Fate of low molecular weight organic substances in an arable soil: From microbial uptake to utilisation and stabilisation

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ABSTRACT

Microbial uptake and utilisation are the main transformation pathways of low molecular weight organic substances (LMWOS) in soil, but details on transformations are strongly limited. As various LMWOS classes enter biochemical cycles at different steps, we hypothesise that the percentage of their carbon (C) incorporation into microbial biomass and consequently stabilisation in soil are different.

Representatives of the three main groups of LMWOS: amino acids (alanine, glutamate), sugars (glucose, ribose) and carboxylic acids (acetate, palmitate) – were applied at naturally-occurring concentrations into a loamy arable Luvisol in a field experiment. Incorporation of 13C from these LMWOS into extractable microbial biomass (EMB) and into phospholipid fatty acids (PLFAs) was investigated 3 d and 10 d after application. The microbial utilisation of LMWOS for cell membrane construction was estimated by replacement of PLFA-C with 13C.

35–80% of initially applied LMWOS-13C was still present in the composition of soil organic matter after 10 days of experiment, with 10–24% of 13C incorporation into EMB at day three and 1–15% at day 10. Maximal incorporation of 13C into EMB was observed from sugars and the least from amino acids. Strong differences in microbial utilisation between LMWOS were observed mainly at day 10. Thus, despite similar initial rapid uptake by microorganisms, further metabolism within microbial cells accounts for the specific fate of C from various LMWOS in soils.

13C from each LMWOS was incorporated into each PLFA. This reflects the ubiquitous utilisation of all LMWOS by all functional microbial groups. The preferential incorporation of palmitate into PLFAs reflects its role as a direct precursor for fatty acids. Higher 13C incorporation from alanine and glucose into specific PLFAs compared to glutamate, ribose and acetate reflects the preferential use of glycolysis-derived substances in the fatty acids synthesis.

Gram-negative bacteria (16:1ω7c and 18:1ω7c) were the most abundant and active in LMWOS utilisation. Their high activity corresponds to a high demand for anabolic products, e.g. to dominance of pentose-phosphate pathway, i.e. incorporation of ribose-C into PLFAs. The 13C incorporation from sugars and amino acids into filamentous microorganisms was lower than into all prokaryotic groups. However, for carboxylic acids, the incorporation was in the same range (0.1–0.2% of the applied carboxylic acid 13C) as that of gram-positive bacteria. This may reflect the dominance of fungi and other filamentous microorganisms for utilisation of acidic and complex organics.

Thus, we showed that despite similar initial uptake, C from individual LMWOS follows deviating metabolic pathways which accounts for the individual fate of LMWOS-C over 10 days. Consequently, stabilisation of C in soil is mainly connected with its incorporation into microbial compounds of various stability and not with its initial microbial uptake.

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1. Introduction

Low molecular weight organic substances (LMWOS) comprise 5–10% of dissolved organic carbon (DOC) in soils (Ryan et al., 2001) and are products of rhizodeposition, above and belowground litter and microbial residue degradation. The main compound classes within the LMWOS are amino acids, sugars (mainly monosaccharides) and carboxylic acids (Fischer et al., 2010).

A main process removing LMWOS from soil solution is microbial uptake, which out-compete physicochemical sorption of LMWOS at mineral surfaces and their leaching from the soil profile, probably by orders of magnitude (Fischer et al., 2010). Microbial removal of LMWOS from solution appears within minutes in the upper soil horizons (Jones et al., 2004). In contrast, the half-life time of C from such LMWOS in the soil is much longer — from several hours to months or even decades (van Hees et al., 2005), because after incorporation in microbial biomass it can further be stabilised in soils. Thus, utilisation within the microbial biomass is assumed to be one of the main factors controlling the LMWOS-C stabilisation in soil on the short- and long-term scales.

To evaluate the contribution of functional microbial groups to LMWOS utilisation 13C or 14C-labelling, coupled with analysis of microbial biomass such as n-alkanes, sugars (Ihde et al., 2001; Engelking et al., 2007; Glaser et al., 2004), phospholipid-derived fatty acids (PLFA) (Frostegard et al., 2011; Zelles, 1997) or DNA-based methods (Ibekwe et al., 2002; Radajewski et al., 2003) can be applied. Coupling PLFA analysis with 13C-labelling has shown that gram-negative (G−) bacteria are more active in the utilisation of plant C (low or high molecular weight) than gram-positive (G+) bacteria, even if the latter group has a higher PLFA content in soil (Garcia-Pausas and Paterson, 2011; Waldrop and Firestone, 2004). Fungi contribute less to the utilisation of plant-derived C than bacteria (Waldrop and Firestone, 2004). In contrast, the use of 13C pulse-labelling of plants to trace 13C in PLFAs has shown that either fungi (Butler et al., 2003) or G− bacteria (Tian et al., 2013) are the most active consumers of rhizodeposits. Incorporation of 13C from labelled straw into PLFAs has shown that fatty acids such as 16:0 (universal fatty acid), 18:1ω9 (bacterial or fungal biomarker), 18:2ω6,9 (typical fungal biomarker) were more 13C-enriched, whereas other 16:1ω5 (fungal or G− bacteria biomarker) or 10Me17:0 (typical actinomycetes biomarker) fatty acids contained negligible amounts of 13C (Williams et al., 2006). Consequently, members of the microbial community are differentially involved in the assimilation of litter- or root-derived C (Williams et al., 2006) and the activity of individual microbial groups appears to depend on the quality of substrate and on environmental conditions such as soil type, season and climate (Bray et al., 2012). Thus, general principles of LMWOS utilisation by individual groups of bacteria and fungi still remain open.

