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-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

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.

Keywords

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

1. Introduction

Over the last decade, numerous studies have demonstrated the role of soil microorganisms in regulating the fate and transformation of organic compounds. Soil microorganisms produce exoenzymes to carry out the primary degradation of plant as well as microbial polymers to monomers. Further transformations of monomers then take place within the microbial cells. Monomeric substances 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.

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

84 in PLFA of living biomass are currently published. The formation of amino sugars from plant
85 biomass is relatively rapid at 6.2–9.0 days (Bai et al., 2013), whereas their turnover times in
86 soil vary between 6.5–81.0 y^{-1} (Glaser et al., 2006). Thus, PLFAs and amino sugars can be
87 used to trace the fate of C within the living microorganisms as well as to estimate the
88 contribution of microbial residues to SOM (Schmidt et al., 2007).

89 Some cell compartments, such as the cytosol, are not specific for various microbial
90 groups, whereas phospholipids are partly specific and consequently can be used to estimate
91 microbial community structure. Thus, PLFAs of bacterial (i16:0, a16:0, i15:0, a15:0, 16:1ω7,
92 18:1ω7) and fungal communities (18:2ω6,9; 18:3ω6,9,12; 16:1ω5) are used to draw
93 conclusions about the qualitative composition of living microbial communities, their
94 contribution to utilisation of C by various origin (plant or microbial) and to understand
95 trophic interactions within the soil (Ruess et al., 2005). In contrast, amino sugars
96 (glucosamine, galactosamine, mannosamine and muramic acid) are usually used to assess the
97 contributions of bacterial and fungal residues to SOM (Engelking et al., 2007; Glaser et al.,
98 2004). Muramic acid is of bacterial origin, whereas glucosamine is derived from both fungal
99 and bacterial cell walls (Glaser et al., 2004). Galactosamine is more abundant in fungal than
100 in bacterial cell walls (Engelking et al., 2007; Glaser et al., 2004).

101 Bacteria and fungi have various chemical composition, which strongly contributes to
102 their turnover rates in soil: for bacteria it consists 2.3-33 days, whereas for fungi it accounts
103 for 130-150 days (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). Despite
104 turnover of microorganisms directly effect the C turnover rates in intercellular compounds
105 (cell membrane and cell wall biomarkers), this relationship has rarely been investigated so
106 far. However, the comparison of C turnover for cell membrane and cell wall components can
107 be used to characterize the contribution of various microbial groups to medium-term C
108 utilisation and to the stabilization of microbially derived C in SOM.

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

133 rotation at the chosen site. The soil type was a loamy haplic Luvisol (IUSS Working group
134 WRB, 2014) and had the following chemical properties in the uppermost 10 cm: total organic
135 C content 1.5%, C/N 10.7, pH 6.6, clay content 22%, CEC 13 $cmol_C\ kg^{-1}$. The annual
136 precipitation is 870 mm and mean annual temperature is +7 °C.
137 In summer 2010, following harvest of the triticale, columns (diameter 10 cm and height 13
138 cm) were installed to a depth of 10 cm. Each column contained 1.5 kg of soil and bulk
139 density was 1.36 g cm^{-3}. The 50 mL of uniformly labelled ^{13}C glucose (99 atom % 13C) was
140 injected into the columns via a syringe at five points inside the column to spread the tracer
141 homogeneously. Syringe was equipped with a special pipe having length 13 cm and
142 perforated along the whole length, while the end of the pipe was sealed to prevent glucose
143 injection below of the column. Each column received 93.4 µmol ^{13}C of tracer (0.06 µmol ^{13}C
144 g^{-1}soil) and similar amounts of non-labeled glucose were applied to the control columns, to
145 make the experimental conditions equal. The concentration was chosen to trace the natural
146 pool of glucose in soil solution (Fischer et al., 2007), rather than stimulate the activity or
147 growth of microorganisms.
148 The experiment was done in four field replicates, which were organized in a randomized
149 block design. Labelled and control columns were present within each block. For the first 10
150 days of the experiment the rainfall was excluded by protective shelter, which was then
151 removed and the experiment was run for 50 days in total. The rainfall was excluded to
152 prevent the added glucose to be leached out from the soil profile, due to processes of
153 microbial uptake go slower in the field conditions, than in the controlled laboratory. After 3,
154 10 and 50 days, separate soil columns (four columns where ^{13}C was applied and four control
155 columns) were destructively sampled. The columns had no vegetation by the collecting time,
156 as well as when the ^{13}C glucose was applied.

