1 **Glucose C turnover in cell compartments and microbial groups in soil**

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Abstract

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

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

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

51   information is especially important for assessing C fluxes in soil and the contribution of C

52   from microbial residues to soil organic matter.

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54   **Keywords**

55   Microbial biomarkers; phospholipid fatty acids; amino sugars; ^{13}C labeling; glucose

56   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 pass into the living microbial pool and are partly mineralised to CO_2, while part is assimilated into cell polymers and ultimately incorporated into soil organic matter (SOM) after cell death (Kindler et al., 2006). Understanding the fate of substances originated from plants and microbial residues into living biomass is therefore crucial for estimating the recycling of carbon (C) in soil and its stabilization as SOM.

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

Organic compounds that are taken up by microorganisms first enter the cytosol (Gottschalk, 1979), which has a high heterogeneity in composition (includes components of various chemical structure and molecular weight). However, due to the heterogeneity of this pool, the calculated C turnover time is a mean of turnover times of various components. The calculated turnover time of intact PLFAs in soil after microbial death is 2.8 days (Kindler et al., 2009), resulting PLFAs are mainly used to characterize the living microorganisms (Frostegard et al., 2011; Rethemeyer, 2004). However, no data concerning turnover time of

83   PLFA C in the living biomass are currently published. The formation of amino sugars from

84   plant biomass is relatively rapid at 6.2–9.0 days (Bai et al., 2013), whereas their turnover

85   times in soil vary between 6.5–81.0 y^{-1} (Glaser et al., 2006). Thus, PLFAs and amino sugars

86   can be used to trace the fate of C within the living microorganisms and estimate their

87   contribution to SOM (Schmidt et al., 2007).

88   Some cell compartments, such as the cytoplasm, are not specific for various microbial

89   groups, whereas phospholipids are partly specific and consequently can be used to estimate

90   microbial community structure. Thus, PLFAs of bacterial (i16:0, a16:0, i15:0, a15:0, 16:1ω7,

91   18:1ω7) and fungal communities (18:2ω6,9; 18:3ω6,9,12; 16:1ω5) are used to draw

92   conclusions about the qualitative composition of living microbial communities, their

93   contribution to utilisation of C by various origin (plant or microbial) and to understand

94   trophic interactions within the soil (Ruess et al., 2005). In contrast, amino sugars

95   (glucosamine, galactosamine, mannosamine and muramic acid) are usually used to assess the

96   contributions of bacterial and fungal residues to SOM (Engelking et al., 2007; Glaser et al.,

97   2004). Muramic acid is of bacterial origin, whereas glucosamine is derived from both fungal

98   and bacterial cell walls (Glaser et al., 2004). Galactosamine is more abundant in fungal than

99   in bacterial cell walls (Engelking et al., 2007; Glaser et al., 2004).

100   Bacteria and fungi have various chemical composition, which strongly contributes to

101   their turnover rates in soil: for bacteria it consists 2.3-33 days, whereas for fungi it accounts

102   for 130-150 days (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). Despite

103   turnover of microorganisms directly effect the C turnover rates in intercellular compounds

104   (cell membrane and cell wall biomarkers), this relationship has rarely been investigated so

105   far. However, the comparison of C turnover for cell membrane and cell wall components can

106   be used to characterize the contribution of various microbial groups to medium-term C

107   utilisation and to the stabilization of microbially derived C in SOM.

108   Combination of PLFAs and amino-sugar biomarkers analyses, as well as cytosolic C

109   measurement with isotope tracing techniques (based on ^{13}C natural abundance or ^{13}C/[14]C

110   labelling) have been used in various studies to characterize organic C utilisation by the

111   microbial community (Bai et al., 2013; Brant et al., 2006). However, to date no systematic

112   studies have compared these contrasting cell compartments in a single soil within a C

113   turnover experiment. Therefore, this study aimed to examine C allocation to various cell

114   compartments following ^{13}C labelling with a ubiquitous monomer, glucose. Glucose has a

115   higher concentrations in the soil solution compared to other low molecular weight organics

116   (Fischer et al., 2007), due to its diverse origin: from cellulose decomposition, presence in

117   rhizodeposition (Derrien et al., 2004; Gunina and Kuzyakov, 2015), and synthesis by

118   microorganisms. It is also used by most of the microbial groups, and, thus, is the most

119   suitable substance for such a study.

120   We analyzed glucose derived ^{13}C partitioning into the cytosol, cell membranes and

121   cell walls, to evaluate the turnover time of C in each pool, and to assess the contribution of

122   bacterial and fungal biomass to SOM. We hypothesized that: 1) turnover times of C in pools

123   follow the order cytosol<PLFA<amino sugars, because substances taken up by cells first are

124   transported by membrane proteins into cytosol, from where they get distributed to other

125   cellular pools and 2) incorporation of ^{13}C glucose should be faster and higher for bacterial

126   than for fungal biomarkers, because bacterial biomass has a faster cell turnover than fungal

127   biomass.

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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 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,

157   10 and 50 days, separate soil columns (four columns where ^{13}C was applied and four control

158   columns) were destructively sampled. The columns had no vegetation by the collecting time,

159   as well as when the ^{13}C glucose was applied.

