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Geochimica et Cosmochimica Acta 174 (2016) 211–221

Geochimica et Cosmochimica Acta

www.elsevier.com/locate/gca

Direct incorporation of fatty acids into microbial phospholipids in soils: Position-specific labeling tells the story

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Received 28 April 2014; accepted in revised form 28 October 2015; Available online 27 November 2015

Abstract

Fatty acids have been used as plant and microbial biomarkers, and knowledge about their transformation pathways in soils and sediments is crucial for interpreting fatty acid signatures, especially because the formation, recycling and decomposition processes are concurrent. We analyzed the incorporation of free fatty acids into microbial fatty acids in soil by coupling position-specific ^{13}C labeling with compound-specific ^{13}C analysis.

Position-specifically and uniformly ^{13}C labeled palmitate were applied in an agricultural Luvisol. Pathways of fatty acids were traced by analyzing microbial utilization of ^{13}C from individual molecule positions of palmitate and their incorporation into phospholipid fatty acids (PLFA).

The fate of palmitate ^{13}C in the soil was characterized by the main pathways of microbial fatty acid metabolism: Odd positions (C-1) were preferentially oxidized to CO_2 in the citric acid cycle, whereas even positions (C-2) were preferentially incorporated into microbial biomass. This pattern is a result of palmitate cleavage to acetyl-CoA and its further use in the main pathways of C metabolism. We observed a direct, intact incorporation of more than 4% of the added palmitate into the PLFA of microbial cell membranes, indicating the important role of palmitate as direct precursor for microbial fatty acids. Palmitate ^{13}C was incorporated into PLFA as intact alkyl chain, i.e. the C backbone of palmitate was not cleaved, but palmitate was incorporated either intact or modified (e.g. desaturated, elongated or branched) according to the fatty acid demand of the microbial community. These modifications of the incorporated palmitate increased with time. Future PLFA studies must therefore consider the recycling of existing plant and microbial-derived fatty acids.

This study demonstrates the intact uptake and recycling of free fatty acids such as palmitate in soils, as well as the high turnover and transformation of cellular PLFA. Knowledge about the intact uptake and use of soil-derived free fatty acids is crucial for interpreting microbial fatty acid fingerprints and their isotopic composition.

1. INTRODUCTION

Lipids are an important constituent of plant biomass. They comprise around 3–10% of aboveground and 0.5–5% of belowground plant biomass and are thus an essential component of plant-derived C input into soils (Bliss, 1962; Ohlrogge and Browse, 1995; Wiesenberg et al., 2004). In addition, microbial biomass contains around 10% lipids, mainly in cell membranes and cell walls (Zelles et al.,

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http://dx.doi.org/10.1016/j.gca.2015.10.032
0016-7037/

1995; Lengeler et al., 1999), and significantly contributes to the lipidic SOM pool. The long-term preservation of some lipid classes, such as alkanes and fatty acids, qualifies them as important biomarkers (White et al., 1997; Otto et al., 2005), which can be used to differentiate vegetation types (Schwark et al., 2002; Wiesenberg and Schwark, 2006; Zech et al., 2012; Bush and McInerney, 2013). Cutin-suberin-derived hydroxylated or poly-carboxylic acids enable aboveground litter input to be differentiated from belowground litter input (Mendez-Millan et al., 2011; Spielvogel et al., 2014).

Microorganisms are able to use lipids as substrates and decompose them to metabolites. They can also build up their own lipids, significantly contributing to the lipid pool of SOM and the modification of initial lipidic compounds (Lichtfouse et al., 1995; Otto et al., 2005). Such transformations of lipid biomarkers need to be considered when applying lipids as biomarkers. Recent studies indicate that plant-derived biomarkers can be modified and overprinted by rhizomicrobial activity (Gocke et al., 2014). For some biomarker classes such as sterols, microbial modifications of plant- or animal-derived lipids are specifically used to trace the microbial community impact (Arima et al., 1969; Bull et al., 1999, 2002). For others classes, like alkanes, approaches used to correct for microbial overprint of plant-derived signals have been developed (Buggle et al., 2010; Zech et al., 2013). Nonetheless, current knowledge on the microbial utilization and transformation of lipids in soils is scarce: It remains unclear whether microorganisms prefer neosynthesis of lipids from low molecular weight precursors or re-utilization of available lipidic compounds, e.g. fatty acids. According to the biochemical principle of the most economic pathways, cells tend to use preformed building blocks for biomass synthesis (Lengeler et al., 1999). This principle may be valid for the majority of metabolites, but was rarely tested, especially not with microorganisms in their natural environment. We therefore hypothesize that lipid precursors released by the decomposition of plant or microbial biomass are the preferred substrates for further lipid synthesis by microorganisms. We traced the utilization of the most abundant fatty acid in soil – palmitic acid (=hexadecanoic acid) – as a microbial substrate for PLFA formation. Palmitic acid is a key compound for plant and microbial fatty acid metabolism. Investigating its microbial utilization and transformation pathways provides a general view of the microbial modification of soil lipids.

To elucidate the metabolic pathways of microbial fatty acids, we used the approach of position-specific labeling. This tool was originally developed in biochemistry to investigate metabolism pathways and has been increasingly used in soil biogeochemistry over the last few years (Fischer and Kuzyakov, 2010; Dijkstra et al., 2011a,b; Apostel et al., 2013; Dippold and Kuzyakov, 2013). This approach enables the fate of individual C positions to be traced through various pools or metabolites and thus helps reconstruct individual transformation steps. In the case of lipids containing non-exchangeable hydrogen, similar metabolic studies can be performed based on deuterium labeling (Kahmen et al., 2011; Gao et al., 2012a,b).