157 The soil was removed from the column, weighed and the water content was determined in a
158 subsample. Soil moisture was determined by drying samples for 24 h at 105 °C and was
159 essentially constant during the experiment, ranging between 21–25% (25.7±1.2 (3 days),
160 23.3±1.3 (10 days), 21.4±0.7 (50 days)). Each soil sample was sieved to <2 mm and divided
161 into three parts. One part was stored frozen (-20ºC) for PLFA analysis, another was cooled
162 (+5°C) (during one week) before the microbial biomass analysis, and the rest was freeze-
163 dried and used for amino-sugar analysis and for measurement of the total amount of glucose
164 derived 13C remaining in the soil.
165
166 *2.2. Bulk soil $\delta^{13}C$ analysis*
167 The soil for the $\delta^{13}C$ analysis was milled and $\delta^{13}C$ values of bulk SOM were determined
168 using a Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit coupled via a ConFlo III
169 interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage IRMS (Thermo
170 Fischer, Bremen, Germany). The amount of glucose derived ^{13}C remaining in the soil was
171 calculated based on a mixing model (Equations 1 and 2), where the amount of C in the
172 background sample in Eq. 1 was substituted according to Eq. 2.

173 $$[C]_{soil} \cdot at\%_{soil} = [C]_{BG} \cdot at\%_{BG} + [C]_{glc} \cdot at\%_{glc} \quad \text{Eq. (1)}$$

174 $$[C]_{soil} = [C]_{BG} + [C]_{glc} \quad \text{Eq. (2)}$$

175 with:

176 $[C]_{soil/BG/glc}$ C amount of enriched soil sample / background soil sample /
177 glucose derived C in soil ($mol \cdot g_{soil}^{-1}$)
178 $at\%_{soil/BG/glc}$ ^{13}C in enriched soil sample / background soil sample /
179 applied glucose (at%)

180 *2.3. Cytosol C pool*

181 The cytosol pool was determined by the fumigation–extraction technique from fresh soil
182 shortly after sampling, according to Wu et al. (1990) with slight changes. Briefly, 15 g fresh
183 soil was placed into glass vials, which were exposed to chloroform during 5 days. After
184 removing the rest of chloroform from the soil, the cytosol C was extracted with 45 mL 0.05
185 M K_2SO_4. As fumigation–extraction technique produces not only soluble components, but
186 also cell organelles and cell particles, we named pool of C in fumigated extracts as "cytosol"
187 only for simplification of terminology. Organic C was measured with a high-temperature
188 combustion TOC-analyser (Analyser multi N/C 2100, Analytik Jena, Germany). The cytosol
189 pool was calculated as the difference between organic C in fumigated and unfumigated
190 samples without correcting for extraction efficiency. After organic C concentrations were
191 measured, the K_2SO_4 extracts were freeze-dried and the $\delta^{13}C$ values of a 30–35 µg subsample
192 were determined using EA-IRMS (instrumentation identical to soil $\delta^{13}C$ determination). The
193 recovery of glucose derived ^{13}C in fumigated and unfumigated samples was calculated
194 according to the above-mentioned mixing model (Eq. 1 and 2). The ^{13}C in the microbial
195 cytosol was calculated from the difference in these recoveries.

196

197 *2.4. Phospholipid fatty acid analysis*

198 The PLFA analysis was performed using the liquid–liquid extraction method of Frostegard et
199 al. (1991) with some modifications (Gunina et al., 2014). Briefly, 6 g of soil were extracted
200 with a 25-mL one-phase mixture of chloroform, methanol and 0.15 M aqueous citric acid
201 (1:2:0.8 v/v/v) with two extraction steps. The 19:0-phospholipid (dinonadecanoylglycerol-
202 phosphatidylcholine, Larodan Lipids, Malmö, Sweden) was used as internal standard one
203 (IS1) and was added directly to soil before extraction (25 µL with 1 µg μL^{-1}). Additional

chloroform and citric acid was added to the extract 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

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).

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)
275

276 $C_{enrichm(t)} = C_{enrichm(0)} \cdot \exp^{-kt}$ Eq. (5)

277 with

278	$C_{enrichm\ (t)}$	^{13}C enrichment of the compartment,	
279		obtained from Eq. 4 at time t	(%)
280	$C_{enrichm\ (0)}$	^{13}C enrichment of the compartment	
281		at time 0	(%)
282	k	decomposition rate constant	(% day^{-1})
283	t	time	(days)

284

285 One-way ANOVA was used to estimate the significance of differences in total ^{13}C
286 recovery and enrichment of non-specified SOM pool, cytosol, PLFAs and amino sugars. The
287 data always represent the mean of four replications ± standard error. The ^{13}C in the non-
288 specified SOM was calculated by subtracting off total ^{13}C measured in the soil, the ^{13}C
289 incorporated into cytosol, PLFAs and amino sugars. To describe decomposition rate of ^{13}C, a
290 single first order kinetic equation was applied to the enrichment of ^{13}C in the pool of cytosol,
291 PLFAs and amino sugars. (Eq. 5) (Kuzyakov, 2011; Parton et al., 1987).