160   The soil was removed from the column, weighed and the water content was determined in a

161   subsample. Soil moisture was determined by drying samples for 24 h at 105 °C and was

162   essentially constant during the experiment, ranging between 21–25% (25.7±1.2 (3 days),

163   23.3±1.3 (10 days), 21.4±0.7 (50 days)). Each soil sample was sieved to <2 mm and divided

164   into three parts. One part was stored frozen (-20ºC) for PLFA analysis, another was cooled

165   (+5°C) (during one week) before the microbial biomass analysis, and the rest was freeze-

166   dried and used for amino-sugar analysis and for measurement of the total amount of glucose

167   derived ^{13}C remaining in the soil.

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169   *2.2. Bulk soil $\delta^{13}C$ analysis*

170   The soil for the $\delta^{13}C$ analysis was milled and $\delta^{13}C$ values of bulk SOM were determined

171   using a Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit coupled via a ConFlo III

172   interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage IRMS (Thermo

173   Fischer, Bremen, Germany). The amount of glucose derived ^{13}C remaining in the soil was

174   calculated based on a mixing model (Equations 1 and 2), where the amount of C in the

175   background sample in Eq. 1 was substituted according to Eq. 2.

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

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

178   with:

179    $[C]_{soil/BG/glc}$ C amount of enriched soil sample / background soil sample /

180		glucose derived C in soil	$(\text{mol} \cdot \text{g}_{soil}^{-1})$
181	$at\%_{soil/BG/glc}$	^{13}C in enriched soil sample / background soil sample /	
182		applied glucose	$(at\%)$

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2.3. Cytosolic C pool

The cytosolic pool was determined by the fumigation–extraction technique from fresh soil shortly after sampling, according to Wu et al. (1990) with slight changes. Briefly, 15 g fresh soil was placed into glass vials, which were exposed to chloroform during 5 days. After removing the rest of chloroform from the soil, the cytosolic C was extracted from the soil with 45 mL 0.05 M K_2SO_4. As fumigation–extraction technique ~~allows to obtain~~ not only soluble components, but also cell organelles and cell particles, we named pool of C in fumigated extracts as cytosol only for simplification of terminology. Organic C was measured with a high-temperature combustion TOC-analyser (Analyser multi N/C 2100, Analytik Jena, Germany). The cytosolic pool was calculated as the difference between organic C in fumigated and unfumigated samples without correcting for extraction efficiency. After organic C concentrations were measured, the 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.

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203 *2.4. Phospholipid fatty acid analysis*

204 The PLFA analysis was performed using the liquid–liquid extraction method of Frostegard et

205 al. (1991) with some modifications (Gunina et al., 2014). Briefly, 6 g soil were extracted with

206 a 25-mL one-phase mixture of chloroform, methanol and 0.15 M aqueous citric acid (1:2:0.8

207 v/v/v) with two extraction steps. The 19:0-phospholipid (dinonadecanoylglycerol-

208 phosphatidylcholine, Larodan Lipids, Malmö, Sweden) was used as internal standard one

209 (IS1) and was added directly to soil before extraction (25 μL with 1 μg μL^{-1}). Additional

210 chloroform and citric acid was added to the extract achieve a separation of two liquid phases,

211 in which the lipid fraction was separated from other organics. Phospholipids were separated

212 from neutral- and glycolipids by soild-phase extraction using a silica column. Alkaline

213 saponification of the purified phospholipids was performed with 0.5 mL 0.5 M NaOH

214 dissolved in dried MeOH, followed by methylation with 0.75 mL BF$_3$ in methanol. The

215 resulting fatty acid methyl esters (FAMEs) were purified by liquid–liquid extraction with

216 hexane (three times). Before the final quality and quantity measurements, internal standard

217 two (IS2) (13:0 FAME) (15 μL with 1 μg μL^{-1}) was added to the samples (Knapp, 1979).

218   All PLFA samples were analysed by gas chromatograph (GC) (Hewlett Packard 5890

219 GC coupled to a mass-selective detector 5971A) (Gunina et al., 2014). A 25 m HP-1

220 methylpolysiloxane column (internal diameter 0.25 mm, film thickness 0.25 μm) was used

221 (Gunina et al., 2014). Peaks were integrated and the ratio to IS2 was calculated for each peak

222 per chromatogram. Substances were quantified using a calibration curve, which was

223 constructed using 29 single standard substances, (13:0, 14:0, i14:0, a14:0, 14:1ω5, 15:0,

224 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,

225 10Me18:0, 18:1ω7, 18:1ω9, 18:2ω6,9, 18:3ω6,9,12, cy19:0, 19:0, 20:0, 20:1ω9, 20:4ω6) at

226 six concentrations. The recovery of extracted PLFA was calculated using IS1 and the PLFA

227 contents of samples were individually corrected for recovery. Based on the measured PLFAs

228 contents, the PLFAs C was calculated for the each single compound.