Knowledge about fatty acid synthesis by microorganisms has mainly been derived from experiments with pure cultures (Lennarz, 1970; Rock et al., 1981; Zelles et al., 1995). Fatty acids can be newly synthesized from precursors such as acetate, or available lipid precursors can be modified by microorganisms (Lennarz, 1970; Rhead et al., 1971; Rethemeyer et al., 2004). If palmitate is provided as a substrate, there are three possible mechanisms by which palmitate C can be used for PLFA synthesis: (1) the resynthesis pathway, i.e. the complete degradation of the molecule to acetyl-CoA units and the subsequent reconstruction of new fatty acids from C2-moieties (Rhead et al., 1971): this pathway is the conventional idea on lipid formation by microorganisms in soils and sediments. (2) Partial step-by-step degradation of the C2-units, starting from the carboxylic group, without total breakdown of palmitate: subsequently, only parts of the molecule are incorporated into newly formed fatty acids (Rhead et al., 1971). (3) untransformed utilization of palmitate because it is the most abundant fatty acid in microorganisms (Rhead et al., 1971; Zelles et al., 1995). Recent results strongly indicate the intact reuse of the core unit of glycerol dialkyl glycerol tetraethers (GDGTs) in marine sediments (Takano et al., 2010). This supports the idea that the recycling of complex lipid metabolites plays a crucial role in sediments and presumably also soils. This re-utilization of intact alkyl chains by microorganisms would preserve the original plant-derived isotopic signature in microbial lipids. Position-specific isotope labeling is the only tool that enables these three pathways of lipid formation and transformation to be distinguished. This approach will deepen our understanding of the microbial transformation of fatty acids in soils and improve our interpretation of lipid isotopic signatures in soils and sediments.

2. MATERIALS AND METHODS

2.1. Experimental site

The field experiment is located in Bavaria, close to Hohenpölz (49.907 N, 11.152 E) at an elevation of 501 m. a.s.l, a mean annual temperature of 6.7 °C and a mean annual precipitation of 874 mm. The agriculturally used field site is managed by a rotation of corn, barley, wheat and triticale. Soil type is a loamy Luvisol with an Ap horizon of 25 cm depth followed by a Bt-Horizon of 10 cm depth and an illite-rich loess-loam as C horizon. It has a pH_{KCl} of 4.88, a pH_{H2O} of 6.49, a total organic C (TOC) content of 1.77%, a total nitrogen (TN) content of 0.19% and a cation exchange capacity of 13 $cmol_C\ kg^{-1}$. Before the experiment started in August 2010, triticale was harvested and the field site was tilled to 10 cm depth for soil homogenization.

2.2. Experiment design

The 12 × 12 m field was subdivided into four quadrants. PVC-tubes (diameter: 10 cm; height: 13 cm) were installed 10 cm deep into the Ap horizon, yielding a soil sample weight between 1 and 1.5 kg for each column. Column

location was randomized within the blocks and each of the four blocks represented one of the four repetitions of each treatment. Consequently, the block could be included as a random variable in statistical evaluation to account for the spatial heterogeneity within the field site.

Tracer-solution was applied with a multipette (Eppendorf, Hamburg, Germany) at 5 injection points per column, each of 2 ml. Uniformly ^{13}C-labeled palmitate as well as position-specific labeled isotopomers (1-^{13}C palmitate, 2-^{13}C palmitate and 16-^{13}C palmitate) were applied (99% ^{13}C, Biotrend Chemicals, Cologne, Germany). In addition, an identical amount of palmitate-C with natural abundance isotope signature was applied on non-labeled background columns. The concentration of ^{13}C was 50 µmol palmitate per column and was identical for each treatment and the backgrounds.

A 7-cm-long needle with closed tip and peripheral holes allowed homogeneous lateral distribution of the tracer solution. Leaching was avoided by injecting the solution only into the upper 2/3 of the column and excluding rainfall by installing a roof above the plots.

2.3. Sampling and sample preparation

The sampling involved harvesting the entire soil column three and ten days after labeling. At both times, the height of the soil inside the column was measured to determine the labeled soil volume. Then, soil was pressed out from the column, fresh weight was determined and the entire soil sample was homogenized manually. Afterwards, a subsample was taken to determine water content and the sample was split: one subsample was freeze-dried and ball milled for bulk isotope analysis, and another subsample was sieved through 2 mm mesh and stored at <5 °C for chloro form-fumigation-extraction (CFE) to extract microbial biomass (Vance et al., 1987; Wu et al., 1990). The remaining soil, also sieved through 2 mm mesh, was stored frozen for analysis of microbial PLFA.

2.4. Bulk soil and microbial biomass analysis

For the analysis of bulk soil C content and δ^{13}C-values, the samples were freeze-dried, ground in a ball mill and 5–6 mg per sample were filled into tin capsules. The samples were measured on the Euro EA Elemental Analyzer (Eurovector, Milan, Italy) coupled with a ConFlo III interface (Thermo Fisher, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fisher, Bremen, Germany). Incorporation of ^{13}C from the applied carboxylic acids into soil was calculated according to the mixing model (Eqs. (1) and (2)), where the C content of the background in Eq. (1) was substituted according to Eq. (2) (Gearing et al., 1991)

$$[C]_{soil\_labeled} \cdot r_{soil\_labeled} = [C]_{soil\_BG} \cdot r_{soil\_BG} + [C]_{Inc\_pal\_13C} \cdot r_{Inc\_pal\_13C} \quad (1)$$

$$[C]_{soil\_labeled} = [C]_{soil\_BG} + [C]_{Inc\_pal\_13C} \quad (2)$$

with:

$[C]_{soil\_labeled/soil\_BG/inc\_pal\_13C}$ C amount in labeled soil sample/background sample/incorporated from applied palmitate (mol g_{soil}^{-1}).

$r_{soil\_labeled/soil\_BG/inc\_pal\_13C}$ ^{13}C atom% of labeled soil sample/background sample/applied palmitate (at.%).