292

293 **3. Results**

294 *3.1. Glucose utilisation and its partitioning within microbial biomass pools*

295 Amino sugar C pool was the largest, due to accumulation of these substances in SOM,
296 whereas pools that mainly characterize living MB showed smaller C contents (Table 1). The
297 cytosol pool (C content 210±7.10 for day 3; 195±14.8 for day 10; 198±19.9 mg C kg^{-1} soil

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. 1).
306 The ^{13}C recovery into PLFA was generally very low and was in the same range as recovery
307 into amino sugars (Fig. 1). The ^{13}C recovery in PLFA showed no clear trend between the
308 sampling points (high standard error) (Fig. 1). In contrast, ^{13}C recovery in amino sugars
309 increased two fold on the 50^{th} 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.

[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.

[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.

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

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.

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;

368 Fischer et al., 2010). Due to the first sampling point in our experiment was 3 days after
369 glucose addition, the obtained data on glucose mineralization can be mainly related to the
370 second stage.

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

380

381 *4.2. Partitioning of ^{13}C-derived glucose between cell compounds*

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

390

391

392

393 *4.3. Cytosol*

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

403

404 *4.4. Phospholipid fatty acids*

405 *4.4.1. Phospholipid fatty acid content and turnover*

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

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

416 dating (Rethemeyer et al., 2005) were between 1 and 80 years. However, these approaches
417 estimate the turnover time of C bound in PLFA, which can be much older than the PLFA
418 molecules due to repeated C recycling before incorporation. In contrast, ^{13}C pulse labeling is
419 an approach that enables direct estimation of the turnover of freshly added C by the initial
420 recovery peak. The approach used in the present study showed that the C turnover time in
421 PLFA is about 47 days (Fig. 02). Accordingly, if the decomposition after cell death is about
422 three days, the PLFA turnover time in living cells is about 44 days. This short turnover time
423 of PLFAs is significantly lower than the C turnover time in the cytosol (Fig. 02, Fig. 05).
424 This is because the membrane is an interacting surface between the cell and the environment
425 and thus, frequent and rapid adaptations of its structure are crucial for active microorganisms
426 (Bossio et al., 1998, Kieft et al.,1997). In contrast, the extracted cytosol pool includes C from
427 both active and dormant microorganisms (Blagodatskaya and Kuzyakov, 2013), and the latter
428 can dilute the ^{13}C signal incorporated into the active pool with non-labelled C, yielding a
429 lower turnover of this pool.

430

431 *4.4.2. Contribution of microbial groups to glucose derived C utilisation*

432 More glucose derived ^{13}C was incorporated into bacterial PLFAs (Fig. 03, top), than
433 into filamentous microorganisms. This can be a consequence of low C loading rates (less than
434 4 mg C g^{-1} soil, see (Reischke et al., 2014)), under which conditions the added C is utilized
435 primarily by bacterial communities, whereas at higher concentrations of applied substrate the
436 dominance of fungi in substrate utilisation is observed (Reischke et al., 2014).

437 The ^{13}C recovery into gram-negative fatty acids was higher (taking both G- groups
438 together) compared to G+ bacterial PLFAs (Fig. 03, top), which might be due to: i) the
439 abundance of their fatty acids, which was higher (Table 1) or ii) glucose uptake activity,

440 which was higher for G- than G+ groups. In contrast, the ^{13}C enrichment (^{13}C recovery
441 related to total C in particular biomarkers) for G- bacterial PLFAs was not higher than that
442 for G+ (Fig. 03, bottom). Thus, the high ^{13}C recovery into G- bacterial biomarkers can mainly
443 corresponds to their high content in the soil, not to higher activity of microbial groups.
444 However, enrichment of PLFAs C by glucose derived ^{13}C is only a proxy of microbial
445 activity and can only partly estimate the real activity of microbial groups. This clearly
446 suggests that the analysis of isotope data after labeling in general requires the calculation and
447 combined interpretation of both the total tracer C recovery as well as the ^{13}C enrichment in
448 the investigated pool.

449 In contrast to our results, a higher recovery of glucose derived ^{13}C into G+ than G-
450 PLFAs was observed in other studies (Dungait et al., 2011; Ziegler et al., 2005). However, in
451 these studies, much higher amounts of C were applied to the soil (15 μg C g^{-1} soil), which
452 stimulated the growth of G+ bacteria. In contrast, under steady-state conditions with low
453 glucose concentrations in soil, G- bacteria were the most competitive group for glucose
454 uptake (Fig. 03).