229   The $^{13}C/^{12}C$ isotope ratios of the single fatty acids were determined by an IRMS Delta

230 PlusTM coupled to a gas chromatograph (GC; Trace GC 2000) via a GC-II/III-combustion

231 interface (all units from Thermo-Fisher, Bremen, Germany) (Gunina et al., 2014). A 15 m

232 HP-1 methylpolysiloxane column coupled with a 30 m HP-5 (5% Phenyl)-

233 methylpolysiloxane column (both with an internal diameter of 0.25 mm and a film thickness

234 of 0.25 μm) were used. The measured $\delta^{13}C$ values of the fatty acids were corrected for the

235 effect of derivative C by analogy to Glaser and Amelung (2002) and were referenced to Pee

236 Dee Belemnite by external standards. The enrichment of ^{13}C in single fatty acids was

237 calculated by analogy to bulk soil and cytosol according to Eq. 1 and 2, following a two-pool

238 dilution model (Gearing et al., 1991).

239

240 *2.5. Amino sugar analysis*

241 Acid hydrolysis was performed to obtain amino sugars from soil and further ion removal was

242 performed according to the method of Zhang and Amelung (1996) with optimization for $\delta^{13}C$

243 determination (Glaser and Gross, 2005). Methylglucamine (100 μL, 5 mg mL^{-1}) was used as

244 IS1 and was added to the samples after hydrolysis. Following iron and salt removal, non-

245 cationic compounds such as monosaccharides and carboxylic acids were removed from the

246 extracts using a cation exchange column (AG 50W-X8 Resin, H$^+$ form, mesh size 100–200,

247 Biorad, Munich, Germany) (Indorf et al., 2012). For final measurement, IS2 – fructose (50

248 μL, 1 mg mL^{-1}) – was added to each sample. The amino sugar content and ^{13}C enrichment

249 were determined by LC-O-IRMS (ICS-5000 SP ion chromatography system coupled by an

250 LC IsoLink to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo-Fischer,

251 Bremen, Germany)) (Dippold et al., 2014). Amino sugars were quantified using a calibration

252 curve, which was constructed using four single standard substances (glucosamine,

253 galactosamine, mannosamine and muramic acid) as external standards at four different

254 concentrations (Dippold et al., 2014).

255

256 *2.6. Calculations and statistical analysis*

257 Factor analysis with the principal component extraction method of mass % of individual

258 PLFAs was done. The final assignment of fatty acids to distinct microbial groups was made

259 by combination the results of factor loadings table with databases about presence of particular

260 fatty acids in microbial groups (Zelles, 1997). Fatty acids which were loaded into the same

261 factor with the same sign (+ or -) and belonged to one group (base of the table provided in

262 Zelles (1997)) were related to one specific microbial group and their PLFA contents were

263 summed. This method enables quality separation of microbial groups within the soils

264 (Apostel et al., 2013; Gunina et al., 2014). The results of the factor analysis are presented in

265 Supplementary Table 1.

266 Recovery of glucose derived ^{13}C (^{13}C$_{rec}$) (means ^{13}C recovery represented as % of

267 total applied ^{13}C) and enrichment (^{13}C$_{enrichm}$) (means ^{13}C recovery represented as % of total C

268 pool) of the cytosol, PLFAs and amino sugars was calculated according to Eq. 3 and 4,

269 respectively. The C turnover time in the cell pools was calculated as $^{1}/_{k}$; the value of k was

270 obtained from Eq. 5.

271 $$^{13}C_{incorp} = \frac{C_{Glc}}{^{13}C_{Applied}} \times 100\%$$ Eq. (3)

272 $$^{13}C_{enrichm} = \frac{C_{Glc}}{^{Total}C_{Pool}} \times 100\%$$ Eq. (4)

273 with

12

274   C_{Glc} amount of glucose derived C incorporated into a distinct cell compartment

275   calculated by equation (1) and (2)   (μmol ^{13}C per column)

276      $^{13}C_{Applied}$ amount of applied glucose ^{13}C   (μmol ^{13}C per column)

277      $^{Total}C_{Pool}$ amount of pool C   (μmol C per column)

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

280   with

281      $C_{enrichm\ (t)}$ ^{13}C enrichment of the compartment,

282   obtained from Eq. 4 at time t   (%)

283      $C_{enrichm\ (0)}$ ^{13}C enrichment of the compartment

284   obtained from Eq. 4 at time 0   (%)

285   k   decomposition rate constant   ($\%\ \text{day}^{-1}$)

286   t   time   (days)

287

288   One-way ANOVA was used to estimate the significance of differences in total ^{13}C

289   recovery and enrichment of cytosol, PLFAs and amino sugars. The data always represent the

290   mean of four replications ± standard error. To calculate the turnover time of C in the cytosol,

291   PLFA and amino sugar pools, a single exponential model was used (Eq. 5) (Kuzyakov, 2011;

292   Parton et al., 1987).

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3. Results

3.1. Glucose utilisation and its partitioning within microbial biomass pools

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

[Table 1]

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

[Fig. 1]

3.2. Turnover time of C in microbial biomass pools

To evaluate C turnover in the cytosol, PLFAs and amino sugars, we calculated the enrichment (% of incorporated ^{13}C relatively to pool C) of each pool by glucose derived ^{13}C. The pool enrichment was the highest for PLFAs and the lowest for amino sugars (Fig. 2).

