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Revision of the Ms. No. Biogeosciences Discuss., doi:10.5194/bg-2016-214, 2016
Glucose C turnover in cell compartments and microbial groups in soil

Dear Prof. Pendall,

we are very thankful for your helpful suggestions and recommendations. We carefully improved the ms according to your comments and incorporated nearly all your suggestions. Please find below the responses to the comments (in green) and the improved ms.
We hope that the ms fulfill the requirements of Biogeosciences.

With kind regards,
Anna Gunina and co-authors.

Editor review
The manuscript has been improved by streamlining the hypotheses and reducing the speculations and assumptions presented in the original version. However, some revisions are still required before the paper can be published. Grammar is still problematic in some sections. See attached pdf for examples of corrections.

- Thank you for the comment. We have corrected the grammar in the ms, and paid the most attention to Abstract and conclusions sections.

Abstract: The abstract should be revised to improve the English and also to make the most important points come across more strongly.

- Thank you for the comment. Abstract was improved, please, see the corrected version provided below.

The authors need to reconcile the terminology to be consistent with the methods, particularly related to the concept of “turnover time”.

- Thank you for the comment. We have corrected the terminology regarding the "turnover time", and made necessary corrections, namely: we corrected equations, and statements in the discussion section.

Line 35: What is meant by "renewal"?

- Thank you for the comment. We wanted to stress that C in a pool of PLFA or amino sugars is replaced by the new ^{13}C (from added glucose), means that C is renewed. The abstract was changed and this term was excluded.

Lines 275-277: what is meant by “per column”? Maybe you mean “per component”?

- Thank you for the comment. All calculations were done to the weight of all soil in the experimental unit, which was "column" in our case. Due to we have collected the columns completely (means all soil where ^{13}C was added was collected during sampling), we calculated the amount of ^{13}C to the weight of all soil in the column, which was 1.5 kg (this information presented in the materials and methods section).

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Lines 304-306 and throughout: Cytosol pool vs cytosolic pool: Be consistent, choose one.

- Thank you for the comment. We have corrected and used only "cytosol" abbreviation.

Lines 308-309 and throughout: “recovery in” or “incorporation into”

- Thank you for the comment. We have checked through the all ms. Term "recovery" is correct. Only in one case L417 the term incorporation is used, due to there we spoke about incorporation of C into PLFA pool (not from pulse-labelling, but due to the uptake of C which is naturally in the soil).

Lines 373-377: How was the “non-specific pool” of SOM determined?

- Thank you for the comment. The ^{13}C in the non-specified SOM was calculated by subtracting off total ^{13}C measured in the soil, the ^{13}C incorporated into cytosol, PLFAs and amino sugars. This information was added into materials and method section.

Lines 481-502: This paragraph is long and rambling and presents some results and calculations in addition to a discussion point. It would benefit the manuscript if the calculations could be put into a supplement and then the main point of the paragraph would be more clear (that amino sugars are slower to incorporate C than the other cell components studied).

- Thank you for the comment. We have put the calculations into the supplementary materials.

Conclusions; This section reads like a lengthy summary of the results. It could be shortened to the main implications of the study.

- Thank you for the comment. The conclusion section was shortened, please see the corrected version of ms, presented below:
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 hypothesis one, the C turnover times were as follows: PLFA (47 days)<cytosol (150 days)<amino sugars. The long C half-life time 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 PLFAs and amino sugars 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 compare to bacterial demonstrates that i) C turnover in filamentous PLFAs is slower compare 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 utilisation than fungi.

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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 important for estimation the contribution of C from microbial residues to SOM.

Figures

Fig. 1: Please indicate what the top brown line indicates; apparently it is the 13C-enriched glucose remaining in the soil?

- Thank you for the comment. Brown line indicates the remaining glucose-derived ^{13}C in the soil.

Fig. 5: Missing?

- We have deleted this figure according to the reviewers suggestions.
We have added figure 5 for the revision by the Editor.