455 The ^{13}C enrichment of bacterial PLFAs decreased from day 3 to day 50, whereas ^{13}C
456 in fungal PLFAs increased (in the case of 16:1ω5) or stayed constant (Fig. 03, bottom). The
457 decrease in ^{13}C enrichment in bacterial fatty acids indicates a partial turnover of bacterial
458 lipid membranes, which is much faster than turnover in fungal membranes. This result is
459 consistent with the turnover time of bacterial biomass in soil (Baath, 1998), which is about 10
460 days, whereas fungal biomass turnover times range between 130–150 days (Rousk and Baath,
461 2007). Consequently, the increase in ^{13}C enrichment in fungal PLFAs at late sampling points
462 indicates that fungi consume the exudation products of bacteria or even dead bacterial
463 biomass (Zhang et al., 2013; Ziegler et al., 2005).

464

465 *4.5. Amino sugars*

466 *4.5.1. Amino sugar content and amino sugar C turnover in total and living microbial cell*
467 *walls*

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

481 The majority of amino sugars, extracted after acid hydrolysis, represent microbial
482 necromass, which does not incorporate any glucose derived ^{13}C, but strongly dilutes the ^{13}C
483 incorporated into the walls of living cells. To estimate the ^{13}C enrichment into amino sugars
484 of living cells, we first calculated the amount of amino sugars in the living MB pool, which
485 consisted 0.87 μmol g^{-1} soil, and was about 11% of the total amino sugar pool (please, see
486 Supplementary calculations for further details). This estimate agrees with that of Amelung et
487 al. (2001a) and Glaser et al. (2004), who reported that the amount of amino sugars in living
488 biomass is one to two orders of magnitude lower than in the total amino-sugar pool. We
489 calculated the ^{13}C enrichment in amino sugars for the first sampling point, assuming that all

490 replaced C is still contained within living MB after three days of glucose C utilisation, and it
491 consisted 0.57% of the C pool. Comparison of these data with the ^{13}C enrichment into PLFAs
492 and the cytosol allowed us to conclude that the enrichment of amino sugar C with glucose
493 derived ^{13}C in living biomass is two-fold lower than the enrichment in PLFAs, and higher
494 than in the cytosol pool. This reflects that microbial C turnover is a phenomenon that is not
495 restricted to the death or growth of new cells, but that even within living cells, highly
496 polymeric cell compounds, including cell walls, are constantly replaced and renewed (Park
497 and Uehara, 2008).

498

499 *4.5.2. Contribution of bacterial and fungal cell walls to SOC*

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

508 Three-fold more glucose derived ^{13}C was recovered in glucosamine than in
509 galactosamine and muramic acid (Fig. 04, top). This correlates with the pool size and
510 indicates that glucosamine is the most dominant amino sugar not only in total amino sugars,
511 but also within the walls of living cells. The galactosamine/muramic acid ratio of the
512 incorporated ^{13}C was six, and consequently was significantly lower than the ratio calculated
513 for the amount of amino sugars (Table 1). This indicates that bacteria are more active in

514 glucose derived ^{13}C utilisation than fungi, a conclusion also supported by the ^{13}C-PLFA data
515 (Fig. 03, top). Thus, even if the composition of amino sugars does not allow a clear
516 conclusion concerning living microbial communities in soil, amino sugar analysis combined
517 with ^{13}C labeling reveals the activity of living microbial groups in terms of substrate
518 utilisation.

519 The calculated ^{13}C enrichment was the highest in muramic acid (Fig. 04, bottom).
520 This is in agreement with the high ^{13}C enrichment of bacterial PLFAs compared to 16:1ω5
521 and fungi (Fig. 03). Due to differences in cell-wall architecture, G+ bacteria contain more
522 muramic acid (approximately four times) than G- bacteria (Lengeler et al., 1999), and thus
523 make a higher contribution to the ^{13}C enrichment of muramic acid.

524 The ^{13}C enrichment of glucosamine was two-fold lower than muramic acid (Fig. 04,
525 bottom). This confirms the hypothesis that glucosamine originates from bacterial as well as
526 fungal cell walls and, consequently, has a mixed enrichment between the fungal
527 galactosamine and bacterial muramic acid.