The second factor controlling LMWOS fate is microbial metabolism: various classes of LMWOS enter different pathways and consequently are utilised differently (Blagodatskaya et al., 2011). Sugars are mainly used directly by the basic glycolysis pathway (Caspi et al., 2012; Keseler et al., 2009), carboxylic acids enter from side branches of the citric acid cycle (Caspi et al., 2012; Keseler et al., 2009), and amino acids enter glycolysis or the citric acid cycle from individual side branches at different steps (Apostel et al., 2013; Knowles et al., 2010). Thus, we assume that universal substances such as sugars, entering glycolysis directly, will be metabolised very rapidly in comparison to carboxylic acids and amino acids entering the citric acid cycle. However, glycolysis also enables entry into many anabolic pathways, i.e. we hypothesise that sugars are used more for anabolism than carboxylic acids, which enter the oxidising citric acid cycle and can be directly metabolised for energy production. The highest diversity in pathways can be expected for amino acids, because they enter basic metabolism at various steps (Apostel et al., 2013). Since carboxylic acid utilisation is substrate-controlled (van Hees et al., 2002), we expect divergence in the utilisation of short- and long-chain acids. Because three classes of LMWOS enter metabolic cycles at various points, we hypothesise that their role in the synthesis of cell components such as PLFAs should be different. Based on the various fates of LMWOS in biomass the conclusions about short-term LMWOS-C stabilisation in soil organic matter (SOM) can be done.

Thus, the overall aim of this study was to estimate the transformation of representatives of three main classes of LMWOS: monosaccharides (glucose and ribose), carboxylic acids (acetate and palmitate) and amino acids (alanine and glutamate) under field conditions, coupling 13C substrate labelling with the analysis of specific cell PLFA biomarkers.

2. Materials and methods

2.1. Experimental design

The field experiment was carried out at an agricultural field trial in Hohenpötz (49° 54′ N, 11° 08′ E, at 500 m a.s.l.). The mean annual temperature was −7 °C and mean annual precipitation was 870 mm. The site is cultivated by a rotation of triticale, wheat and barley. The soil is an arable loamy haplic Luvisol (IUSS Working group WRB, 2007) and had the following characteristics: pH 6.6, total C content 1.5%, C/N 10.7, CEC 13 cmol−1 kg−1, clay content 22%.

In August 2010, following harvest of the triticale and harrowing, columns (i.e. plastic tubes with 13 cm height and 10 cm diameter) were inserted to a depth of 10 cm and six, 13C uniformly-labelled substances: alanine, glutamate, glucose, ribose, sodium acetate and palmitate were injected with a syringe into separate columns. The injection was done in 5 points, which allowed spreading the 13C homogeneously inside the column. The injection was done as a single pulse-labelling. The amounts of applied tracer were: alanine 96.3, glutamate 91.6, glucose 93.4, ribose 91.8, acetate 95.8 and palmitate 49.5 μmol 13C column−1. The amount of added C was kept as low as possible and constant for all columns, including the controls, where similar amounts of non-labelled C were applied (735 μmol C column−1). Each column contained 1.5 kg soil. The field experiment had a randomised block design with four blocks, which represented four field replicates. Preventing rainfall by using a protective roof excluded leaching through the columns for the 10 days of the experiment. Due to the absence of leaching and uptake by plants, we assumed that all losses of 13C from soil are connected to LMWOS mineralisation to CO2. After day three and day 10, separate soil columns were destructively sampled. We assumed that on the day three the maximum incorporation of LMWOS-C into microbial biomass should occur, and on the day 10 the maximal differences in LMWOS-C utilisation within the microbial metabolism can be observed. The soil was removed from the column, weighed and the water content was determined in a subsample. The soil water content was between 22 and 25% for both time points. Each soil sample was sieved to 2 mm and divided into two portions. One was cooled (−5 °C) for microbial biomass analysis and another was stored frozen (−20 °C) until PLFA analysis.

2.2. Bulk soil 13C analysis

The soil for the 13C analysis was freeze-dried, milled and 13C values of bulk SOM were determined using a Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit coupled via a Conflo III interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). The amount of applied 13C in the soil was calculated based on the mixing model.
Combined hexane aliquots were dried under N\textsubscript{2}, and re-dissolved in an amount of 13C in fumigated and unfumigated samples was calculated by the mixing model (Equations (1) and (2)). Enrichment of 13C on GC-C-IRMS was calculated in analogy to bulk soil and EMB according to Equations (1) and (2), using a calibration curve, which was constructed using standard substances as external standards at six different concentrations (see Table 1, Supplementary material). All peaks per sample were corrected for the recovery of the first internal standard.

2.3. Microbial biomass

Extractable microbial biomass (EMB) was determined by the fumigation-extraction technique in fresh soil shortly after sampling. Briefly, 15 g fresh soil was placed into glass vials, which were exposed to chloroform for 120 h. After complete removal of chloroform, the EMB was extracted from the soil with 45 mL 0.05 M K\textsubscript{2}SO\textsubscript{4}. Organic C was measured with a high-temperature combustion TOC-analyser (Analyser multi N 2100, Analytik Jena, Germany) and the EMB was calculated as the difference between organic C in fumigated and unfumigated samples without further correction factors. After analysis of the organic C concentration, the liquid samples were freeze-dried and \delta\textsuperscript{13}C values of 40 \mu g sub-samples were determined using EA-IRMS (described above). The amount of 13C in fumigated and unfumigated samples was calculated by the mixing model (Equations (1) and (2)). Enrichment of 13C in the EMB was calculated from the difference of these values.