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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 turnover within the microbial cells, the fate of ^{13}C-labeled 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 (~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 three times slower than that of cell membranes. The C turnover time in the cytosol (150 days) was three times longer than in PLFAs (47 days). Consequently, even though the cytosol pool has the fastest processing rates compare 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 3.5x greater than for galactosamine, showing a more rapid turnover of bacterial cell wall components compare to fungal. Thus, bacteria utilise 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

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^{13}C enrichment

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Удалено: Amino sugars originate mainly from microbial residues, thus longer experimental periods are required for estimation of C turnover times in that pool.

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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.

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Keywords

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

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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 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.

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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

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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 6.5–81.0 y^{-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, 16:1ω7, 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.

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Combination of PLFAs and amino-sugar biomarkers analyses, as well as cytosol 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 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) 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 labeling 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

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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 % 13C) 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^{-1}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.

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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 (-20ºC) 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 13C remaining in the soil.

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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:

$[C]_{soil/BG/glc}$	C amount of enriched soil sample / background soil sample / glucose derived C in soil	($mol \cdot g_{soil}^{-1}$)
$at\%_{soil/BG/glc}$	^{13}C in enriched soil sample / background soil sample / applied glucose	(at%)

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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 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 cytosol C was extracted with 45 mL 0.05 M K_2SO_4. As fumigation–extraction technique produces 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 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 (Eq. 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 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 (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

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chloroform and citric acid was 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- 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 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 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

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of 0.25 µm) were 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 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 $\delta^{13}C$ 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 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).

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253 *2.6. Calculations and statistical analysis*

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

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

268 $$^{13}C_{rec} = \frac{C_{Glc}}{^{13}C_{Applied}} \times 100\% \qquad \text{Eq. (3)}$$

269 $$^{13}C_{enrichm} = \frac{C_{Glc}}{^{Total}C_{Pool}} \times 100\% \qquad \text{Eq. (4)}$$

270 with

271 C_{Glc} amount of glucose derived C incorporated into a distinct cell compartment
272 calculated by equation (1) and (2) (µmol ^{13}C per column)
273 $^{13}C_{Applied}$ amount of applied glucose ^{13}C (µmol ^{13}C per column)
274 $^{Total}C_{Pool}$ amount of pool C (µmol C per column)

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

with

$C_{enrichm\ (t)}$	^{13}C enrichment of the compartment, obtained from Eq. 4 at time t	(%)
$C_{enrichm\ (0)}$	^{13}C enrichment of the compartment at time 0	(%)
k	decomposition rate constant	(% day^{-1})
t	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 ± standard error. The ^{13}C in the non-specified SOM was calculated by subtracting off total ^{13}C measured in the soil, the ^{13}C incorporated into cytosol, PLFAs and amino sugars. 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).

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3. Results

3.1. Glucose utilisation and its partitioning within microbial biomass pools

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

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298 for day 50) as well as nearly all PLFA groups (Suppl. Table 2) remained constant during the
299 experiment.

300 **[Table 1]**

301

302 The highest recovery of ^{13}C was found for cytosol pool (15–25% of applied ^{13}C),
303 whereas the lowest was reported for amino sugars (0.8–1.6% of applied ^{13}C) (Fig. 01). The
304 recovery of glucose derived ^{13}C in the cytosol pool decreased over time, with the largest
305 decline from day 3 to day 10, and then remained constant for the following month (Fig. 01).
306 The ^{13}C recovery into PLFA was generally very low and was in the same range as recovery
307 into amino sugars (Fig. 01). The ^{13}C recovery in PLFA showed no clear trend between the
308 sampling points (high standard error) (Fig. 01). In contrast, ^{13}C recovery in amino sugars
309 increased two fold on the 50th day experiment ($p<0.05$).

310 **[Fig. 01]**

311

312 *3.2. Turnover time of C in microbial biomass pools*

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

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[Fig. 02]

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).

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 increased 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.

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[Fig. 03]

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.

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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 ratio of galactosamine/muramic acid, calculated for the incorporated ^{13}C, was about six. This is much

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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.

[Fig. 04]

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 variations between studies in the portion of remaining glucose C.