14

318   Based on the decrease of ^{13}C enrichment over time (Fig. 2), the C turnover in the cytosol and

319   PLFAs was calculated as 151 and 47 days, respectively. The C turnover time in the amino-

320   sugar pool could not be calculated by this approach because the maximum enrichment had

321   not yet been reached, and consequently a decomposition function could not be fitted.

322   **[Fig. 2]**

323

324   *3.3 Phospholipid fatty acids*

325   Fatty acids of bacterial origin dominated over those of fungal origin within the living

326   microbial community characterized by PLFA composition (Table 1). The PLFA content of

327   most groups did not change significantly during the experiment, reflecting steady-state

328   conditions for the microbial community (see Suppl. Table 2).

329   Glucose derived ^{13}C was incorporated in higher portions into bacterial than into fungal

330   PLFAs (Fig. 3, top). Remarkably, the ^{13}C enrichment decreased over time for all bacterial

331   PLFAs, whereas it increased or remained constant for 16:1ω5, fungi and filamentous,

332   bacterial actinomycetes (Fig. 3, bottom), indicating differences in C turnover in single-celled

333   organisms compared to filamentous organisms.

334   **[Fig. 3]**

335

336   *3.4. Amino sugars*

337   The content of amino sugars followed the order: muramic acid < galactosamine <

338   glucosamine (Table 1). The glucosamine/muramic acid ratio varied between 17 and 55,

339   whereas the galactosamine/muramic acid ratio ranged between 12 and 19 (Table 1). This

340   provides evidence that bacterial residues were dominant in the composition of microbial

341   residues in SOM.

342   The recovery of glucose derived ^{13}C into amino sugars increased in the order:

343   muramic acid = galactosamine < glucosamine (Fig. 4, top) reflecting partly their pool sizes.

344   The ^{13}C recovery showed no increase from day 3 to day 50 for any amino sugars. The ratios

345   of glucosamine/muramic acid and galactosamine/muramic acid, calculated for the

346   incorporated ^{13}C, were about six. This is much lower than the ratio observed for the pools of

347   amino sugars. The ^{13}C enrichment did not increase from day 3 to day 50 for any of the amino

348   sugars. The highest enrichment was observed for muramic acid and the lowest for

349   galactosamine (Fig. 4, bottom). The ^{13}C enrichment in amino sugars was 10–20 times lower

350   than for PLFA.

351   **[Fig. 4]**

352

353   **4. Discussion**

354   *4.1. Glucose decomposition*

355   The amount of glucose derived ^{13}C remaining in soil after 50 days was in the range 80 %

356   which was higher than reported by other studies. Glanville et al. (2012) observed that 50% of

357   glucose C remained in SOM after 20 days; Wu et al. (1993) reported that 55% of glucose

358   derived ^{14}C remained after 50 days; Perelo and Munch (2005) reported the mineralisation of

359   50% of ^{13}C glucose within 98 days. The amounts of applied C (Bremer and Kuikman, 1994;

360   Schneckenberger et al., 2008), as well as differences in microbial activity (Bremer and

361   Kuikman, 1994; Schimel and Weintraub, 2003) in the investigated soils, explain the

362   variation between studies in the portion of remaining glucose C.

363   The highest mineralization of glucose derived [13]C (20 %) was found within the first

364   three days after tracer application (Fig. 1), whereas at day 50 mineralization was much

365   slower. Glucose is decomposed in soil in two stages (Gunina and Kuzyakov, 2015): during

366   the first one, part of glucose C is immediately mineralized to CO_2 and part is incorporated

367   into the microbial compartments and second one, when C incorporated into MB is further

368   transformed and is used for microbial biosynthesis and mineralization of glucose-C to CO_2

369   occurs much slower (Bremer and Kuikman, 1994). This first stage takes place in the first day

370   after substrate addition and is 30 times faster than the 2nd stage (Gregorich et al., 1991). Due

371   to the first sampling point in our experiment was 3 days after glucose addition, the obtained

372   data on glucose mineralization can be mainly related to the second stage.

373   A significant portion of glucose derived C was stored in the non-specific pool in SOM

374   (Fig. 1), e.g., as microbial storage compounds and other cellular building blocks, which can

375   contribute to C accumulation in microbial residues (Wagner GH, 1968; Zelles et al., 1997;

376   Lutzow et al., 2006). This part cannot be extracted by the methods applied in this study. The

377   amino sugar method detects only the peptidoglycan and chitin proportions of the cell walls,

378   whereas other constituents can not be determined by this method (Glaser et al., 2004).

379   Chloroform fumigation only partially extracts the cytosolic cell compounds, as high

380   molecular weight compounds, which interact with the soil matrix, cannot be extracted with

381   low molarity salt solution.

