad (5)$$

where $C_{enrichm(t)}$ is the ^{13}C enrichment of the compartment, obtained from Eq. (4) at time t (%), $C_{enrichm(0)}$ is the ^{13}C enrichment of the compartment at time 0 (%), k is the decomposition rate constant (% d^{-1}), and t stands for time (days).

One-way ANOVA was used to estimate the significance of differences in total ^{13}C recovery and enrichment of non-specified SOM pool, cytosol, PLFAs and amino sugars. The data always represent the mean of four replications ± SE. The ^{13}C in the non-specified SOM was calculated by subtracting ^{13}C incorporated into cytosol, PLFAs and amino sugars from total ^{13}C measured in the soil. To describe decomposition rate of ^{13}C, a single first-order kinetic equation

was applied to the enrichment of ^{13}C in the pool of cytosol, PLFAs and amino sugars (Eq. 5) (Kuzyakov, 2011; Parton et al., 1987).

3 Results

3.1 Glucose utilization and its partitioning within microbial biomass pools

The amino-sugar C pool was the largest, due to accumulation of these substances in SOM, whereas pools that mainly characterize living MB showed smaller C contents (Table 1). The cytosol 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 (Table S2) remained constant during the experiment.

The highest recovery of ^{13}C was found for cytosol pool (15–25 % of applied ^{13}C), whereas the lowest was reported for amino sugars (0.8–1.6 % of applied ^{13}C) (Fig. 1). The recovery of glucose-derived ^{13}C in the cytosol pool decreased over time, with the largest decline 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 2-fold on the 50th day of the experiment ($p<0.05$).

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 in ^{13}C enrichments over time (Fig. 2), the C turnover times in the cytosol and PLFAs were 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.

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 Table S2).

Higher ^{13}C recovery was found in bacterial than in fungal PLFAs (Fig. 3, top). Remarkably, the ^{13}C enrichment decreased over time for all bacterial PLFAs, whereas it in-

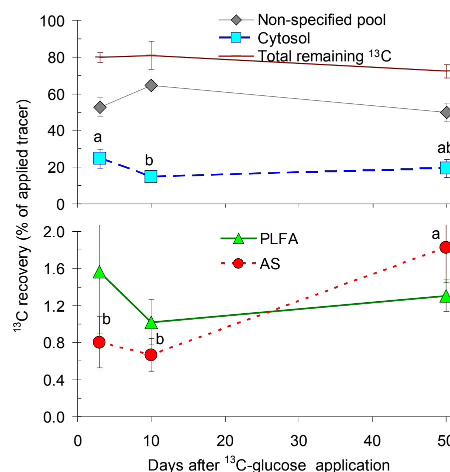

Figure 1. Partitioning of glucose-derived ^{13}C in SOM presented as the ^{13}C recovery (% of initially applied ^{13}C) between the following pools: non-specified SOM (calculated as total ^{13}C recovery subtract ^{13}C recovery in cytosol, PLFAs and amino sugars), cytosol, PLFAs and amino sugars. Brown line indicates the total remaining glucose-derived ^{13}C glucose in the soil and is a sum of ^{13}C in non-specified SOM, cytosol, 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 (SEs). The SEs for the amino sugars are not fully shown.

creased or remained constant for 16 : 1ω5, fungi and actinomycetes (Fig. 3, bottom), indicating differences in C turnover in single-celled organisms compared to filamentous organisms.

3.4 Amino sugars

The content of amino sugars followed the order muramic acid < galactosamine < glucosamine (Table 1). The galactosamine / muramic acid ratio ranged between 12 and 19 (Table 1), showing 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), partly reflecting their pool sizes. The ^{13}C recovery showed no increase from day 3 to day 50 for any amino sugars. The ratio of galactosamine / muramic acid, calculated for the incorporated ^{13}C, was 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.

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.