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The highest mineralization of glucose derived ^{13}C (20 %) was found within the first three days after tracer application (Fig. 01), 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 2nd stage (Gregorich et al., 1991). Due

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368 to the first sampling point in our experiment was 3 days after glucose addition, the obtained
369 data on glucose mineralization can be mainly related to the second stage.

370 A significant portion of glucose derived C was stored in the non-specific pool in SOM
371 (Fig. 01), e.g., as microbial storage compounds and other cellular building blocks, which can
372 contribute to C accumulation in microbial residues (Wagner GH, 1968; Zelles et al., 1997;
373 Lutzow et al., 2006). This part cannot be extracted by the methods applied in this study. The
374 amino sugar method detects only the peptidoglycan and chitin proportions of the cell walls,
375 whereas other constituents can not be determined (Glaser et al., 2004). Chloroform
376 fumigation only partially extracts the cytosol cell compounds, and high molecular weight
377 components, which interact with the soil matrix, cannot be extracted with low molarity salt
378 solution.

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380 *4.2. Partitioning of ^{13}C-derived glucose between cell compounds*

381 To estimate the residual amount of C derived from applied ^{13}C-labelled low molecular weight
382 organic substances (LMWOS), the ^{13}C in SOM or in the total MB pool is frequently
383 determined. This approach, however, does not allow the portions of ^{13}C incorporated into
384 stable and non-stable C pools to be estimated, because the ^{13}C in SOM includes the sum of
385 ^{13}C in living biomass and ^{13}C in microbial residues. Furthermore, the living MB contains cell
386 compartments with a broad spectrum of C turnover times. The approach applied in the
387 present study allows the partitioning of glucose derived C in living MB to be estimated, as
388 well as the contribution of LMWOS-C to SOM composition.

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392 *4.3. Cytosol*

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

402

403 *4.4. Phospholipid fatty acids*

404 *4.4.1. Phospholipid fatty acid content and turnover*

405 Phospholipid fatty acid C comprised 0.27% of the soil organic carbon (SOC). The ^{13}C
406 recovery into PLFAs, in case of constant PLFAs content during the experiment, reflects
407 microbial activity under steady-state conditions (growth and death of microorganisms occur
408 with the same rates) and processes of the exchange and replacement of existing PLFAs C
409 within living cells.

410 Few studies have estimated the C turnover time in PLFAs or the turnover time of
411 PLFAs themselves in soil, as very few options exist to estimate these parameters under
412 steady-state conditions. The turnover time of ^{13}C-labelled PLFAs contained in dead microbial
413 cells, was 2.7 days (Kindler et al., 2009). The PLFAs turnover times estimated in the field
414 conditions using a C_3/C_4 vegetation change (Amelung et al., 2008; Glaser, 2005) or ^{14}C
415 dating (Rethemeyer et al., 2005) were between 1 and 80 years. However, these approaches

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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 recovery peak. The approach used in the present study showed that the C turnover time in PLFA is about 47 days (Fig. 02). 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. 02, Fig. 05). 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.

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4.4.2. Contribution of microbial groups to glucose derived C utilisation

More glucose derived ^{13}C was incorporated into bacterial PLFAs (Fig. 03, 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 utilisation is observed (Reischke et al., 2014).

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The ^{13}C recovery into gram-negative fatty acids was higher (taking both G- groups together) compared to G+ bacterial PLFAs (Fig. 03, 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

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related to total C in particular biomarkers) for G- bacterial PLFAs was not higher than that for G+ (Fig. 03, 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, enrichment 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. 03).

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. 03, 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 130–150 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).

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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; 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 three 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 enrichment into amino sugars of living cells, we first calculated the amount of amino sugars in the living MB pool, which consisted 0.87 µmol g^{-1} soil, and was about 11% of the total amino sugar pool (please, see Supplementary 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

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Удалено: 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

Удалено: was 0.87/7.70 (total AS (µmol g^{-1} soil))*100 = 11%.