528

529 **5. Conclusions**

530 Tracing the ^{13}C labelled glucose through cytosol, PLFAs and amino sugars is a prerequisite
531 for understanding the fate of organic substrates in soil and can be used to estimate C turnover
532 times in various microbial cell compartments. In contradiction to hypothesis one, the C
533 turnover times were as follows: PLFA (47 days)<cytosol (150 days)<amino sugars. The long
534 C half-life time in cytosol can be explained by efficient C recycling and cytosol
535 heterogeneous composition, which involves compounds with different turnover rates. Due to
536 significant part of amino sugar pool was in the composition of microbial residues, the ^{13}C
537 enrichment of this pool was still increasing at the end of the experiment, which reflects the

538 slowest C turnover time here. An approximate calculation of ^{13}C enrichment of amino sugars
539 in the living biomass accounted for 0.57% of pool size, which was lower than for PLFAs.
540 This reflects that C turnover in cell wall components is slower than in membrane
541 components.

542 Both PLFAs and amino sugars analysis showed the prevalence of bacterial
543 biomass/bacterial residues in investigated soil. Much higher recovery and enrichment by
544 glucose-^{13}C was found in bacterial than in fungal PLFAs. A lower ^{13}C enrichment of
545 filamentous PLFAs compare to bacterial demonstrates that i) C turnover in filamentous
546 PLFAs is slower compare to bacterial and ii) filamentous organisms might consume bacterial
547 biomass and utilize products of its metabolism. The ratio of galactosamine/muramic acid for
548 incorporated ^{13}C evidences that bacteria were more active in glucose utilisation than fungi.
549 The ^{13}C enrichment was the highest for muramic acid and the lowest for galactosamine,
550 demonstrating that the turnover of bacterial cell wall components is more rapid than fungal.

551 Consequently, the combination of ^{13}C labeling with the subsequent analysis of several
552 microbial cell compartments and biomarkers is a unique approach to understanding C
553 partitioning within microbial cells and the microbial communities in soil. This knowledge is
554 not only crucial for assessing C fluxes and recycling in soil, but is also important for
555 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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814 **Table 1** Amount of microbial biomass compartments, their C content, PLFA content of
815 microbial groups and composition of microbial residues in investigated soil. G-1 and G-2 are
816 gram-negative group one and two, respectively; G+1 and G+2 are gram positive group one
817 and two, respectively; Ac – actinomycetes; 16:1ω5 - saprotrophic fungi. Data present mean
818 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 Fungi	5.3 271
Galactosamine/muramic acid			12 - 19
Galactosamine/muramic acid literature data for pure cultures*)		Bacteria Fungi	2.8 59

819 *Data are taken from Glaser et al. (2004).
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827 **Table captions**

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

833

834 **Figure captions**

835 **Fig. 01** Partitioning of glucose derived ^{13}C in SOM presented as the ^{13}C recovery (% of
836 initially applied ^{13}C) between the following pools: non-specified SOM (calculated as total ^{13}C
837 recovery subtract ^{13}C recovery in cytosol, PLFAs and amino sugars), cytosol, PLFAs and
838 amino sugars. Brown line indicates the total remaining ^{13}C derived glucose in the soil and is a
839 sum of ^{13}C in non-specified SOM, cytosol, PLFAs and amino sugars. Small letters reflect
840 differences between the sampling points for the distinct pool. Data present mean (n=4) and
841 bars present standard errors (SE). The SE for the amino sugars are not fully shown.