2.4. Phospholipid fatty acid analysis

2.4.1. PLFAs extraction and purification

Analysis of PLFAs was performed using the liquid—liquid extraction method of Frostegard et al. (1991) with some modifications. Firstly, 25 \mu g 1 mg mL\textsuperscript{-1} 19:0-phospholipid (dinonadecanoylglycerol-phosphatidylycholine) was added to each sample as an internal initial standard to calculate the recovery of the phospholipid extraction and purification (Frostegard et al., 1991). Extraction of 6 g soil was performed twice, firstly with 18 mL and secondly with 6 mL of a one-phase mixture of chloroform, methanol and 0.15 M citric acid (1:2:0.8 v/v/v). Six mL of chloroform and 6 mL of 0.15 M citric acid were added to the extract to generate a two-phase solution and the sample was shaken. The lower phase was then separated and an additional liquid—liquid extraction was performed with 12 mL chloroform. The combined chloroform phase was reduced to 0.5 mL and the phospholipids were separated from the neutral and glycolipids using a solid phase extraction method with activated Silica gel (Silica gel Merck, particle size 0.063–0.200 mm). After transfer to the column, the first fraction (neutral lipids) was eluted with 5 mL chloroform and the second fraction (glycolipids) with 20 mL acetone and the PLFAs were obtained by a four-fold elution with 5 mL methanol. The methanol phase was reduced to 0.5 mL and dried under nitrogen flow.

For the alkaline saponification, 0.5 mL 0.5 M NaOH in dried MeOH (Sigma–Aldrich, assay ≥99.9%) was added and samples were heated to 100 °C for 10 min. The free fatty acids were methylated with 0.75 mL BF\textsubscript{3} in methanol (10%, 1.3 M, Fluka) for 15 min at 80 °C. For hydrolysing the excess BF\textsubscript{3}, 0.5 mL saturated NaCl solution was added. Fatty acid methyl esters (FAMEs) were extracted three times with 1 mL hexane by liquid—liquid extraction. Combined hexane aliquots were dried under N\textsubscript{2}, and re-dissolved in 185 mL toluene with the addition of 15 mL of a second internal standard (IS2) (13:0 FAME at 1 mg mL\textsuperscript{-1}) (Knapp, 1979).

2.4.2. PLFAs quantification on GC-MS

All PLFAs samples were analysed using a Hewlett Packard 5890 gas chromatograph coupled to a mass-selective detector 5971A. A 25 m HP-1 methylpolysiloxane column (internal diameter 0.25 mm, film thickness 0.25 \mu m) was used. A single 1 \mu L injection was analysed with an initial temperature of 80 °C, which was then ramped to 164 °C at 10 °C min\textsuperscript{-1}, then to 230 °C at 0.7 °C min\textsuperscript{-1} and finally to 300 °C at 10 °C min\textsuperscript{-1} at a constant flow rate of 2.4 mL min\textsuperscript{-1}. 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 standard substances as external standards at six different concentrations (see Table 1, Supplementary material). All peaks per sample were corrected for the recovery of the first internal standard.

2.4.3. Analysis of \delta\textsuperscript{13}C on GC-C-IRMS

The 13C/12C isotope ratios of the single fatty acids were determined by IRMS Delta PlusTM (Thermo Finnigan, Bremen, Germany) coupled to a gas chromatograph (GC; Trace GC 2000, Thermo Finnigan) via a GC-II/III-combustion interface. A 15 m HP-1 methylpolysiloxane column coupled with a 30 m HP-5 (5% Phenyl)-methylpolysiloxane column (both had an internal diameter of 0.25 mm and a film thickness of 0.25 \mu m) were used. A single 1.5 \mu L injection was analysed with an initial temperature of 80 °C, which was then ramped to 180 °C at 7 °C min\textsuperscript{-1}, then to 185 °C at 0.3 °C min\textsuperscript{-1} followed by holding for 3 min, then to 204 °C at 0.5 °C min\textsuperscript{-1} and holding for 1.5 min, then to 300 °C at 15 °C min\textsuperscript{-1} and holding for 10 min and finally to 80 °C at 50 °C min\textsuperscript{-1} at a constant flow rate of 2 mL min\textsuperscript{-1}. Detailed information about the instrumental set-up is described in (Sauheitl et al., 2005). Online referencing of 13C values was performed by the injection of several reference gas pulses directly into the IRMS during measurement (Glaser and Amelung, 2002). Measured 13C values of the PLFAs were corrected for the effect of derivative C similar to Glaser and Amelung (2002) and referenced on Pee Dee Belemnite by external standards. The enrichment of 13C in single PLFAs was calculated in analogy to bulk soil and EMB according to Equations (1) and (2), following a two-pool dilution model (Gearing et al., 1991).

2.5. Calculations and statistical analysis

All soil, EMB and PLFAs data were tested with nested mixed effect ANOVA with block as a random factor. Substances were nested in class, namely alanine and glutamate to amino acids, glucose and ribose to sugars, acetate and palmitate to carboxylic acids. Statistica 7.0 software was used.