The determination of microbial biomass C and ^{13}C incorporation was performed according to a modified protocol described in Gunina et al. (2014). Briefly, two subsets of 15 g soil were taken to determine microbial C and its δ^{13}C values. One subsample was directly extracted, containing the extractable SOC pool, whereas the other was first fumigated with chloroform before extraction, and consequently contained the extractable SOC and the microbial biomass C pool (Malik et al., 2013). The C content of the extracts was determined on a TOC analyser multi C/N® 2000 (Analytik Jena, Jena, Germany). The remaining extracts were then freeze-dried for δ^{13}C measurements. ^{13}C incorporation into fumigated and unfumigated samples was calculated according to the mixing model (Eqs. (1) and (2)). Microbial biomass and palmitate ^{13}C incorporated into microbial biomass was calculated according to Wu et al. (1990) with an extraction factor of 0.45.

2.5. PLFA δ^{13}C analysis

Phospholipids were extracted and purified by a modified method of Frostegård et al. (1991), which is described in detail in Gunina et al. (2014). Modifications included the use of 6 g of soil for extraction, a doubled liquid–liquid extraction and a very slow elution of polar lipids from the activated silica column with four times 5 ml methanol. Before extraction, 25 µl of a 1 M solution of phosphatidyl choline-dinonadecanoic acid was added as internal standard 1 (IS 1). For measurements on a gas chromatograph (GC), the fatty acids were saponified to free fatty acids and derivatized into fatty acid methyl esters (FAME) following the description by Knapp (1979). Before transferring the samples to autosampler vials, 15 µl of tridecanoic acid methyl ester (1 µg µl^{-1} in toluene) was added as internal standard 2 (IS 2). External standards consisting of the 27 fatty acids listed in Supplementary Table 1 together with the phospholipid IS 1 were prepared with fatty acid contents of 1, 4.5, 9, 18, 24 and 30 µg, respectively, and derivatized and measured together with each sample batch.

FAME-contents were measured on a GC–MS (GC 5890 with MS 5971A, Agilent, Waldbronn, Germany) with a 30 m DB1-MS column, in the selected ion mode. The relation between the area of each FAME and the area of the IS 2 was calculated and a linear regression based on the six external standards was used for quantification. The recovery of each sample was determined based on the area of the initially added 25 µg of IS 1, and the amount of each fatty acid was corrected by the recovery.

δ^{13}C-values were analyzed on a GC-C-IRMS consisting of the autosampler unit AS 2000, the Trace GC 2000 by ThermoQuest, the Combustion Interface III combustion unit and the isotope-ratio mass spectrometer Delta^{Plus} (Thermo Fisher, Bremen, Germany). Volumes of 1.5 µl

were injected in splitless mode (splitless time: 1 min) into a liner (Type TQ(CE) 3 mm ID TAPER) at 250 °C. Gas chromatography was accomplished with a combination of two capillary columns: a 30 m DB5-MS and a 15 m DB1-MS (both: internal diameter 0.25 mm, film thickness 0.25 μm; Agilent), a constant He-flux (99.996% pure) of 2 ml min^{-1} and the temperature program presented in Supplementary Table 2. CO_2 reference gas (99.995% pure) was injected for 20 s into the detector four times throughout the measurement to identify any detection drift. The $\delta^{13}C$-value of the second reference gas peak was calibrated on IAEA standards and fixed on the calibrated value (−40‰). $\delta^{13}C$-values of all PLFA samples were measured four times. The chromatograms were integrated and the $\delta^{13}C$-value was generated by the software ISODAT NT 2.0.

Linear regressions were calculated from reference gas peaks surrounding the fatty acid peaks for drift correction within the chromatogram (Apostel et al., 2013) and chromatographic drift was corrected according to the slope of this regression. To correct for amount-dependent ^{13}C isotopic fractionation during measurements (Schmitt et al., 2003) and for the addition of C during derivatization, we calculated linear and logarithmic regressions of the external standard $\delta^{13}C$-values to their area. If both regressions were significant, that with the higher significance was applied. As the $\delta^{13}C$-value for the derivatization agents was unknown, the correction was performed according to Glaser and Amelung (2002) (Eq. (5))

$$C_{FS}(at\%) = \frac{N(C)_{FAME}}{N(C)_{FS}} \cdot (C_{FAME-DK}(at\%) - (m_{lin/\ln} \cdot A_{FAME} + t_{lin/\ln})) + C_{EA-FS}(at\%) \quad (3)$$

with:

C_{FS}(at.%) corrected ^{13}C amount of the fatty acid [at.%].
C_{FAME}(at.%) drift-corrected ^{13}C amount of the FAME [at.%].
$m_{lin/ln}$ slope of linear/logarithmic regression [at.% Vs^{-1}].
$t_{lin/ln}$ y-intercept of linear/logarithmic regression [at.%].
A_{FAME} area of FAME [Vs].
$N(C)_{FAME}$ number of C atoms in FAME.
$N(C)_{FS}$ number of C atoms in fatty acid.
$C_{EA\text{-}FS}$(at.%) measured ^{13}C-value of fatty acid [at.%].

2.6. Calculation of ^{13}C incorporation into PLFA

The amount (mol) of ^{13}C incorporated into an individual fatty acid was calculated according to the isotope mixing model (Gearing et al., 1991):

$$[C]_{PLFA\_labeled} \cdot r_{PLFA\_labeled} = [C]_{PLFA\_BG} \cdot r_{PLFA-BG} + [C]_{Inc\_pal\_13C} \cdot r_{app\_pal\_13C} \quad (4)$$

$$[C]_{PLFA\_labeled} = [C]_{PLFA\_BG} + [C]_{Inc\_pal\_13C} \quad (5)$$

with:

$[C]_{PLFA\_labeled/PLFA\_BG/inc\_pal\_13C}$ C amount of PLFA in labeled sample/background sample/incorporated from applied palmitate (mol g_{soil}^{-1}).
$r_{PLFA\_labeled/PLFA\_BG/inc\_pal\_13C}$ ^{13}C atom% of PLFA in labeled sample/background sample/applied palmitate (at.%).