Compartment	mg component C kg^{-1} soil	mg kg^{-1} soil	Ratio
Cytosol	201.0 ± 7.1	–	
Phospholipid fatty acids	39.4 ± 4.7	51.9 ± 6.2	
Specific phospholipid fatty acids			
G−1	8.9 ± 3.6	11.6 ± 4.6	
G−2	5.6 ± 0.8	7.4 ± 1.1	
G+1	5.9 ± 1.2	7.9 ± 1.6	
G+2	0.7 ± 0.3	1.0 ± 0.4	
Ac	2.3 ± 0.7	3.0 ± 1.0	
16 : 1ω5	1.7 ± 0.3	2.2 ± 0.3	
Fungi	1.0 ± 0.2	1.3 ± 0.2	
Bacteria/fungi			6–8.5
Amino sugars	560.7 ± 68.2	1393.8 ± 170.0	
Glucosamine	460.7 ± 79.3	1146.5 ± 197.3	
Galactosamine	90.9 ± 11.3	226.3 ± 28.2	
Muramic acid	9.1 ± 1.8	21.1 ± 4.1	
Glucosamine / muramic acid			17–55
Glucosamine / muramic acid (literature data for pure cultures*)		Bacteria	5.3
		Fungi	271
Galactosamine / muramic acid			12–19
Galactosamine / muramic acid (literature data for pure cultures*)		Bacteria	2.8
		Fungi	59

* Data are taken from Glaser et al. (2004).

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 mineralization 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 variations between studies in the portion of remaining glucose C.

The highest mineralization of glucose-derived ^{13}C (20 %) was found within the first 3 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 second stage (Gregorich et al., 1991; Fischer et al., 2010). 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 cannot be determined (Glaser et al., 2004). Chloroform fumigation only partially extracts the cytosol cell compounds, and high-molecular-weight components, which interact with the soil matrix, cannot be extracted with low-molarity salt solution.

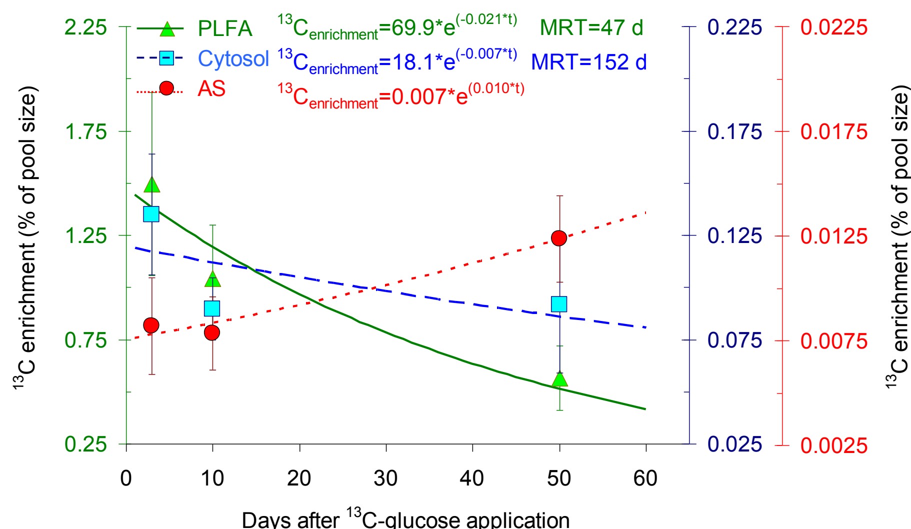

Figure 2. ^{13}C enrichment in the cytosol, PLFA and amino-sugar cell pools as well as functions to calculate the C turnover times in these microbial cell pools. The left y axis represents the PLFA pool, the first right y axis the cytosol, and the second y axis the amino-sugar pool. Data present mean ($n = 4$) and bars present standard errors.