842

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

847

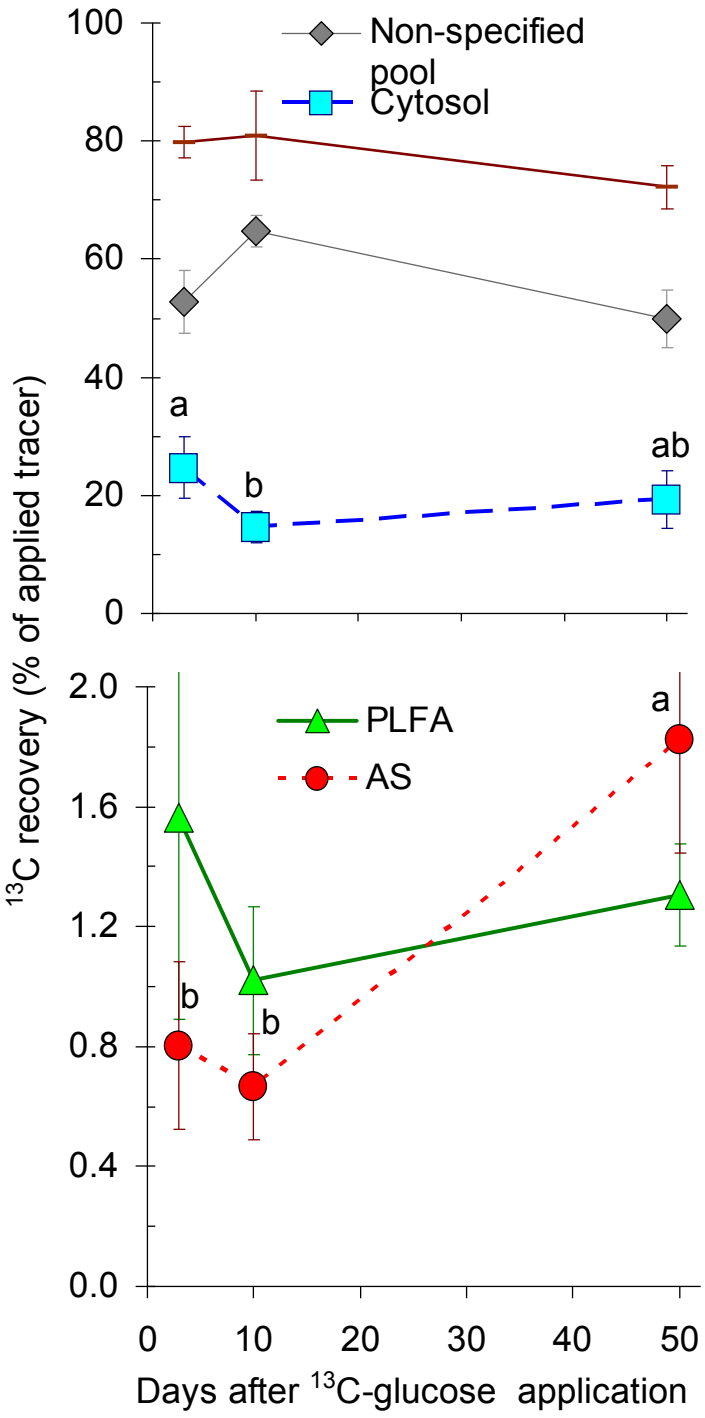
848 **Fig. 03** Recovery of glucose derived ^{13}C (top) and ^{13}C enrichment (bottom) of the microbial
849 PLFAs. Note that the values for 16:1ω5 and fungi are scaled-up 10 times (secondary Y axis)
850 compared to those of other groups (Y axis at the left). Data present mean (n=4) and bars

present standard errors. Small letters reflect differences between the microbial groups for ^{13}C recovery and ^{13}C enrichment from glucose; letters a-d are for day three, l-o are for day 10, x-z are for day 50.

Fig. 04 Recovery of glucose derived ^{13}C (top) and ^{13}C enrichment (bottom) of amino sugars and muramic acid. Letters reflect significant differences in the recovery and ^{13}C enrichment from glucose ^{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.

887 Figure 01.



888 Figure 02.

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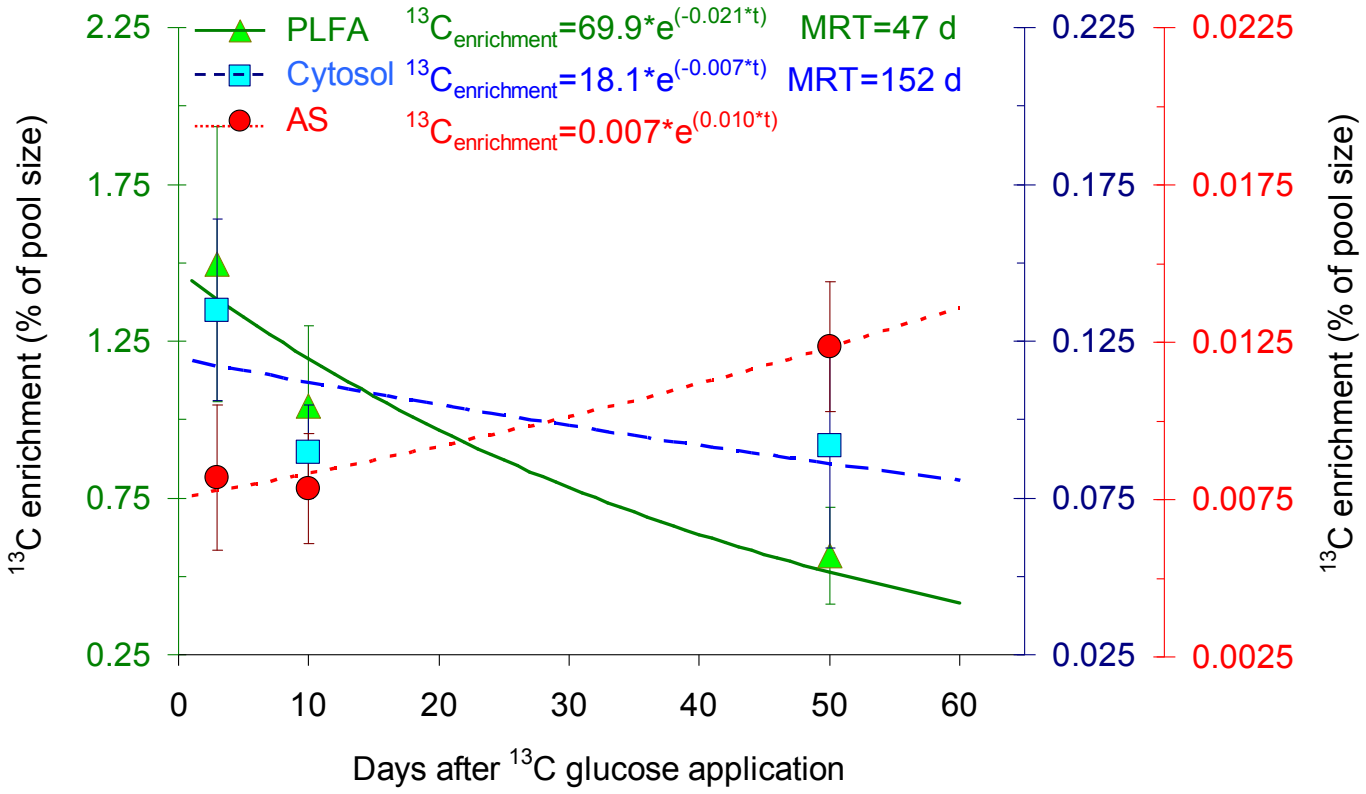