These equations can be solved for $[C]_{inc\_pal\_13C}$, which quantifies the mol of palmitate-derived ^{13}C, which was incorporated into the respective fatty acid. This mol of incorporated ^{13}C was then divided by the amount of applied ^{13}C and multiplied with 100%. This yields the percentage of ^{13}C incorporation into the respective PLFA from the applied palmitate, labeled at an individual position. This division by the amount of applied ^{13}C is crucial for comparison between the individual positions: it corrects for the minimal differences in ^{13}C enrichment in the individual tracer solutions of the position-specific labeled isotopomeres. As, for intact incorporation of palmitate into PLFA, all palmitate C positions need to be recovered in the phospholipid bound palmitate, the position with the lowest incorporation reflects the potential intact uptake and incorporation of the applied fatty acid into the PLFA.

2.7. Statistics

The values presented show mean ± standard error of mean (±SEM) of the four field replicates of each sample. Measured values were tested for normal distribution using the Kolmogorov Smirnoff test, for homogeneous variances using Levene's test and screened for outliers using the Nalimov test (Gottwald, 2000). Factorial ANOVA was calculated using Statistica (version 6.0, Statsoft GmbH, Hamburg, Germany). If assumptions such as normal distribution or homogeneous variances were not met, the result of the ANOVA was confirmed by non-parametric Kruskal–Wallis ANOVA before performing a Tukey HSD post hoc test for unequal sample size.

3. RESULTS

3.1. Incorporation of palmitate ^{13}C in soil and microbial C pools

At day 3 uniformly labeled palmitate ^{13}C was decomposed to 30%, and 70% of the added ^{13}C were recovered in soil (see Fig. 1, uniformly labeled treatment shown as line, respective pools see Table 1). From day 3 to day 10 half of that soil palmitate was furthermore decomposed and only 33% of the palmitate ^{13}C still remained in the soil (Fig. 1).

At each sampling day, the C-1 position of palmitate was preferentially oxidized but this phenomenon was more strongly pronounced at day 10. The ^{13}C loss from palmitate between day 3 and day 10 was higher for the carboxylic C-1 position of palmitate compared to the alkyl-C positions C-2 and C-16 (see Fig. 1).

The position-specific pattern of soil incorporation was similarly reflected by the ^{13}C incorporation into microbial biomass. The carboxylic group C-1 showed lowest incorporation, i.e. was preferentially oxidized (Fig. 1). In contrast, the PLFA showed no clear preference for incorporation of individual palmitate positions but seemed to incorporate all

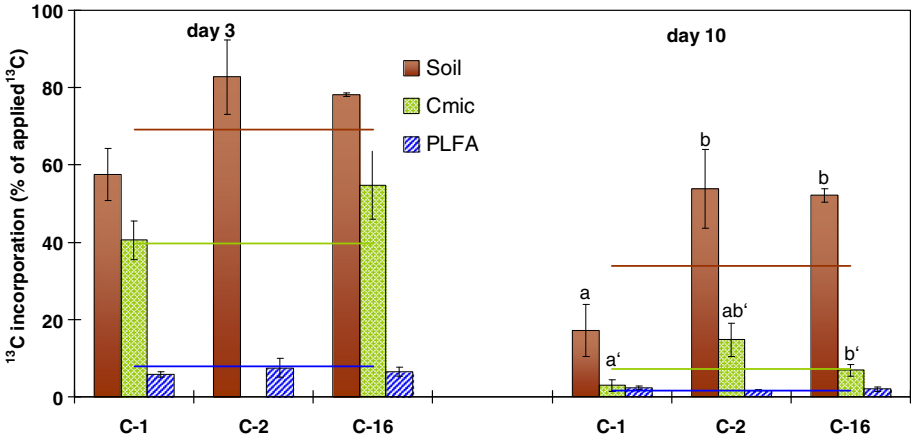

Fig. 1. Recovery of position-specifically ^{13}C labeled palmitate in soil, microbial biomass and in the sum of PLFA (Σ-PLFA), 3 (left) and 10 days (right) after substance application. Bars represent means ± SEM ($n = 4$) for the individual labeled C positions, and lines represent the mean of the uniformly labeled palmitate. Letters indicate significant differences ($p < 0.05$) between recovery in bulk soil (a), microbial biomass (a′) and in Σ-PLFA (a″).

Table 1
Total organic C (TOC), microbial biomass C (C_{mic}) and the sum of all measured PLFA in mg C per g soil (dry weight).

		TOC	C_{mic}	Σ_{PLFA}
Pool size (mg C g^{-1} soil)	Day 3	15.60 ± 0.60	0.521 ± 0.022	0.064 ± 0.005
	Day 10	16.39 ± 0.30	0.463 ± 0.038	0.051 ± 0.003

positions equally, indicating intact incorporation (Fig. 1). There was, however, a strong decrease of ^{13}C content incorporated in PLFA from day 3 to day 10 (Figs. 1 and 2), which reflects the short half-life time of microbial PLFA in soils (Rethemeyer et al., 2004; Frostegard et al., 2011).

3.2. Incorporation of C from palmitate into individual PLFA

At day 3, highest incorporation of palmitate ^{13}C was found in palmitate (Fig. 2). Comparing the 3 labeled positions of palmitate, lowest incorporation, and thus the maximum of intact palmitate uptake and incorporation is around 4% of the applied palmitate (Fig. 3).

^{13}C incorporation of the straight-chain fatty acids dominated over ^{13}C incorporation into more complex fatty acids (Fig. 2). With increasing time, the simple straight-chain fatty acids decreased in their incorporation whereas more complex fatty acids such as cyclopropyl or 10Methyl-branched fatty acids even increased their absolute ^{13}C incorporation (Fig. 2). However, this incorporation of ^{13}C occured mainly by renewal of the existing fatty acid pool despite new formation of fatty acids due to microbial growth, because most of the fatty acids showed no increase of their concentration over time (Table 2).