Significant differences between individual data points were tested with the HSD post-hoc test for unequal n at a 95% significance level. For the repetitive measurements of 13C values, a Nalimov outlier test with significance levels of 95% (when four replicates were available) or 99% (when three replicates were available) was performed. PLFAs were classified into corresponding microbial groups by a factor analysis with a principal component extraction method (for factor loadings of the PLFA fingerprint, see Supplementary material, Table 2). We excluded ubiquitous fatty acids (i.e. unsaturated, straight-chain fatty acids) from the factor analysis and those which were at the detection limit. The classified data were compared with the literature for single fatty acids, to ascertain functional groups of the microorganism (Zelles, 1997). Incorporation of 13C into individual fatty acids was summed to 13C values, a Nalimov
create incorporation of individual microbial groups. The data were presented as incorporation of $^{13}$C ($^{13}$C$_{inc}$) into PLFAs (equation (3)) and also as the replacement of $^{12}$C by $^{13}$C ($^{13}$C$_{C_{repl}}$) (equation (4)) in PLFA.

$$^{13}$C$_{inc} = \frac{^{13}$C$_{PLFA}}{^{13}$C$_{Applied}} \times 100\% \quad (3)$$

$$^{12}$C$_{/^{13}$C$_{repl}} = \frac{^{13}$C$_{C_{repl}}}{^{13}$C$_{Total}} \times 100\% \quad (4)$$

As far as PLFA content (data not shown) and PLFA composition (data not shown) were not affected by substrate addition, the LMWOS were very likely used by the same microbial community in each treatment. Thus, the differences in utilisation of LMWOS can mainly be attributed to individual pathways of the substrates or to specific use by individual microbial groups.

3.2. Microbial utilisation of LMWOS

The three classes of LMWOS were decomposed and incorporated into EMB and PLFAs differently (Fig. 1). Between 35 and 45% of $^{13}$C from both amino acids remained in SOM. $^{13}$C from glutamate contained in the EMB decreased during one week by about eight-fold ($p < 0.05$). In contrast, the proportion of $^{13}$C from alanine incorporated into EMB stayed constant from day three to day 10.

Decomposition of monosaccharides was lower than that of amino acids: 65–80% of the applied tracer was still present in the soil after 10 days (Fig. 1).

Decomposition of carboxylic acids was about 50% at day 10 and thus, was comparable to that of amino acids. Incorporation of $^{13}$C into EMB was nearly constant for the two time points for palmitate, whereas it significantly decreased for acetate (Fig. 1).

The proportion of $^{13}$C from all substances in EMB on day three was in the range from 10 to 24% of applied $^{13}$C and was similar for all substances except glucose and palmitate ($p < 0.05$). In contrast, more $^{13}$C from monosaccharides (mainly from glucose) remained in EMB on day 10 compared to amino acids (glutamate) and carboxylic acids. This reflects the universal role of sugars as a C source for microorganisms.

Only 1–3% of the applied $^{13}$C from the five LMWOS (except palmitate) was used for the synthesis of cell membrane components, evaluated by $^{13}$C in total PLFA. Palmitate-C was the only exception: more than half of its incorporation into EMB was recovered in the PLFAs after three days and half on day 10 (Fig. 1). Although PLFAs comprised only about 5% of the EMB, they incorporated a comparatively high percentage of LMWOS-C, namely, 10% of the microbiologically used C.

Table 1

<table>
<thead>
<tr>
<th>Microbial groups</th>
<th>Abbr.</th>
<th>Fatty acids (FAs)</th>
<th>FAs content (µg g$^{-1}$ dry soil)</th>
<th>Relative abundance FAs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram negative 1</td>
<td>G-1</td>
<td>16:1w7c + 18:1w7c</td>
<td>9.06 (0.77)</td>
<td>7.03 (0.53)</td>
</tr>
<tr>
<td>Gram negative 2</td>
<td>G-2</td>
<td>18:1w9c + a15:0</td>
<td>5.96 (0.54)</td>
<td>6.12 (0.43)</td>
</tr>
<tr>
<td>Gram positive 1</td>
<td>G+1</td>
<td>16:0 + i15:0 + i17:0</td>
<td>3.26 (0.23)</td>
<td>2.35 (0.20)</td>
</tr>
<tr>
<td>Gram positive 2</td>
<td>G+2</td>
<td>i15:0</td>
<td>3.38 (0.35)</td>
<td>3.24 (0.30)</td>
</tr>
<tr>
<td>Gram positive 3</td>
<td>G+3</td>
<td>i14:0 + i14:0</td>
<td>0.49 (0.11)</td>
<td>0.62 (0.15)</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>Ac</td>
<td>10Me16:0 + 10Me18:0</td>
<td>3.72 (0.31)</td>
<td>3.60 (0.26)</td>
</tr>
<tr>
<td>VAM</td>
<td>VAM</td>
<td>16:1w5c</td>
<td>1.85 (0.16)</td>
<td>2.06 (0.18)</td>
</tr>
<tr>
<td>Fungi</td>
<td>F</td>
<td>18:2w6:9</td>
<td>0.70 (0.09)</td>
<td>1.03 (0.11)</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Pr</td>
<td>20:4w6:9</td>
<td>0.60 (0.09)</td>
<td>0.53 (0.05)</td>
</tr>
</tbody>
</table>

Table 1 Absolute and relative abundance (absolute in µg per g dry soil and relative in % of total PLFAs) of the fatty acids of the microbial groups, classified by factor analysis (factor loadings see Supplementary Table 2). Data presents mean and standard error. No significant differences between day three and day 10 were observed for fatty acids content or relative abundance of fatty acids for the single microbial groups.
due to the higher abundances of the respective PLFAs at day 10 relative to day three (Table 1).

In general, alanine-C was preferred over glutamate-C for PLFAs synthesis, and significant differences were observed mainly on day 10 for the G− group 2, G+ group 1 and 2 bacteria, actinomycetes and fungi. At day 10, 13C replacement by alanine-derived 13C was significantly higher than glutamate for each of these groups (Fig. 2, bottom).