890 Figure 03.

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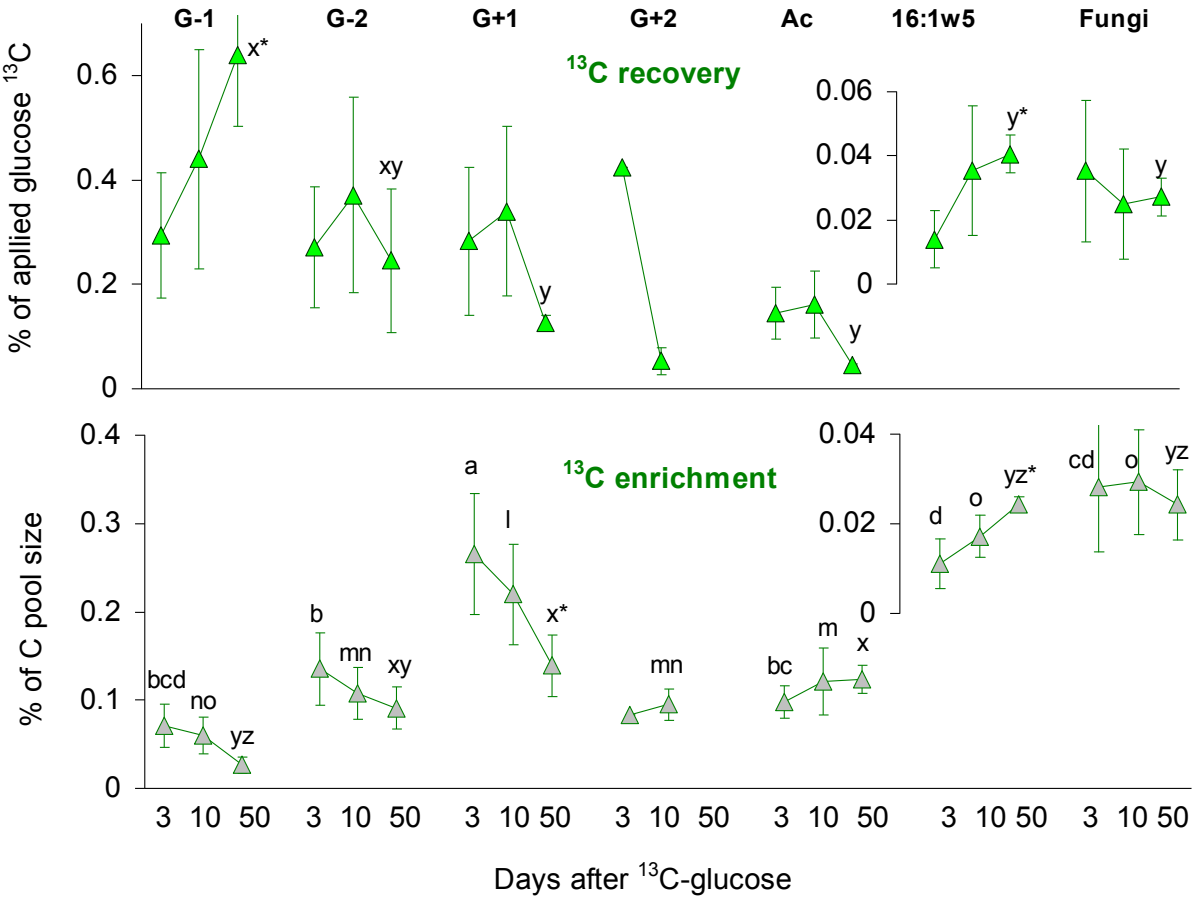
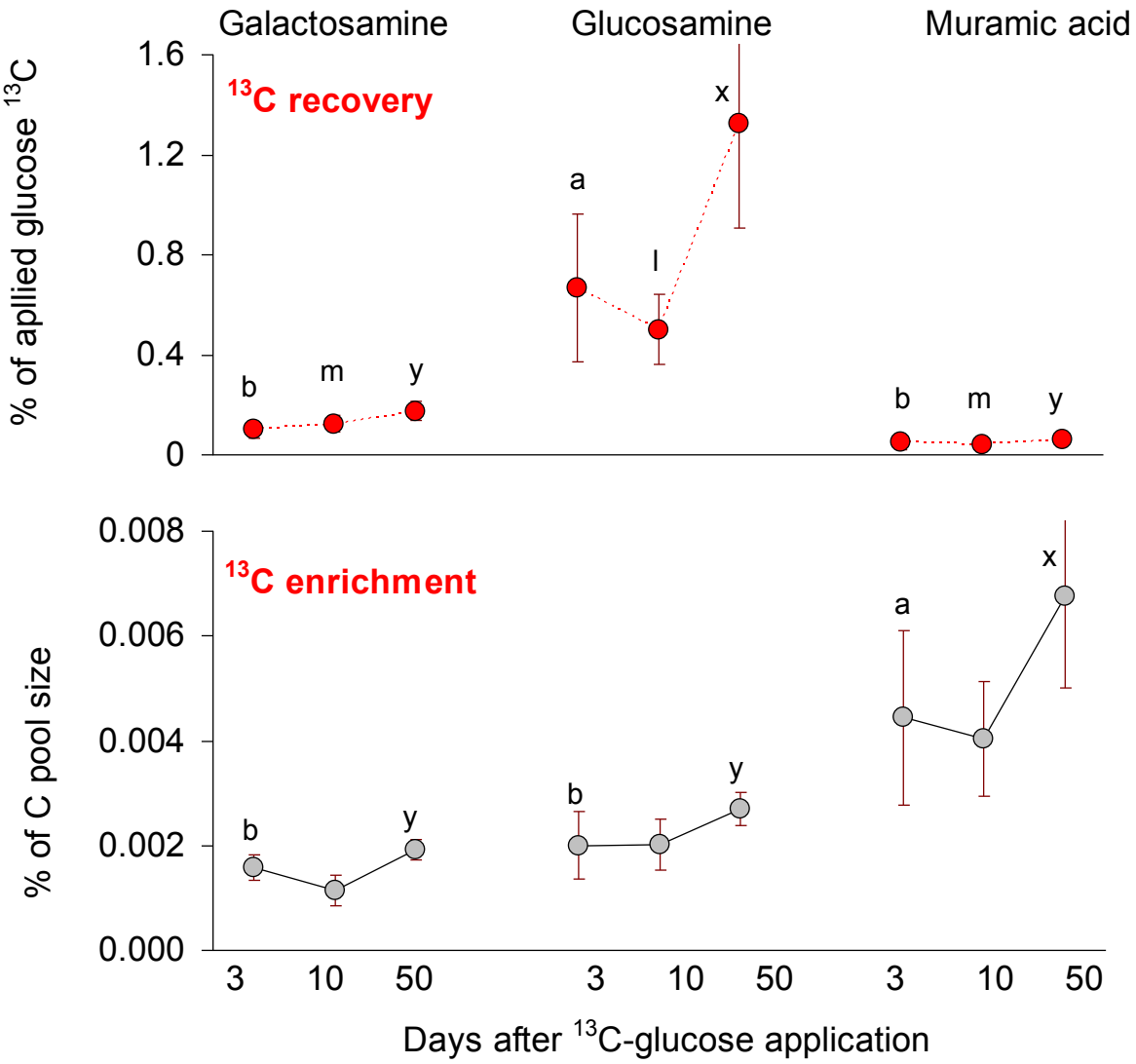


Figure 04.


Galactosamine
Glucosamine
Muramic acid
13C recovery
% of apllied glucose 13C
1.6
1.2
0.8
0.4
0
b
m
y
a
l
x
b
m
y
13C enrichment
% of C pool size
0.008
0.006
0.004
0.002
0.000
b
y
b
y
a
x
3
10
50
3
10
50
3
10
50
Days after 13C-glucose application

938 Figure 05.
939