This detailed information on individual fatty acids was summarized by pooling the fatty acids into biochemical classes in Fig. 3. Sixteen-carbon fatty acids, shorter fatty acids (14 and 15 carbons) and longer fatty acids (17 to 20 carbons) were grouped into straight chain even, straight chain odd, desaturated and branched fatty acids. This grouped fatty acid pattern demonstrates that newly formed fatty acids based on palmitate ^{13}C became more diverse from day 3 to day 10 after palmitate application. This increase in diversity of newly formed fatty acids leads to an approaching of the newly-formed ^{13}C fatty acid pattern to the pattern of microbial fatty acids in soil (Fig. 3).

In Fig. 4 the effect of decreasing ^{13}C incorporation from day 3 to day 10 was removed from the results by presenting the proportion of ^{13}C incorporation to the sum of ^{13}C incorporated into all PLFA at the respective day. Thus,

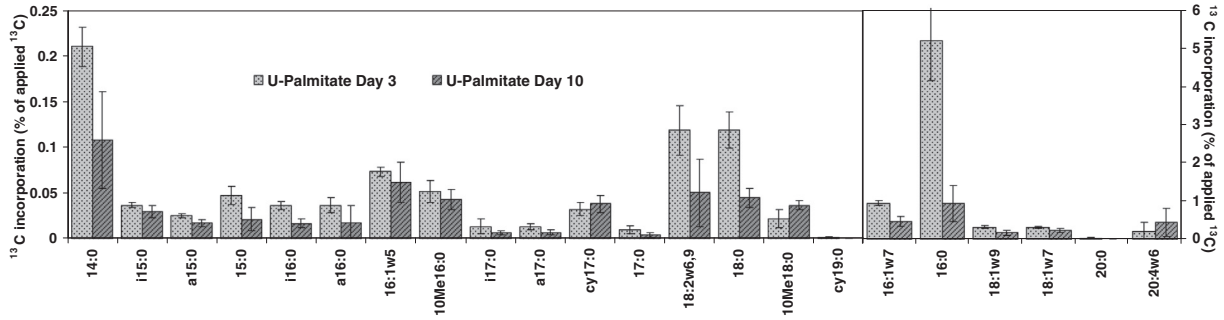

Fig. 2. Recovery of uniformly ^{13}C labeled palmitate in the individual PLFA, 3 and 10 days after ^{13}C application. Experimental averages (means ± SEM, $n = 4$) are presented.

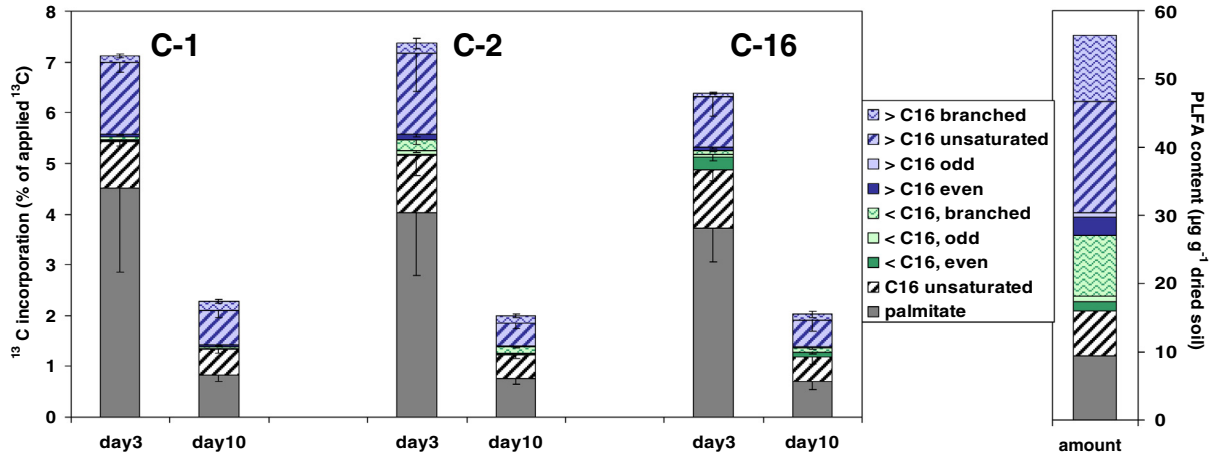

Fig. 3. Classes of phospholipid-derived fatty acids extracted from soil (right) and recovery of position-specifically ^{13}C labeled palmitate (left) in the different fatty acid classes, 3 and 10 days after ^{13}C application. Experimental averages (means ± SEM, $n = 4$) are presented.

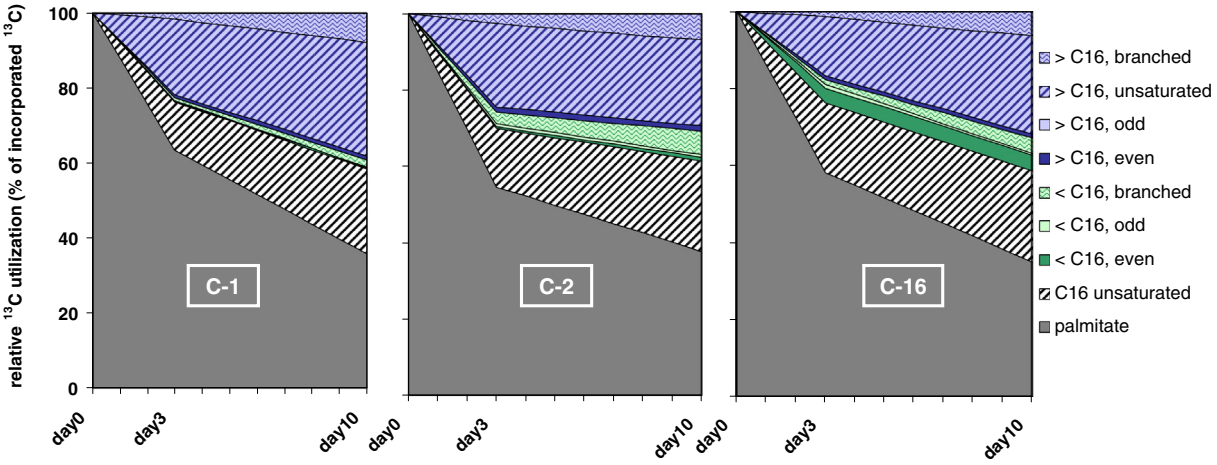

Fig. 4. Relative recovery of individual positions of palmitate ^{13}C in fatty acid classes. Experimental averages (means ± SEM, $n = 4$) are presented.