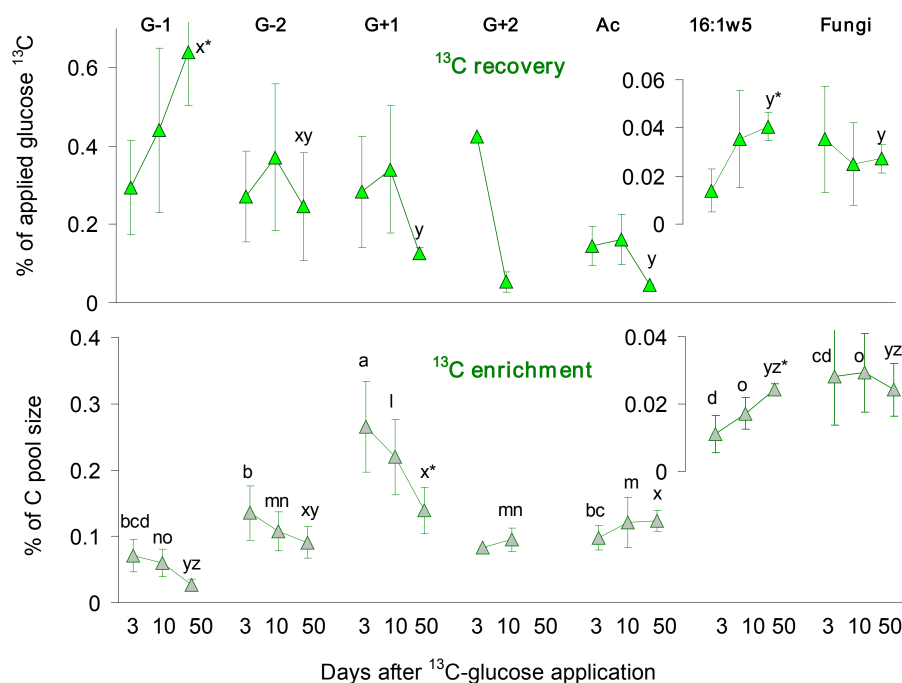

Figure 3. Recovery of glucose-derived ^{13}C (top) and ^{13}C enrichment (bottom) of the microbial PLFAs. Note that the values for $16:1\omega5$ and fungi are scaled up 10 times (secondary y axis) compared to those of other groups (y axis on the left). Data present the mean ($n = 4$) and bars present 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 3, (l–o) are for day 10, and (x–z) are for day 50.

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. Fur-

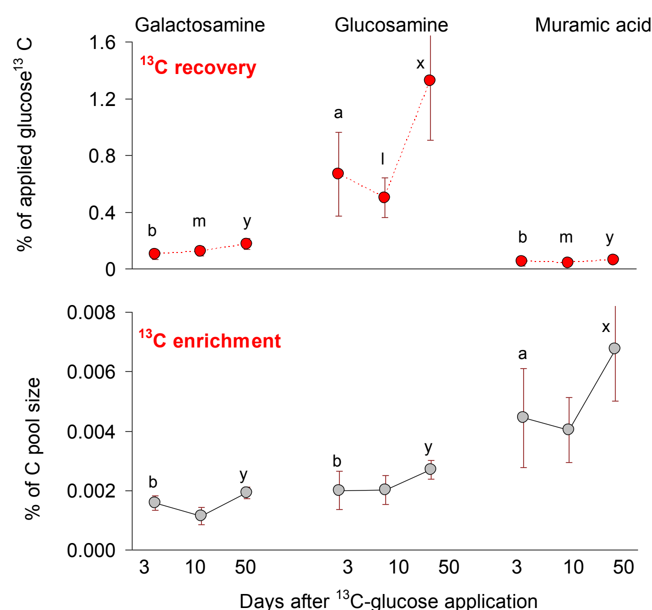

Figure 4. Recovery of glucose-derived ^{13}C (top) and ^{13}C enrichment (bottom) of amino sugars and muramic acid. Letters reflect significant differences in the recovery and ^{13}C enrichment from glucose ^{13}C into amino sugars on a particular day; letters (a–b) are for day 3, (l–m) are for day 10, and (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.

thermore, 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 cytosol 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 was lower than MB C turnover time calculated using a conversion factor (2.22) 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, with 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 the 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 PLFA C 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 labelling is an approach that enables direct estimation of the turnover of freshly added C by the initial recovery 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 3 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 (Figs. 2, 5). This is because the membrane is an 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 cytosol 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 utilization

More glucose-derived ^{13}C was incorporated into bacterial PLFAs (Fig. 3, top), than into 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 utilization 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),