382

383   *4.2. Partitioning of ^{13}C-derived glucose between cell compounds*

384   To estimate the residual amount of C derived from applied ^{13}C-labelled low molecular weight

385   organic substances (LMWOS), the ^{13}C in SOM or in the total MB pool is frequently

386   determined. This approach, however, does not allow the portions of ^{13}C incorporated into

387 stable and non-stable C pools to be estimated, because the ^{13}C in SOM includes the sum of

388 ^{13}C in living biomass and ^{13}C in microbial residues. Furthermore, the living MB contains cell

389 compartments with a broad spectrum of C turnover times. The approach applied in the

390 present study allows the partitioning of glucose derived C in living MB to be estimated, as

391 well as the contribution of LMWOS-C to SOM composition.

392

393 *4.3. Cytosol*

394 We calculated the ^{13}C enrichment of the cytosolic microbial C pool, extracted after

395 chloroform fumigation. The estimated turnover time of C in this pool was about 151 days.

396 This value lies close to the previously reported range of 87–113 days, for the same pool for

397 soils incubated for 98 days with ^{13}C glucose (Perelo and Munch, 2005), but were lower than

398 MB C turnover time calculated using a conversion factor (2.22) - 82 days, for soils incubated

399 for 60 days with ^{14}C glucose (Kouno et al., 2002). The long C turnover time in cytosol is

400 related to the high heterogeneity of this pool, which includes compounds with various

401 molecular masses (Malik et al., 2013) and functions, having different turnover times. Thus, C

402 turnover time in 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 within

410 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   incorporation peak. The approach used in the present study showed that the C turnover time

421   in PLFA is about 47 days (Fig. 2). 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. 2). This is

424   because the membrane is the interacting surface between the cell and the environment and

425   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 cytosolic pool includes C

427   from both active and dormant microorganisms (Blagodatskaya and Kuzyakov, 2013), and the

428   latter can dilute the ^{13}C signal incorporated into the active pool with non-labelled C, yielding

429   a 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. 3, top), than

433   filamentous microorganisms. This can be a consequence of low C loading rates (less than 4

434   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. 3, 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. 3, 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, replacement 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. 3).

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. 3, 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 PLFA can only be explained by their

472   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 two 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 recovery into amino sugars of

484   living cells, we first calculated the amount of amino sugars in the living MB pool based on

485   the fatty acids content. Assuming that PLFAs are present only in living biomass, and that the

486   ratio of fatty acids to amino sugars in living biomass is about 0.23 (Lengeler et al., 1999), we

487   estimated the amount of amino sugars in living MB to be 0.20 µmol g^{-1} soil fatty acids/0.23 =

488   0.87 µmol g^{-1} soil. The estimated percentage of amino sugars in living biomass from the total

489   amino sugar pool was 0.87/7.70 (total AS (µmol g^{-1} soil))*100 = 11%. This estimate agrees

490   with that of Amelung et al. (2001a) and Glaser et al. (2004), who reported that the amount of

491   amino sugars in living biomass is one to two orders of magnitude lower than in the total

492   amino-sugar pool. We calculated the ^{13}C enrichment in amino sugars for the first sampling

493   point, assuming that all replaced C is still contained within living MB after three days of

494   glucose C utilisation. Total tracer recovery into amino sugars consisted of 0.00071 µmol

495   glucose derived ^{13}C in amino sugars g^{-1} soil/0.87 (µmol amino sugars g^{-1} soil)*7 (mean

496   amount of C atoms in amino sugars)*100 = 0.57% of the C pool. Comparison of these data

497   with the ^{13}C enrichment into PLFAs and the cytosol allowed us to conclude that the

498   replacement of the amino sugar C with glucose derived ^{13}C in living biomass is two-fold

499   slower than the replacement in PLFAs, and faster than in the cytosolic pool. This reflects that

500   microbial C turnover is a phenomenon that is not restricted to the death or growth of new

501   cells, but that even within living cells, highly polymeric cell compounds, including cell walls,

502   are constantly replaced and renewed (Park and Uehara, 2008).

503

504   *4.5.2. Contribution of bacterial and fungal cell walls to SOC*

505   Glucosamine was the dominant amino sugar in the soil, whereas muramic acid was the least

506   abundant (Table 1), which agrees with most literature data (Engelking et al., 2007; Glaser et

507   al., 2004). To conclude about the proportions of bacterial and fungal residues in the SOM, the

508   ratio of galactosamine/muramic acid (Glaser et al., 2004) was calculated (Table 1) and

509 showed bacteria to be the dominant within the soil microbial community. The bacterial origin

510 of microbial residues in the soil is supported by: 1) the dominance of bacterial PLFA

511 biomarkers 2) the environmental conditions of the site, namely, long-term agricultural use,

512 which promotes the dominance of bacterial communities.

513   Three-fold more glucose derived ^{13}C was incorporated into glucosamine than into

514 galactosamine and muramic acid (Fig. 4, top). This correlates with the pool size and indicates

515 that glucosamine is the most dominant amino sugar not only in total amino sugars, but also

516 within the walls of living cells. The galactosamine/muramic acid ratio of the incorporated ^{13}C

517 was six, and consequently was significantly lower than the ratio calculated for the amount of

518 amino sugars (Table 1). This indicates that bacteria are more active in glucose derived ^{13}C

519 utilisation than fungi, a conclusion also supported by the ^{13}C-PLFA data (Fig. 3). Thus, even

520 if the composition of amino sugars does not allow a clear conclusion concerning living

521 microbial communities in soil, amino sugar analysis combined with ^{13}C labeling reveals the

522 activity of living microbial groups in terms of substrate utilisation.