Fig. 4 presents the transformations of incorporated palmitate by microbial PLFA formation. The portion of desaturated fatty acids was already high at day 3 and did not increase significantly from day 3 to day 10 (Fig. 3). This shows that palmitate desaturation is a fast process. Nonetheless, incorporation of palmitate ^{13}C into elongated (and even more so into branched fatty acids) significantly increased from day 3 to day 10 for each of the C positions (Fig. 4). This reflects slower kinetics of complex elongation or branching versus rather simple desaturation processes – which can even occur if fatty acid is bound to the PLFA in the membrane (Aguilar et al., 1998).

3.3. Incorporation of individual palmitate C positions into various fatty acids

C-1 of palmitate is only negligibly incorporated into fatty acids shorter than 16 carbons (Fig. 3 and Fig. 4). In addition, the carboxylic C-1 position of palmitate was not incorporated into any odd-numbered PLFA (Fig. 3 and Fig. 4). This indicates the preference for palmitate as a direct precursor for microbial phospholipid synthesis, i.e. the even-numbered C-1 group was removed from the intact fatty acid to shorten the alkyl chain.

Comparing C-2 and C-16 positions (both are even-numbered C positions in the alkyl chain) showed that C-16 was incorporated in significant amounts into even-numbered fatty acids smaller than C16, but that this was not the case for C-2 (Fig. 3 and Fig. 4). This supports the view of splitting-off terminal positions of palmitate to shorten the alkyl chain.

In general, palmitate was modified according to the demand of the microbial community, i.e. the de novo formed, ^{13}C-derived fatty acid pattern aligned with the PLFA fingerprint of the microbial community present in soil.

Table 2
Amounts of PLFA in nmol fatty acid per g soil (dry weight) for day 3 and day 10 after label applications; means ± SEM ($n = 4$) of all labeled columns are presented.

Abbreviation	Fatty acid name	Fatty acid amount (nmol g^{-1} soil)	
		Day 3	Day 10
i14:0	Iso-Tetradecanoic Acid	4.63 ± 0.49	4.19 ± 0.25
a14:0	Anteiso-Tetradecanoic Acid	1.27 ± 0.41	0.47 ± 0.10
14:1ω5c	9-Tetradecenoic Acid	2.67 ± 1.37	2.58 ± 0.91
14:0	Tetradecanoic Acid	6.94 ± 1.01	5.09 ± 0.45
i15:0	Iso-Pentadecanoic Acid	18.67 ± 2.71	12.69 ± 1.50
a15:0	Anteiso-Pentadecanoic Acid	14.46 ± 1.80	10.70 ± 0.84
15:0	Pentadecdanoic Acid	2.90 ± 0.83	2.19 ± 0.36
i16:0	Iso-Hexadecanoic Acid	7.93 ± 1.28	5.61 ± 0.63
a16:0	Anteiso-Hexadecanoic Acid	3.20 ± 1.00	1.52 ± 0.49
16:1ω7c	9-Hexadecenoic Acid	17.84 ± 2.05	12.64 ± 1.07
16:1ω5c	11-Hexadecenoic Acid	11.62 ± 1.68	10.06 ± 1.30
16:0	Hexadecanoic Acid (Palmitic Acid)	39.45 ± 5.33	30.20 ± 2.39
10Me16:0	10-Methyl-Hexadecanoic Acid	9.00 ± 1.16	6.89 ± 0.72
i17:0	Iso-Heptadecanoic Acid	3.58 ± 0.93	2.80 ± 0.39
a17:0	Anteiso-Heptadecanoic Acid	3.93 ± 0.94	2.35 ± 0.30
cy17:0	Cyclopropyl-Heptadecanoic Acid	6.68 ± 1.08	4.53 ± 0.41
17:0	Heptadecanoic Acid	2.86 ± 0.89	1.19 ± 0.24
18:2ω6,9	9,12-Octadecadienoic Acid	4.78 ± 0.94	4.25 ± 0.51
18:3ω6,9,12	6,9,12-Octadecatrienoic Acid	3.92 ± 1.21	2.92 ± 0.80
18:1ω9c	11-Octadecenoic Acid	20.39 ± 1.84	15.30 ± 0.55
18:1ω7c	9-Octadecenoic Acid	31.02 ± 3.67	22.87 ± 1.09
18:0	Octadecanoic Acid	6.70 ± 1.09	5.92 ± 0.46
10Me18:0	10-Methyl-Octadecanoic Acid	5.79 ± 0.88	4.94 ± 0.44
cy19:0	Cyclopropyl-Octadecanoic Acid	8.77 ± 0.85	6.01 ± 0.69
20:4ω6c	5,8,11,14-Eicosatetraenoic Acid	3.41 ± 0.93	2.26 ± 0.54
20:1ω9c	11-Eicosenoic Acid	2.92 ± 0.95	2.24 ± 0.57
20:0	Eisocanoic Acid	2.66 ± 0.86	1.51 ± 0.38

4. DISCUSSION

4.1. Utilization and turnover of palmitate by the soil microbial community

Short-chain low molecular weight organic acids are well-used microbial substrates (Jones et al., 2003). Our study showed that long-chain carboxylic acids such as palmitate are also good substrates in soils and are used in high proportions by the microbial community (Fig. 1). In specific pathways, such long-chain carboxylic acids can function as direct precursors for lipid formation, which promotes their incorporation into microorganisms (Fig. 1).