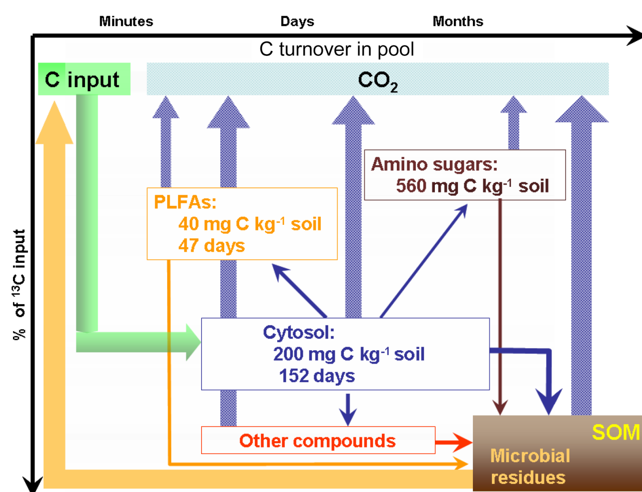

Figure 5. Dynamic relationships between microbial glucose utilization and C turnover times in cytosol, cell membrane and cell wall components.

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 correspond to their high content in the soil, not to higher activity of microbial groups. However, enrichment of PLFA 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 labelling in general requires the calculation and combined interpretation of both the total tracer C recovery and 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 decrease 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 and 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 PLFAs, can only be explained by their high proportion in microbial residues/necromass (Glaser et al., 2004; Liang et al., 2008). 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 three reasons: (1) cell walls are polymers that require a rather complex biosynthesis of the amino-sugar fibres, (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, it is necessary to conduct long-term experiments.

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 enrichment into amino sugars of living cells, we first calculated the amount of amino sugars in the living MB pool, which constituted 0.87 µmol g^{-1} soil, and was about 11 % of the total amino-sugar pool (please see Supplement calculations for further details). 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 3 days of glucose C utilization, and it constituted 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 enrichment of amino-sugar C with glucose-derived ^{13}C in living biomass is 2-fold lower than the enrichment in PLFAs, and higher than in the cytosol pool. This reflects the fact 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 the 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), showing 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 and (2) the environmental conditions of the site, namely long-term agricultural use, which promotes the development of bacterial communities.

Three-fold more glucose-derived ^{13}C was recovered in glucosamine than in 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 6 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 utilization than fungi, a conclusion also supported by the ^{13}C-PLFA data (Fig. 3, top). 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 labelling reveals the activity of living microbial groups in terms of substrate utilization.

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 $16:1\omega5$ and fungi (Fig. 3). Due to differences in cell wall architecture, G+ bacteria contain more muramic acid (approximately 4 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 2-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 the fungal galactosamine and 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 and can be used to estimate C turnover times in various microbial cell compartments. In contradiction to the first hypothesis, the C turnover times were as follows: PLFA (47 days) < cytosol (150 days) < amino sugars. The long C half-life in cytosol can be explained by efficient C recycling and cytosol heterogeneous composition, which involves compounds with different turnover rates. Due to significant part of amino-sugar pool was in the composition of microbial residues, the ^{13}C enrichment of this pool was still increasing at the end of the experiment, which reflects the slowest C turnover time here. An approximate calculation of ^{13}C enrichment of amino sugars in the living biomass accounted for 0.57 % of pool size, which was lower than for PLFAs. This reflects that C turnover in cell wall components is slower than in membrane components.

Both PLFA and amino-sugar analysis showed the prevalence of bacterial biomass/bacterial residues in investigated soil. Much higher recovery and enrichment by glucose-^{13}C was found in bacterial than in fungal PLFAs. A lower ^{13}C enrichment of filamentous PLFAs compared to bacterial demonstrates that (i) C turnover in filamentous PLFAs is slower compared to bacterial and (ii) filamentous organisms might consume bacterial biomass and utilize products of its metabolism. The ratio of galactosamine / muramic acid for incorporated ^{13}C evidences that bacteria were more active in glucose utilization 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 labelling 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 also important for estimating the contribution of C from microbial residues to SOM.

6 Data availability

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

The Supplement related to this article is available online at doi:10.5194/bg-14-271-2017-supplement.

Author contributions. Yakov Kuzyakov and Bruno Glaser designed the experiments and Michaela Dippold and Anna Gunina carried them out. Anna Gunina prepared the manuscript with contributions from all co-authors.

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