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replaced C is still contained within living MB after three days of glucose C utilisation, and it consisted 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 two-fold lower than the enrichment in PLFAs, and higher than in the cytosol 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 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), 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 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. 04, 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

Удалено: . Total tracer recovery into amino sugars consisted

Удалено: 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

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glucose derived ^{13}C utilisation than fungi, a conclusion also supported by the ^{13}C-PLFA data (Fig. 03, 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 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. 04, bottom). This is in agreement with the high ^{13}C enrichment of bacterial PLFAs compared to 16:1ω5 and fungi (Fig. 03). 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. 04, 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 hypothesis one, the C turnover times were as follows: PLFA (47 days)<cytosol (150 days)<amino sugars. The long C half-life time 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

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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 PLFAs and amino sugars 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 compare to bacterial demonstrates that i) C turnover in filamentous PLFAs is slower compare 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 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 important for estimation the contribution of C from microbial residues to SOM.

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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.

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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).

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Table captions

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

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Figure captions

Fig. 01 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 ^{13}C derived 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 (SE). The SE for the amino sugars are not fully shown.

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Fig. 02 ^{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.

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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

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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 ^{13}C 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 relationships between microbial glucose utilization and C turnover times in cytosol, cell membrane and cell wall components.

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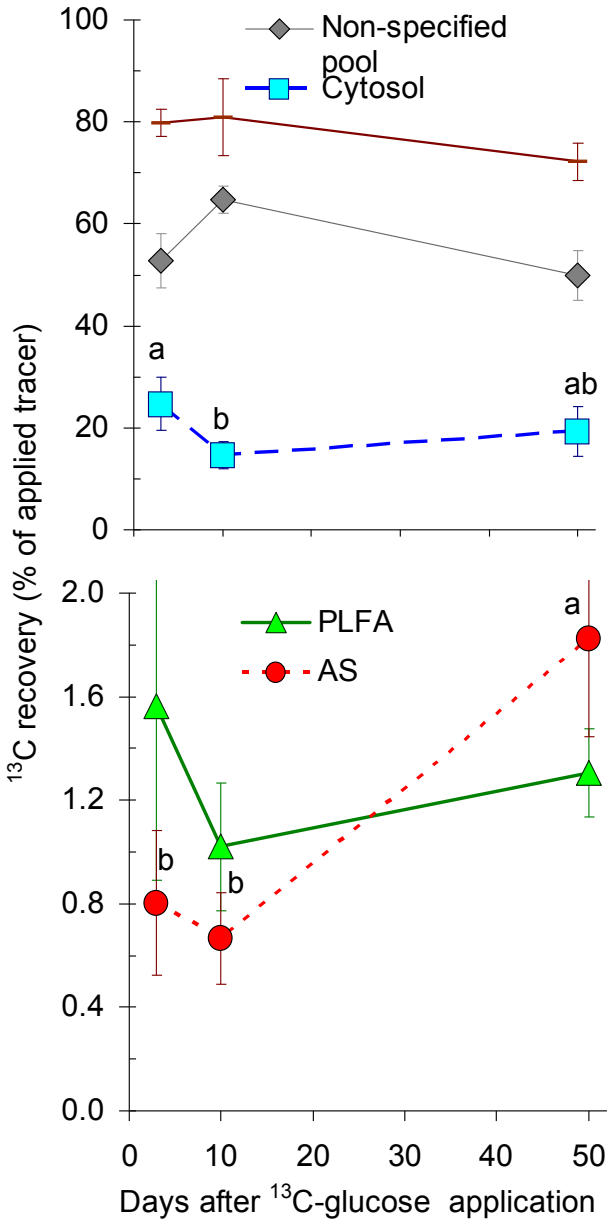
886 Figure 01.



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887 Figure 02.