523   The calculated ^{13}C enrichment was the highest in muramic acid (Fig. 4, bottom). This

524 is in agreement with the high ^{13}C enrichment of bacterial PLFAs compared to 16:1ω5 and

525 fungi (Fig. 3). Due to differences in cell-wall architecture, G+ bacteria contain more muramic

526 acid (approximately four times) than G- bacteria (Lengeler et al., 1999), and thus make a

527 higher contribution to the ^{13}C enrichment of muramic acid.

528   The ^{13}C enrichment of glucosamine was two-fold lower than muramic acid (Fig. 4,

529 bottom). This confirms the hypothesis that glucosamine originates from bacterial as well as

530 fungal cell walls and consequently has a mixed enrichment between that of the fungal

531 galactosamine and the bacterial muramic acid.

532

23

5. Conclusions

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

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

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

557 galactosamine, demonstrating that the turnover of bacterial cell wall components is more

558 rapid than fungal.

559 Consequently, the combination of ^{13}C labeling with the subsequent analysis of several

560 microbial cell compartments and biomarkers is a unique approach to understanding C

561 partitioning within microbial cells and the microbial communities in soil. This knowledge is

562 not only crucial for assessing C fluxes and recycling in soil, but is also of special importance

563 concerning the contribution of C from microbial residues to SOM.

564

565 **Author contribution**

566 Y. Kuzyakov and B. Glaser designed the experiments and M. Dippold and A. Gunina carried

567 them out. A. Gunina prepared the manuscript with contributions from all co-authors.

568

569 **Data availability**

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

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805   **Table 1** Amount of microbial biomass compartments, their C content, PLFA content of

806   microbial groups and composition of microbial residues in investigated soil. G-1 and G-2 are

807   gram-negative group one and two, respectively; G+1 and G+2 are gram positive group one

808   and two, respectively; Ac – actinomycetes; 16:1ω5 - saprotrophic fungi. Data present mean

809   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

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

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Table captions

819   **Table 1** Amount of microbial biomass compartments, their C content, content of microbial

820   groups and composition of microbial residues in investigated soil. G-1 and G-2 are gram-

821   negative group one and two, respectively; G+1 and G+2 are gram positive group one and

822   two, respectively; Ac – actinomycetes; 16:1ω5 - saprotrophic fungi.

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824   **Figure captions**

825   **Fig. 01** Partitioning of glucose derived ^{13}C in SOM presented as the ^{13}C recovery (% of

826   initially applied ^{13}C found in soil) between the following pools: non-specified SOM

827   (calculated as total ^{13}C recovery subtract ^{13}C recovery in cytosol, PLFAs and amino sugars),

828   cytosolic, PLFAs and amino sugars. Small letters reflect differences between the sampling

829   points for the distinct pool. Data present mean (n=4) and bars present standard errors (SE).

830   The SE for the amino sugars are not fully shown.

831

832   **Fig. 02** ^{13}C enrichment in the cytosolic, PLFA and amino-sugar cell pools as well as

833   functions to calculate the C turnover times in these microbial cell pools. The left y-axis

834   represents the PLFA pool, the first right y-axis, the cytosolic and the second y-axis, the