Utilisation of 13C from sugars for PLFAs formation showed different trends in bacterial and fungal groups and much higher absolute 13C incorporation compared to amino acids (Fig. 3, top). Between 0.01 and 0.70% of initially applied sugars 13C was found in various taxonomic groups after three days, whereas only

Fig. 1. 13C recovery (in % of applied 13C) from six LMWOS in soil, extractable microbial biomass and PLFAs, three and 10 days after substances application. Data present mean (n = 4) and bars present standard errors. Letters above the error bars reflect significant differences (p < 0.05) for 13C recovery in soil and extractable microbial biomass separately for day three and 10. Stars reflect differences between the day three and 10 within the substances separately for the investigated pools (tested with nested mixed effect ANOVA). Significant differences for 13C recovery in soil were observed only on day 10; on day three there were no significant differences.

Fig. 2. 13C incorporation from both amino acids (in % of applied 13C) into PLFAs (top) and percent of 13C replacement (in % of PLFA-C) (bottom) of microbial groups three and 10 days after alanine and glutamate application. Data present mean (n = 4) and bars present standard errors. Small letters reflect differences between the microbial groups for incorporation or replacement of 13C from amino acid; please read the letters separately for day three and 10. Stars show differences between the days within a substance. Hash symbols show differences within a day between substances.

Fig. 3. 13C incorporation from both monosaccharides (in % of applied 13C) into PLFAs (top) and percent of 13C replacement (in % of PLFA-C) (bottom) of microbial groups three and 10 days after glucose and ribose application. Data present mean (n = 4) and bars present standard errors. Small letters reflect differences between the microbial groups for incorporation or replacement of 13C from monosaccharide; please read the letters separately for day three and 10. Stars show differences between the days within a substance. Hash symbols show differences within a day between substances.
0.001–0.25% of $^{13}$C was recovered from amino acids. The incorporation of $^{13}$C from sugars into all microbial groups remained constant between days three and 10. G+ bacterial species used glucose-C more efficiently than ribose-C whereas G– group 1 preferred ribose. Among the filamentous microorganisms, fungi did not differ from 16:1 o 5 in glucose-$^{13}$C incorporation into PLFAs. In general, the microbial specialisation for individual monosaccharides, as building blocks for PLFAs, was visible within bacterial but not within eukaryotic groups.

Incorporation of $^{13}$C from both carboxylic acids into PLFAs of G– bacteria group 1 was higher than from other LMWOS (except ribose) (Fig. 4, top). Other bacterial groups used $^{13}$C from carboxylic acids less efficiently than $^{13}$C from sugars and amino acids for PLFAs synthesis. Filamentous microorganisms (actinomycetes and fungi, but also 16:1 o 5) exceeded the prokaryotic groups of G+ bacteria in incorporation of $^{13}$C from the most complex substrate, palmitate, into PLFAs.

In general, incorporation of LMWOS-C in bacterial species was higher than that in eukaryotes.

4. Discussion

4.1. Incorporation of LMWOS into SOM and microbial biomass

4.1.1. Amino acids

The decomposition of alanine and glutamate in our experiment was similar to other literature data (Jones et al., 2005; Kuzyakov, 1997) and less than 50% of the applied $^{13}$C remained in the soil after 10 days. The half-life of alanine and glutamate-derived C reported for field conditions was 18 and three days, respectively (Glanville et al., 2012). This is much longer than in our experiment and we observed a similar decomposition of glutamate and alanine C within 10 days. These contrasting results might be attributable to differences in total microbial activity or community structure (Jones et al., 2005) in the studied soils as well as due to methodological differences. Similar $^{13}$C amounts from glutamate and alanine, remaining in the soil on day 10, corresponds to similar microbial decomposition of differently charged amino acids (Jones and Hodge, 1999).

The high amount of $^{13}$C incorporated into the EMB at day three (Fig. 1) corresponds with the rapid and efficient uptake of free amino acids as intact molecules (Dippold and Kuzyakov, 2013; Geisseler et al., 2010; Jones and Hodge, 1999). After uptake, amino acids can either be oxidised for energy production, directly incorporated into proteins (Geisseler et al., 2010) or used in other metabolic pathways (Fig. 5) (Dippold and Kuzyakov, 2013; Knowles et al., 2010). The incorporation of alanine-C into EMB remained constant between two time points, whereas glutamate-C significantly decreased on day 10, which shows the more rapid mineralisation of glutamate-C. Similarly, glutamate was utilised more rapidly than glycine and lysine over a broad concentration range (Jones and Hodge, 1999). This corresponds to the different entry point of these amino acids into metabolism (Knowles et al., 2010). Alanine enters the basic cellular metabolism at the connecting step between glycolysis and the citric acid cycle (Apostel et al., 2013; Caspi et al., 2012). Thus, it is easily distributed throughout all anabolic pathways for the synthesis of cell components, e.g., gluconegenesis, protein synthesis, fatty acid synthesis and ribonucleotide synthesis (Fig. 5). In contrast, glutamate directly enters the citric acid cycle as oxoglutarate (Knowles et al., 2010). This demands energy for gluconegenesis and therefore, fatty acid synthesis pathways will not be used if other more appropriate substrates are available. Thus, alanine-C is preferentially incorporated into the more stable components of microbial cells—the cell walls and the membranes—compared to glutamate. In contrast, glutamate plays a central role in the amino acid cycle, and oxoglutarate produced from glutamate by transamination will be rapidly decompose to CO$_2$ (Vinolas et al., 2001).

4.1.2. Sugars

The half-life of glucose-C in our experiment (25% decomposed within 10 days) is within the range of previous studies: Glanville et al. (2012) reported a decomposition of 50% of glucose-C after 20 days, Saggar et al. (1999) measured a glucose-C decomposition of 51–66% within 35 days and Schneckenberger et al. (2008) observed 26–44% mineralisation of $^{13}$C from glucose within 22 days.