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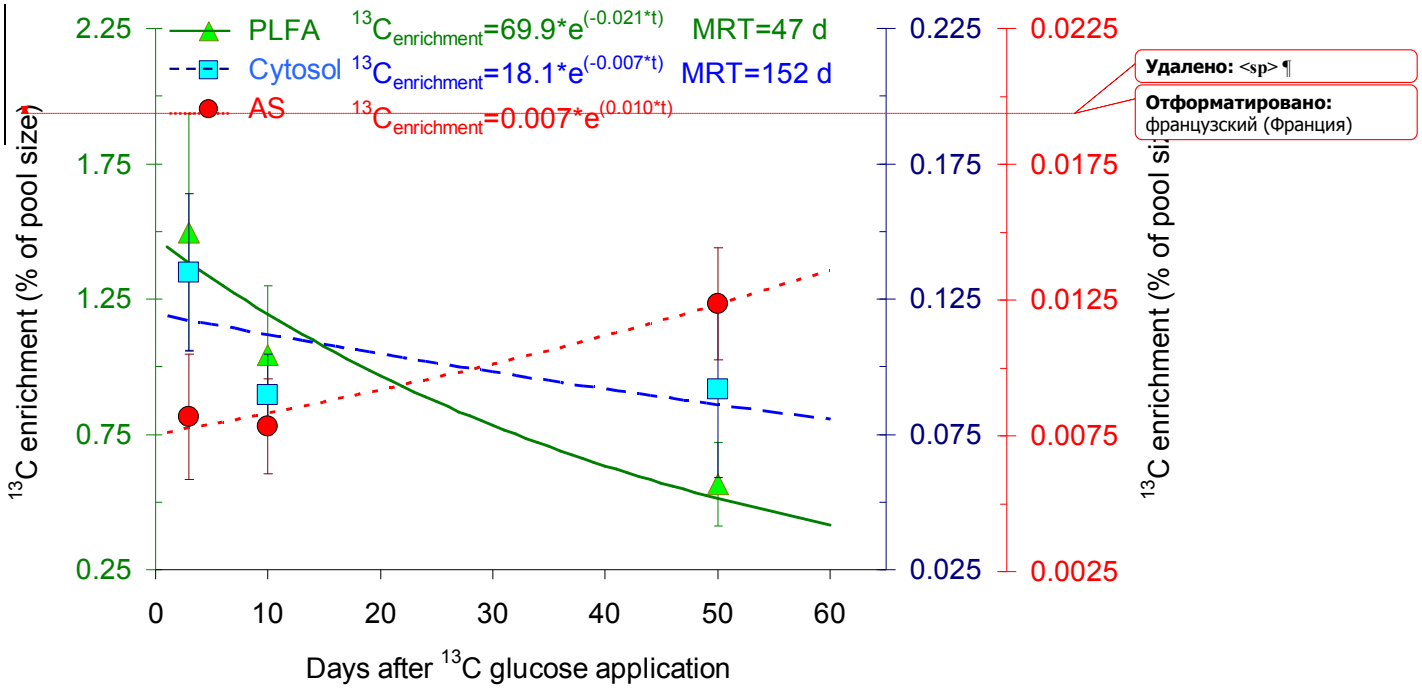

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889 Figure 03.