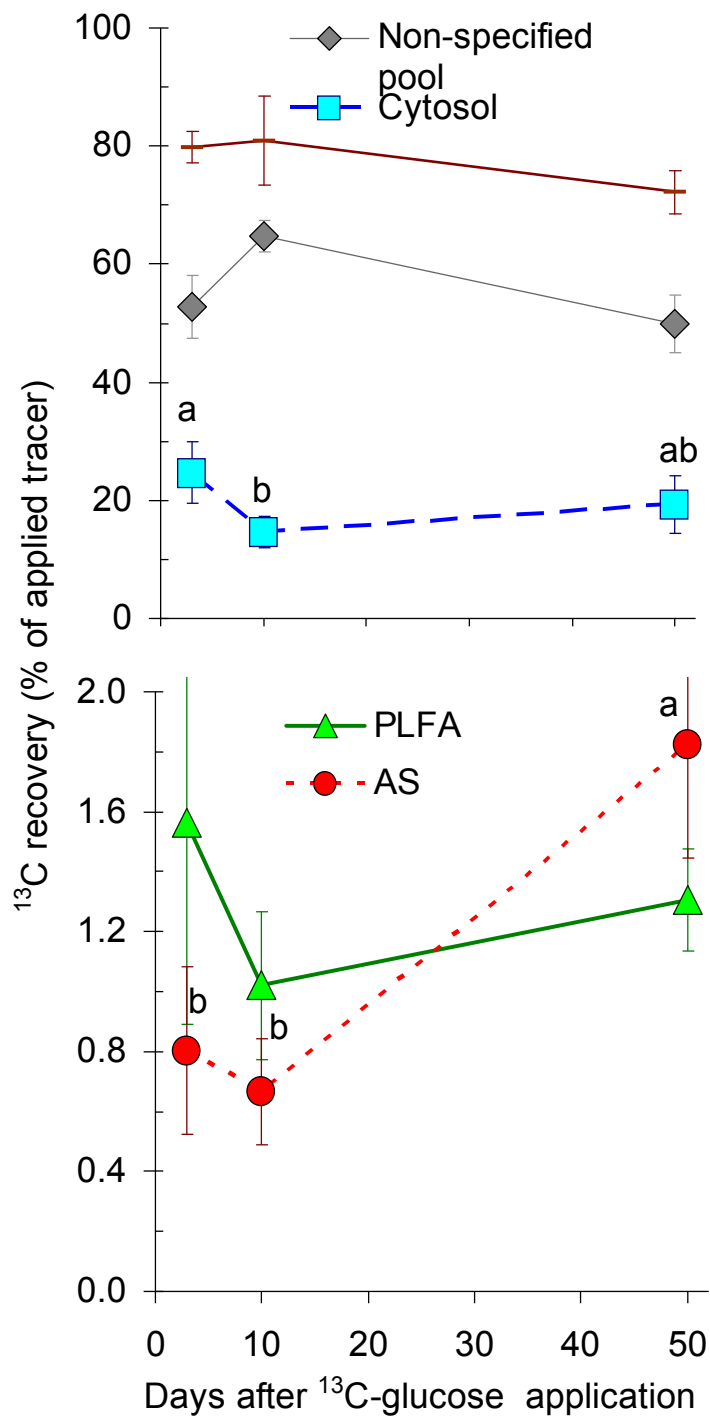835   amino-sugar pool. Data present mean (n=4) and bars present standard errors.

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837   **Fig. 03** Recovery of glucose derived ^{13}C (top) and ^{13}C enrichment (bottom) of the microbial

838   PLFAs. Note that the values for 16:1ω5 and fungi are scaled-up 10 times (secondary Y axis)

839   compared to those of other groups (Y axis at the left). Data present mean (n=4) and bars

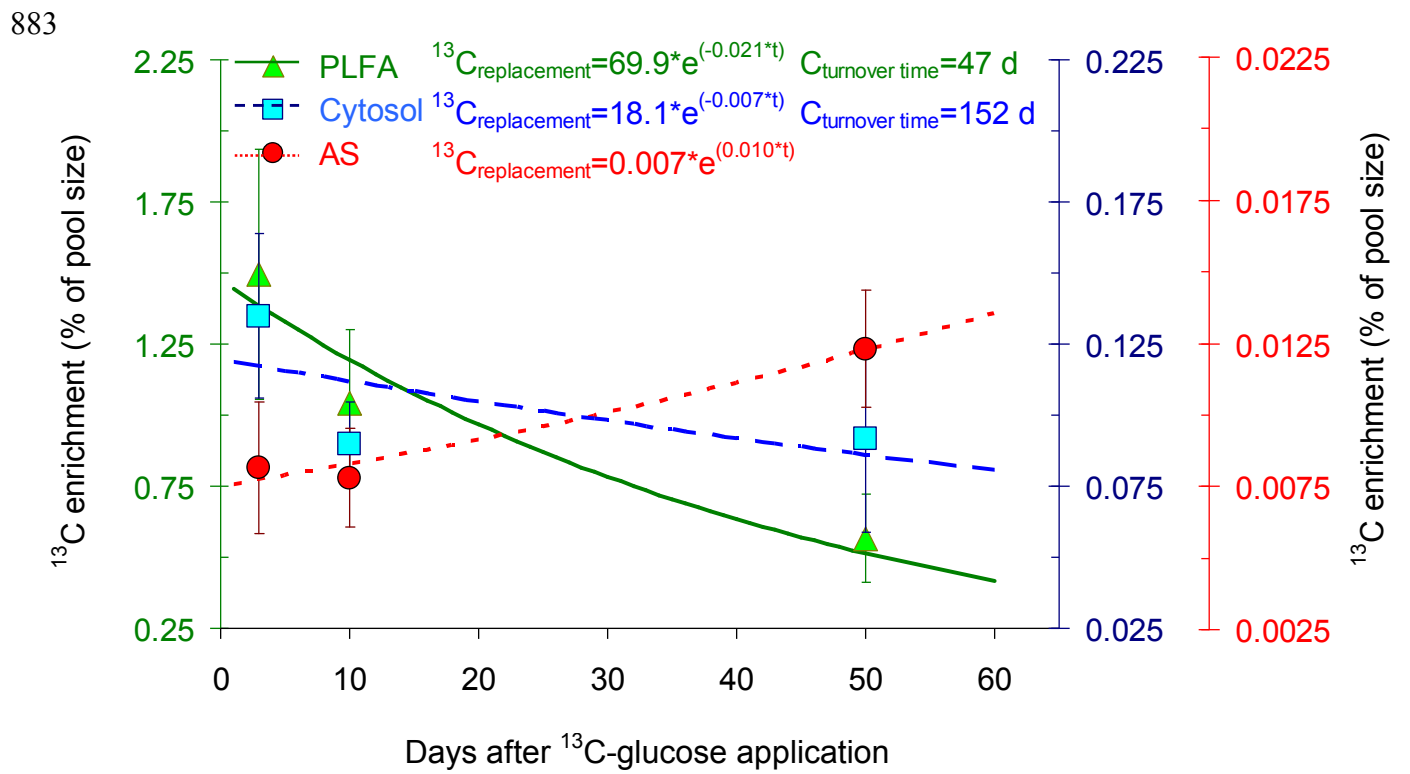840   present standard errors. Small letters reflect differences between the microbial groups for ^{13}C