The incorporation of a significant proportion of applied $^{13}$C from sugars into EMB in our experiment is in agreement with the model of short-term glucose utilisation (Nguyen and Guckert, 2001). In this model, glucose taken up from solution is initially allocated to an intermediate pool and thereafter can be respired or used as a structural component. Thus, when the demand for cellular products is high, glucose C will be preferentially transferred to anabolic pathways rather than be oxidised for energy production.

The lower decomposition and incorporation of ribose (a pentose monosaccharide) compared to the hexose glucose on day three may be attributed to the metabolism of pentoses, which occurs mainly via the pentose-C. Similarly, pathway. This pathway leads to incorporation into various cell components such as DNA or other ribonucleotides (Fig. 5) but supposedly to a lower incorporation of ribose into PLFAs compared to glucose. Preferential incorporation
of ribose into biosynthetic ribonucleotide products has further to be proven by substance-specific analysis, e.g., by stable isotope probing of DNA (Radajewski et al., 2003). However, similar decomposition and incorporation of both monosaccharides into the EMB on day 10 suggest that all monosaccharides are generally good precursors for water-soluble metabolite pool in cells and that hexasse and pentose pathways are closely linked. Starting from this intermediate pool, the transfer of this C towards biosynthetic pathways can favour the synthesis of either intracellular, dissolved compounds or structural, mainly polymeric materials depending on the type of monosaccharide and the cellular C demand.

4.1.3. Carboxylic acids

Similar to amino acids and monosaccharides, the rapid uptake of acetate out-competes its physicochemical sorption in soils (Fischer et al., 2010). Within the microbial biomass, acetate C can be subjected to “arrest metabolism” and stored in cells before use (Fischer and Kuzyakov, 2010). Acetate can also be transformed into carbohydrates, amino acids (Sorensen and Paul, 1971) and other cell components and thus is fixed in diverse microbial products. Incorporation of acetate into EMB was less than that of glucose, which confirms that acetate was used for respiration (ca. 80–90%) rather than for new cell biomass production (Jones and Edwards, 1998; van Hees et al., 2002). This occurs due to the oxidation of a high proportion of acetate C into the citric acid cycle. Furthermore, acetate must be activated prior to incorporation into the key metabolic pathways (van Hees et al., 2002). In addition to the transformation of acetate to anabolic products is thus unfavourable, as long as microorganisms have access to more freely available substrates. An exception to this pathway, however, is fatty acid synthesis, where acetate is a direct precursor.

Palmitate is an anion of a short-chain fatty acid, the most dominant fatty acid in bacteria and fungi (Lawlor et al., 2000) and is a precursor for the synthesis of more complex fatty acids. Due to its high molecular weight and long aliphatic chain, we hypothesised that its decomposition is much slower than the decomposition of short chain carboxylic acids such as acetate. However, this hypothesis was not confirmed in our experiments. Previous studies showed that the degradation of palmitate was more rapid than that of similar or longer fatty acids (Moucawi et al., 1981). It was estimated that 41% of oleic and 31% of stearic acids were decomposed within four weeks (Moucawi et al., 1981), whereas 50% of palmitate decomposed within 10 days (Fig. 1).

In contrast to net decomposition, the incorporation of $^{13}$C from palmitate into EMB was the lowest (Fig. 1) and significantly different from sugars. This might be due to hydrophobic interactions of palmitate with SOM that lead to the lower uptake into microbial biomass. However, if taken up, it was preferentially incorporated in PLFAs, but not used for the synthesis of other microbial compounds. Incorporation into PLFAs was higher than for any other LMWOS, which is in accordance with their direct precursor role for PLFA formation.

In general, our results reflect that the uptake and utilisation of six LMWOS within three days was quite similar and comparable with the literature (Glanville et al., 2012). Substance-specific differences in incorporation into EMB were the most visible only at day 10 (Supplementary Table 2), when the total amount of incorporation C in soils is mostly controlled by the metabolic pathways of individual substance classes within microbial cells than any preferential uptake during the first few days. Therefore, we conclude that the medium-term divergence of the fate of LMWOS-C depends on its initial form entering the soil.

4.2. Microbial community composition

The constant composition of PLFAs (Table 1) after the addition of very low amount of LMWOS-C shows that microbial community structure could be under steady-state conditions (Blagodatskaya et al., 2007, 2009). This corresponds to other studies with similar amounts of applied C (Brant et al., 2006). Incorporation of $^{13}$C into microbial biomass and individual microbial groups reflected by this way the typical utilisation of these substances under natural soil conditions – i.e. a microorganisms under maintenance metabolism.

The main classes of decomposers for the six substances were G– and G+ bacteria. G– bacteria are very common in the rhizosphere, which reflects their preference for LMWOS common in rhizosphere hotspots (sugars, carboxylic acids, amino acids). In contrast, G+ bacteria are abundant in bulk soil (Soderberg et al., 2004).

The soil environment of this study, with aerobic conditions, a neutral soil pH as well as the above- and below-ground litter remaining after the harvest, provides optimum conditions for the development of actinomycetes, which are assumed to be important for the primary degradation of recalcitrant SOM (McCarthy and Williams, 1992). In contrast, present environmental conditions and loadings of small amounts of complex substrates did not support fungal growth (Reischke et al., 2014), which explains low abundance of fungal biomarkers as well as low activity of fungal biomass in LMWOS utilisation in our experiment.