The preferential oxidation of C-1 palmitate is a result of palmitate allocation into basic C metabolism, e.g. its use as an energy source and general anabolic C source. Therefore, it is successively oxidized by fatty acid β-oxidation to acetyl-CoA (2 C atoms) units (Caspi et al., 2008; Keseler et al., 2009). When the terminal C-1 and C-2 from palmitate are cleaved off form the alkyl-chhain, they form an acetate unit. Such an acetate unit is transformed similarly to acetate taken up from soil via the citric acid cycle (Fischer and Kuzyakov, 2010; Dippold and Kuzyakov, 2013), i.e. C-1 is preferentially oxidized compared to C-2. C-2 gets only oxidized after several rounds of cycling through the citric acid cycle and can during many of these transformation steps be allocated towards the anabolism, which results in a lower overall mineralization of this position to CO_2.

The difference in ^{13}C incorporation between microbial biomass and soil constitutes the portion of ^{13}C palmitate which was not accessible for microbial uptake but remained extracellular, e.g. as SOM-associated palmitate. The increase of this extracellular ^{13}C from day 3 to day 10 after label application, however, suggests that microbial residues from dead cells containing incorporated palmitate ^{13}C contribute significantly to this extracellular ^{13}C. The high discrimination between C-1 and C-2 at day 10 confirms this interpretation, because cellular metabolism of acetyl-CoA causes a preferred C-2 incorporation into microbial biomass (Fischer and Kuzyakov, 2010). Extracellular terminal oxidation processes can further contribute to this increasing discrimination between C-1 and C-2 from day 3 to day 10. This terminal oxidation of carboxylic acids to odd and even alkanes has been previously described for plants and microorganisms (Dennis and Kolattukudy, 1992; Park, 2005; Ladygina et al., 2006), and specific as well as unspecific decarboxylases can contribute to the decarboxylation of carboxylic acids in soils (Hofrichter et al., 1998). Extracellular transformations are less relevant for well available, low molecular weight organic substances (Dippold and Kuzyakov, 2013). Whether hydrophobic substances, such as palmitate, are modified extracellularly remains open and can be clarified only by combining selective inhibition of microbial, intracellular processes with position-specific lipid ^{13}C labeling (Dippold and Kuzyakov, 2013).

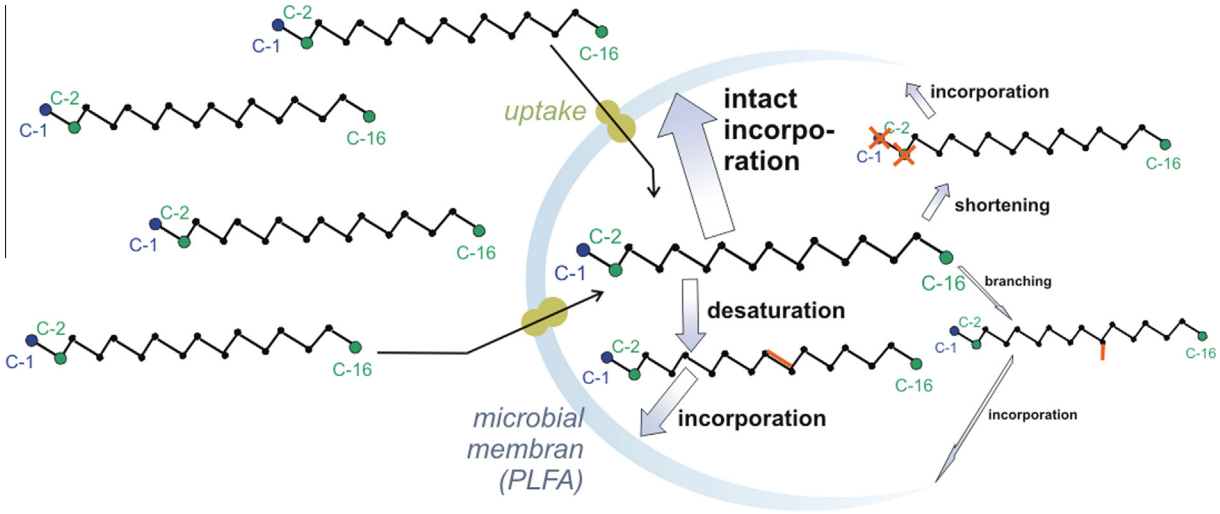

Fig. 5. Microbial utilization of fatty acids: uptake, intact incorporation into PLFA and incorporation into PLFA after modification were identified in this study and are illustrated in this scheme.

4.2. Pathways of fatty acid formation and transformation in soil

Gunina et al. (2014) compared incorporation of several, well water dissolvable, low molecular weight organic substances and palmitate into microbial biomass and PLFA. They found a much higher proportion of palmitate incorporation into PLFA than incorporation of ^{13}C from low molecular weight compounds. They interpreted this preferential fatty-acid derived ^{13}C allocation into the PLFA as a hint for an intact use of palmitate as a PLFA precursor. In this study, the initially added ^{13}C palmitate was successively transformed to more diverse spectra of fatty acids. Comparing those transformed fatty acids with the PLFA profile of the soil (Fig. 2) shows that within 10 days the newly transformed fatty acids approached the composition of the PLFA profile and consequently the demand of the microbial community.

The utilization of intact palmitate and the following modifications confirm the idea of a high recycling of existing fatty acids by soil microorganisms after intact uptake and subsequent modification of the alkyl chain: (i) almost no palmitate C-1 and C-2 is incorporated into even-numbered fatty acids smaller than palmitate, e.g. tetradecanoic acid. This suggests that the terminal acetate (C-1 and C-2) of palmitate is merely cleaved off to form tetradecanoic acid, whereas the basic C skeleton containing the C-16 position remains intact. (ii) No palmitate C-1 is present in odd-numbered fatty acids (Fig. 4), suggesting that the terminal C-1 is oxidized during the formation of odd-numbered fatty acids from even-numbered palmitate. (iii) C-1, C-2 and C-16 are incorporated in similar amounts into desaturated fatty acids (Fig. 2c). This suggests that the unsaturated, straight-chain palmitic acid is merely desaturated – or elongated and desaturated – for the formation of desaturated C_{16} and C_{18} fatty acids (Fig. 5).