841 recovery and ^{13}C enrichment from glucose; letters a-d are for day three, l-o are for day 10, x-z

842 are for day 50.

843 **Fig. 04** Recovery of glucose derived [13]C (top) and ^{13}C enrichment (bottom) of amino sugars

844 and muramic acid. Letters reflect significant differences in the recovery and ^{13}C enrichment

845 from glucose into amino sugars on a particular day; letters a-b are for day three, l-m are for

846 day 10, x-y are for day 50. No significant differences were observed between the three

847 sampling days. Data present mean (n=4) and bars present standard errors.

848 **Fig. 05** Dynamic relationship of microbial utilization of glucose and turnover of cytosol, cell
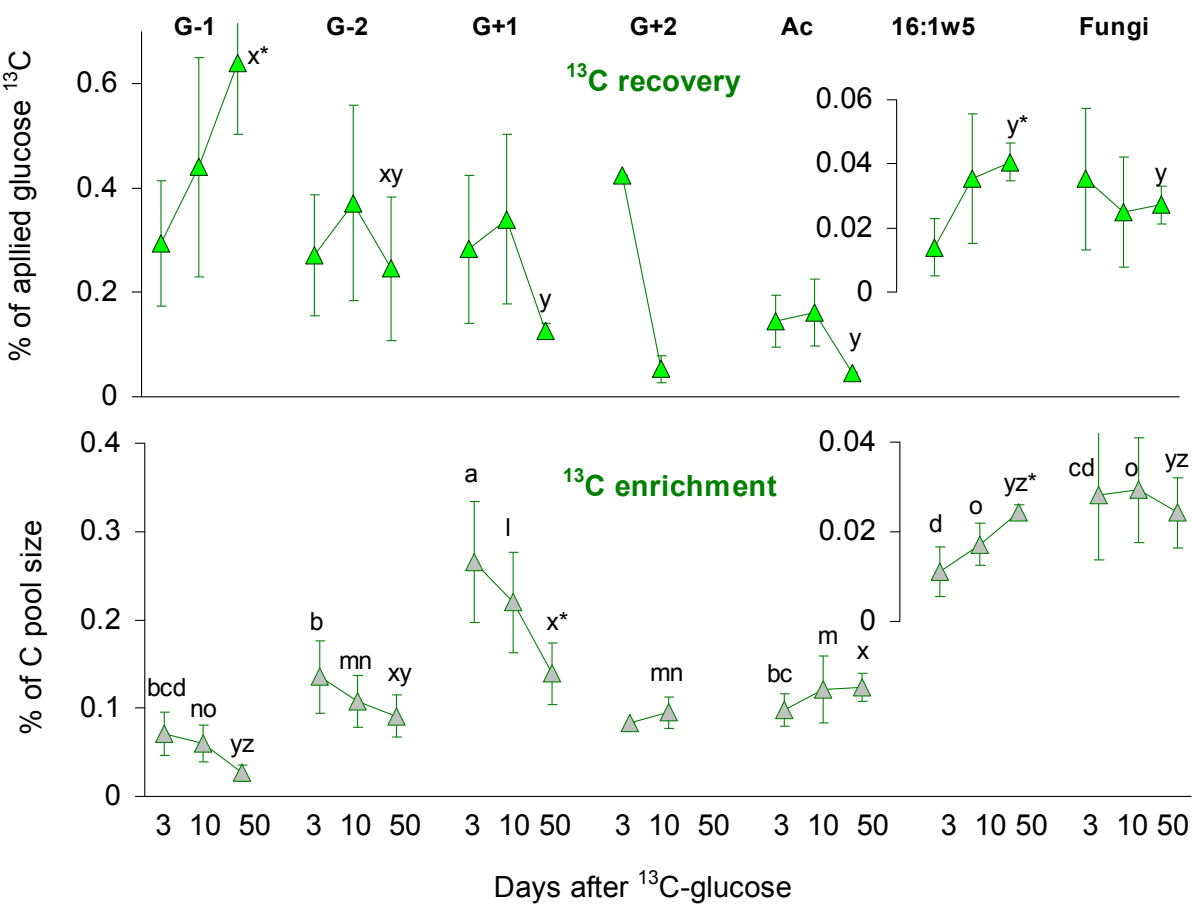
849 membrane and cell wall components.

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