We detected relevant amounts of 16:1ω5 fatty acid, which can be used to characterise the VAM fungi or gram-negative bacteria (Olsson, 1999; Zelles, 1997). Results of the factor analysis do not attribute the 16:1ω5 to the group 1 or group 2 of G– bacteria, moreover they were loaded up similarly with fungi (Supplementary Table 2). Second the VAM are usually abundant in soils, because they form a symbiotic relationship with up to 80% of land plants (Madan et al., 2002). Third 16:1ω5 behaved similarly with fungi in utilisation of investigated LMWOS (especially for carboxylic acids). All these factors support the interpretation that 16:1ω5 reflects VAM in this soil. For ensuring the interpretation of
16:1ω5 as VAM fatty acid, the simultaneous analysis of 16:1ω5 in PLFAs and neutral lipids should be done, otherwise the interpretation of 16:1ω5 as VAM should be done with caution.

4.3. Incorporation of LMWOS into PLFAs

4.3.1. Amino acids

The observed dominant role of bacteria in amino acid utilisation is in agreement with previous studies, which found that the relative incorporation of 13C from glutamate (the added amount was 50 μg C g⁻¹ soil) into bacteria was high, whereas incorporation into fungi was significantly lower (Brant et al., 2006; Rinnan and Bååth, 2009). Actinomycetes utilised amino acids in a forest soil, similar to G+ bacteria (Brant et al., 2006). Our results with agricultural soil support those of Brant et al. (2006) and allow the conclusion that amino acid turnover is dependent on microbial community structure, a major factor controlling microbial activity (Jones et al., 2005).

We demonstrated a preferred incorporation of alanine than glutamate into PLFAs and a higher replacement of PLFA-C by alanine 13C than glutamate 13C (Fig. 2, bottom). Despite a similar uptake of alanine and glutamate into EMB, their contrasting incorporation into PLFAs shows differences in intracellular metabolism: alanine C is directly converted to acetyl-CoA, the direct precursor of fatty acids, whereas complex energy-consuming pathways are needed to transform glutamate C into acetyl-CoA (Fig. 5). In addition, the formation of acetyl-CoA from alanine causes a loss of one-third of its C backbone compared to a three-fifths loss of C from glutamate if converted to acetyl-CoA (Apostel et al., 2013). This also contributes to the lower incorporation of glutamate-C into PLFAs. Thus, our results confirm those of previous studies on metabolic tracing, showing that intracellular metabolism is the master process that determines the fate of amino acid C in soils (Apostel et al., 2013; Dippold and Kuzyakov, 2013; Knowles et al., 2010).

4.3.2. Sugars

The preference of bacteria for glucose utilisation compared to fungi, corresponds with the dominance of bacteria biomarkers, but can also be attributed to high competitive ability of bacteria to take up glucose compared to fungi (Moore et al., 2005) especially if low concentrations of LMWOS are applied (Reischke et al., 2014). This was revealed previously by a higher relative glucose incorporation into bacteria (Brant et al., 2006). The preferential incorporation of sucrose into bacterial fatty acids (16:1ω7 and 18:1ω7) was also reported by Nottingham et al. (2009), who noted the importance of the 16:1ω7 biomarker in the control of priming effects. Thus, G– bacteria (corresponding to our G–1 group) represent a group whose growth is based on easily available substrates and which are the most competitive for LMWOS in many ecosystems (Treonis et al., 2004). Hence, the majority of studies show that bacteria are the most relevant group for the uptake and degradation of easily available substrates e.g., following the initial stage of litter decomposition, whereas fungi decompose more complex substrates that remain at later stages (Moore-Kucera and Dick, 2008). However, studies based on nuclear magnetic resonance showed the significant utilisation of 13C from glucose for the formation of unsaturated triacylglycerols, typical storage metabolites of eukaryotes (Lundberg et al., 2001). Based on these results, it has been suggested that fungi are the most active organisms in glucose degradation. Interpretations in our study are based on membrane lipids — a substance class whose structure, function and biosynthetic pathways are similar between many prokaryotes and eukaryotes. Thus, a comparison of the utilisation pattern is probably more reliable if functional and biosynthetically comparable compounds are included (Rinnan and Bååth, 2009).

The uptake pattern of ribose was relatively similar to that of glucose (Fig. 3, top), with predominant utilisation by G– bacteria. This primary incorporation of pentose by G– bacteria was also characterised by 13C-xyllose utilisation (Waldrop and Firestone, 2004), a similar structure and thus presumably a similar uptake and metabolism. The high percentage incorporation of ribose 13C into EMB compared to the relatively small amount of 13C detected in specific PLFAs can be explained by the use of ribose for the synthesis of other cell polymers. After modification in the pentose-phosphate pathway and phosphorylation, ribose is likely to become a subunit for ribonucleotides and less used for fatty acid biosynthesis (Fig. 5). Ribonucleotides are extracted after chloroform fumigation and this can explain high 13C incorporation in the microbial pool. Only G–1, the most active group in LMWOS utilisation, incorporated high amounts of ribose into PLFAs, i.e. pentose-phosphate intermediates. This accounts for high intracellular turnover of this most active microbial group.

4.3.3. Carboxylic acids

Acetate is an ubiquitous substrate in soil: it is the main product of lignin degradation, the main substance of plant litter anaerobic decomposition (Kusel and Drake, 1999), present at high concentrations in cattle slurry (Laughlin et al., 2009) and is known as direct precursor for the formation of fatty acids.

The amount of acetate incorporated into membranes of G– bacteria 1 (16:1ω7c and 18:1ω7c) was 5-fold higher than for most of the other PLFAs (Fig. 4, top). Similar to the other LMWOS, this might be a result of their higher abundance within the microbial community and their rapid uptake of LMWOS. A similar high recovery of 13C from acetate in 16:1ω7c and 18:1ω7c PLFAs was reported in experiments with anoxic brackish sediment (Boschker et al., 2001). Our experiment with well-aerated agricultural soil showed that the high competitiveness of these G– bacteria for acetate does not depend on the oxygen supply. In addition, experiments with sediment and groundwater samples showed that only few genera were involved in acetate degradation (Pombo et al., 2005). The 16:1ω7c PLFA has been suggested as a biomarker for acetate-oxidising sulphate-reducing bacteria. This anaerobic degradation can only occur in O₂-deficient microhabitats such as aggregate cores and is very unlikely to play a relevant role in freshly tilled soil.