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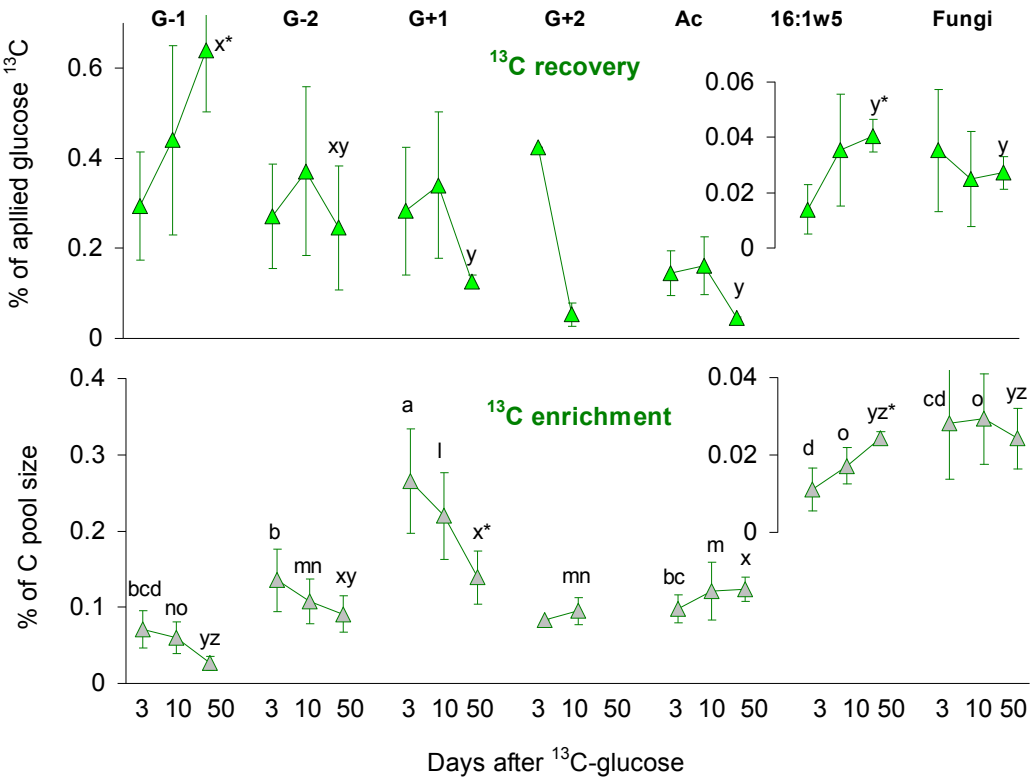

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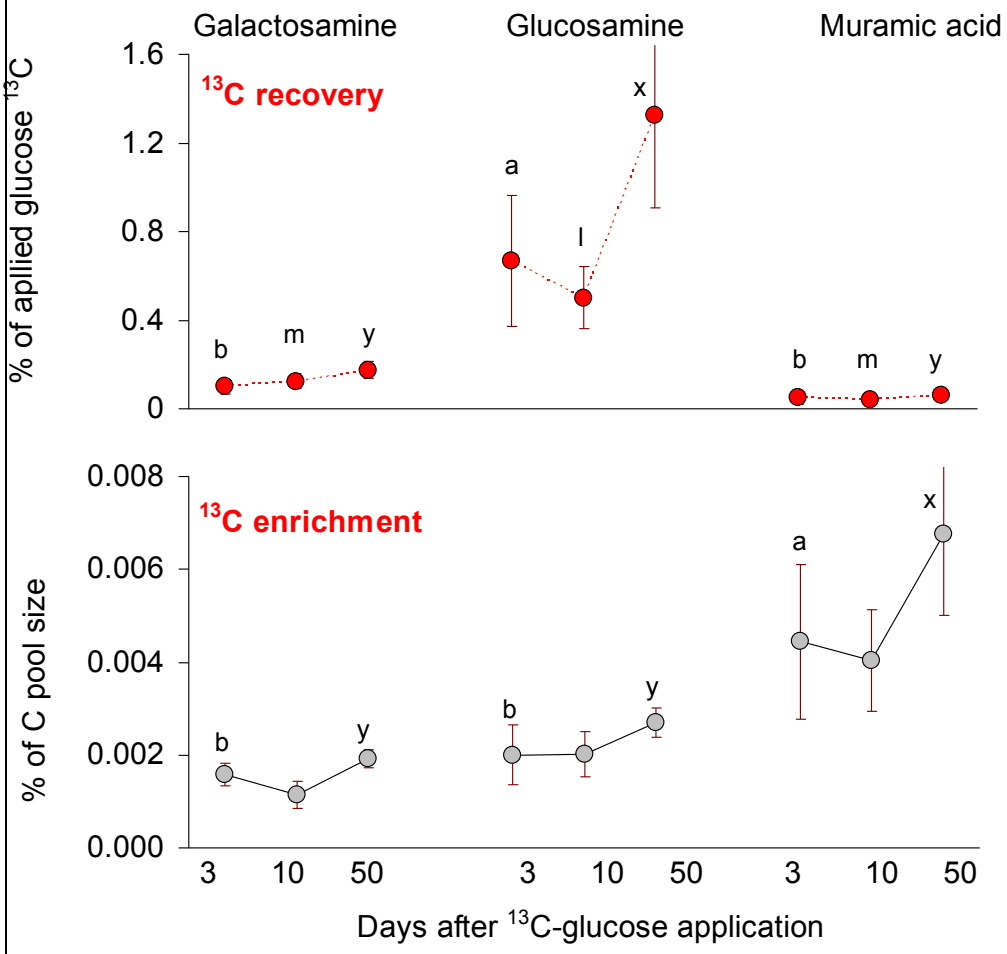
Figure 04.