Fatty acids that characterise G+ bacteria (such as i15:0, i16:0, i17:0, a17:0) were also enriched, but to a much lower degree than G– bacterial fatty acids (Fig. 4, top). Similar results were obtained with anoxic brackish sediments, where 10Me16:0, cy17:0, i15:0 and a15:0 PLFAs were enriched in 13C from acetate and were related to sulphate-reducing bacteria (Boschker et al., 2001). Carboxylic acids were the only substrate class where fungal uptake and incorporation could compete with those of prokaryotic, G+ groups. Thus, although fungi are less competitive for most LMWOS, they prefer acidic substrates within the LMWOS (Rinnan and Bååth, 2009). This correlates with their preference for acidic soil conditions, where acidic (non-neutralised), more complex substrates, dominate (Haider, 1996).

A comparison of palmitate and acetate utilisation in soils is important because acetate is a direct microbial precursor for palmitate synthesis. There are three pathways for the incorporation of palmitate into phospholipids: 1) partial step-by-step degradation of C₂-units without total breakdown of palmitate can occur and subsequently, only parts of the molecule are used for further biosynthesis (Rhead et al., 1971); 2) the resynthesis pathway includes the complete degradation of the molecule to acetyl-CoA and the following synthesis of new fatty acids by a series of enzymatic
reactions (Rhead et al., 1971); 3) the alternative is the utilisation of palmitate directly without further transformation, because it is the most abundant fatty acid in microorganisms and might be slightly modified by elongation or desaturation. According to the first pathway, palmitate should have a similar behaviour to acetate at least concerning the relative fate of $^{13}$C-PLFA/$^{13}$C-EMB ratios. Indeed, the $^{13}$C-PLFA/$^{13}$C-EMB ratio was higher for palmitate than for any other investigated LMWOS. This indicates at least partial use of palmitate without breakdown for PLFA synthesis (the third pathway), whereas complete or partial breakdown pathways have a lower importance.

Comparably to the other LMWOS, there was a higher total incorporation of $^{13}$C from palmitate in bacteria than in fungi (Fig. 4, top) and the G+ bacterial PLFAs (Fig. 4, top) were most enriched. This shows the high activity and cellular turnover of this bacterial group (Fig. 4, bottom). Notably, palmitate $^{13}$C incorporation into actinomycetes (10Me16:0, 10Me18:0) was higher than acetate $^{13}$C. Comparison of single cell G+ bacteria with their corresponding filamentous microorganisms, the actinomycetes, shows the preference of osmotrophic groups for highly water-soluble, simple substrates. In contrast, filamentous organisms respond slower, but with a similar incorporation of less water-soluble, more complex carboxylic acids, such as palmitate. Thus, a lack of mobility and defined filamentous organisation of microorganisms lead to a lowered competitiveness for simple LMWOS and a slow turnover within microbial biomass (Rousk and Baath, 2007). In contrast, if the focus is set on long-term C uptake, filamentous organisms show a higher incorporation of C from complex, less-available substrates (Brant et al., 2006).

5. Conclusions

This study compared and revealed the role of three classes of LMWOS — amino acids, sugars and carboxylic acids in short-term microbial utilisation in soil. The similar LMWOS uptake at day three, but differences in microbial incorporation at day 10, reflects that instead of initial uptake, the intracellular metabolism accounted for the observed differences in LMWOS fate in soils.

Amino acids were taken up by soil microorganisms in similar amounts on day three, but much less glutamate than alanine remained in EMB on day 10 (compare to day three). This reflects that substrates with direct incorporation into the oxidising citric acid cycle, such as glutamate, are preferentially oxidised for energy production compared to alanine, which enters glycolysis. The high and rapid glucose uptake by microbial biomass is connected with the fact that glucose is the most abundant sugar in the soils and ribose is taken up more slowly. More sugar $^{13}$C (mainly from glucose) was incorporated into EMB than from amino acids (glutamate) and carboxylic acids, which reflects the preference of glycolysis substrates for anabolic utilisation compared to catabolism. For carboxylic acids (acetate), the $^{13}$C in EMB declined by a factor of two from day three to day 10, also reflecting the preferred catabolic oxidation of substances entering the citric acid cycle.

$0.8$–$2\%$ of the initial applied $^{13}$C were used for the formation of cell membranes i.e., for total PLFAs with no differences between amino acids, sugars and acetate. $8\%$ of $^{13}$C from palmitate detected in PLFAs was a result of its direct use as a precursor for PLFA formation.

The PLFAs analysis showed bacteria (especially G+ ) were highly competitive for LMWOS uptake. The contribution of fungi to LMWOS-C utilisation was less than that of bacteria, due to the low amount of fungi as well as their low competitiveness for water-soluble, easily available and easily degradable substances. Fungi can only compete with some bacterial groups in the uptake of acidic substrates like acetate or palmitate. In general, more complex substrates such as palmitate are preferred by filamentous microorganisms. Thus, metabolism and C partitioning within microbial cells between catabolism and anabolism affect the fate of individual LMWOS in soil. This can be attributed to the step where they enter the basic C metabolism and consequently, to their individual metabolic pathways.

Our results demonstrate that to better understand short as well as long-term stabilisation of C from LMWOS in soil, we should focus on differences in microbial metabolism of LMWOS and the subsequent microbial products formed, rather than their initial uptake.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.06.029.

References