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937 Figure 05.
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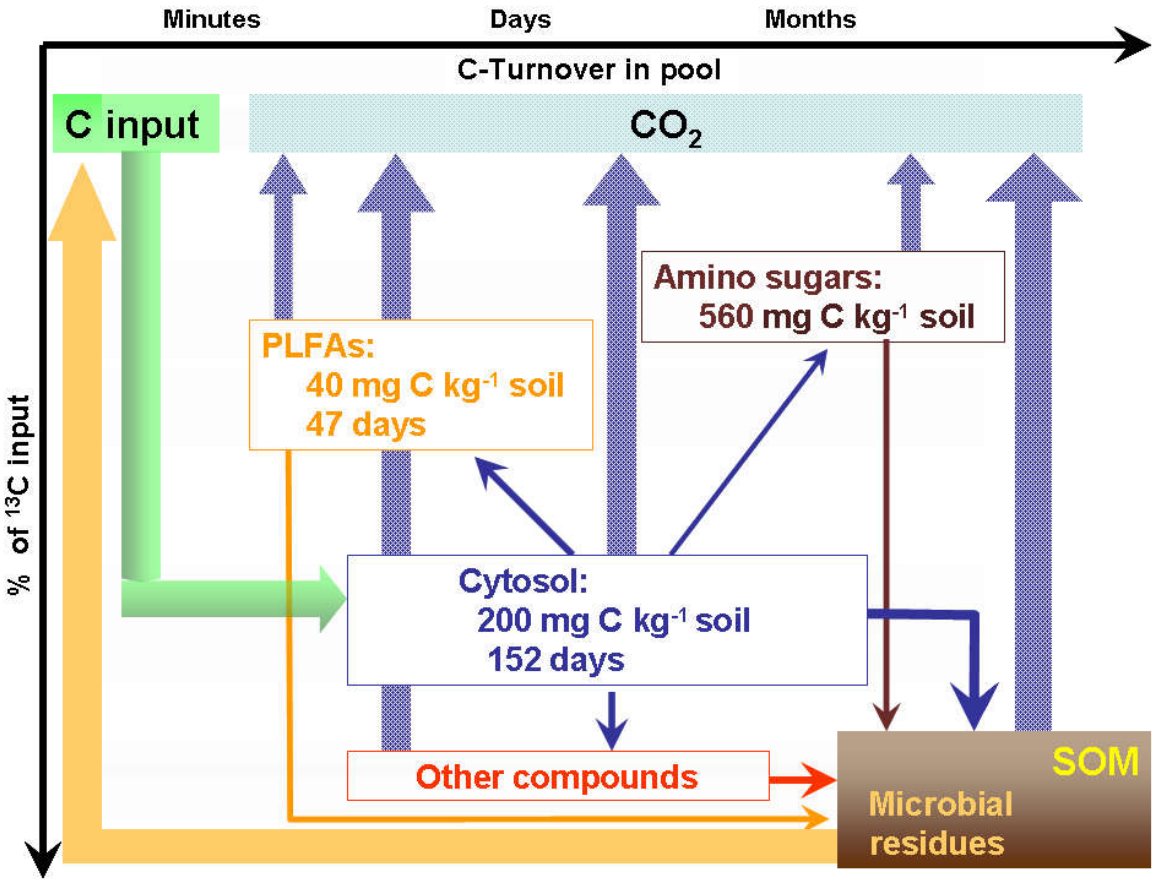

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