Position-specific ^{13}C labeling cannot distinguish whether these modifications occur as free fatty acids or bound to the PLFA. Therefore, the isotopic label in intact phospholipids and free fatty acids has to be measured at much shorter time intervals than those chosen for this study. Irrespective of the detailed biochemical mechanism, this study proved: (1) an intensive recycling of the present fatty acid pool in soils and (2) intact uptake and subsequent modification of the free fatty acids in soil. Similar results were suggested for the recycling of isoprenoid unites by marine archeal communities (Takano et al., 2010). Especially for non-growing microbial communities under maintenance conditions, like in this study (see Table 2), internal recycling of fatty acids is likely: the utilization of direct precursors helps save energy and C and is thus a biochemically preferred mechanism, at least in C limited environments like soils and sediments.

4.3. Consequences of recycling and reutilization of fatty acids in soils and sediments for biomarker applications

Whereas n-alkanes can be corrected for microbial contribution (Buggle et al., 2010; Zech et al., 2013), this step is rarely performed for fatty acids. It is unclear whether microbial enzymatic systems modifying n-alkanoic acids such as palmitate are highly specific enzymes (working only intracellularly), or whether unspecific modification of long-chain plant-derived free fatty acids may occur as well. This study cannot definitely conclude whether and to which extent potential modifications of free fatty acids other than palmitate take place and which conditions affect the rate of such transformations. It is likely that in sediments, which have a much lower microbial activity than topsoils, the microbial utilization and modification of free fatty acids is rather low and may be irrelevant compared to the microbially active topsoil used in this study. Nonetheless, most sedimentary archives contain microbial hotspots, e.g. close to roots, where microbial transformations of synsedimentary deposited fatty acids may occur (Gocke et al., 2014). Clarifying the relevance of this post-sedimentary overprint

of fatty acid patterns will require further investigations in soils and sediments by position-specific labeling of long-chain plant-derived fatty acids and deuterium- and ^{13}C-based approaches checking for intramolecular variations of the isotopic profile within fatty acids. Intensive recycling and exchange of leaf waxes, revealed by deuterium labeling, is already proven for plant waxes in living plant tissue (Gao and Huang, 2013) but remains to be investigated for plant-derived fatty acids in soils and sediments.

Position-specific palmitate labeling in this study demonstrated that medium-chain fatty acids such as palmitate are rapidly transformed and modified in soils. Pure culture studies confirm that these fatty acid modifications occur within living cells if environmental conditions (e.g. temperature) change (Aguilar et al., 1998). This calls for further information about the impact of internal fatty acid turnover to help interpret PLFA fingerprints (Frostegard et al., 2011).

PLFA are assumed to have a half-life between one day and one week (Ranneklev and Baath, 2003; Rethemeyer et al., 2004; Kindler et al., 2009). In contrast, the turnover of the bacterial microbial community in C-limited soils and sediments is assumed to occur 2–3 times per year (Moore et al., 2005; Rousk and Baath, 2007; Waring et al., 2013). However, a broad range of turnover times strongly deviating between fast-growing r-strategists and slow-growing K-strategists (Blagodatsky et al., 1994; Blagodatskaya et al., 2007) is stated and a novel dual-isotope labeling approaches based on ^{13}C-^{18}O-DNA analysis suggest deviating growth rates for individual microbial groups depending on the C supply (Mau et al., 2014). An intensive intracellular turnover of PLFA, found in this study, explains the much faster turnover of PLFA than of the entire cell. Malik et al. (2013) confirmed this conclusion, showing that the turnover of microbial biomass compounds decreased with increasing molecular size. The high reutilization and recycling of C in this study may also reflect the incubation conditions: the microbial community studied here was under maintenance conditions with rather low amounts of available C for microbial growth. Alternating environmental conditions (pH, water content,...) and especially a higher C availability may significantly affect results and can decrease recycling and increase neosynthesis of microbial fatty acids. Irrespective of substrate and incubation conditions, non-homogeneous ^{13}C incorporation into PLFA has to be considered for pulse labeling studies.

5. CONCLUSIONS

This is the first study investigating the transformation of fatty acids by soil microorganisms based on position-specific labeling. Palmitate was partially cleaved to acetyl-CoA and subsequently partially oxidized in the citric acid cycle, but was also used for the formation of microbial biomass. Transformations due to basic microbial C metabolism caused preferential incorporation of palmitate C-2 into microbial biomass, whereas palmitate C-1 was oxidized to a greater extent.

Compound-specific ^{13}C analysis of microbial PLFA revealed that palmitate was preferentially used as a precursor for PLFA formation. This demonstrated the recycling of the existing fatty acid pool in soils as intact precursors for PLFA formation. After uptake fatty acids can be incorporated unmodified or can be modified according to the fatty acid demand of the microbial community. This modification was increasingly observed from day three to day ten after palmitate application, with desaturation outpacing elongation or branching. In summary, ^{13}C labeling of palmitate provides new evidence for an intensive recycling of fatty acids, taken up intact by microorganisms, but also shows the internal transformations of these fatty acids. Such recycling and reutilization needs to be considered when interpreting microbial fatty acid profiles and their ^{13}C signature in soils. This calls for collecting further data on fatty acid recycling and on the consequences for ^{13}C and ^{2}H isotopic signatures in various soils and sediments and under various environmental conditions to take a step forward in understanding fatty acid biomarker fingerprints.

This new view on fatty acid recycling and transformations improves the interpretation of labeling experiments and microbial lipid transformations in soils. The final verification of the intact incorporation and transformations of fatty acids needs a combination of position-specific ^{13}C labeling with position-specific analysis in the metabolites.

ACKNOWLEDGEMENTS

We thank the Deutsche Forschungsgemeinschaft (DFG) for funding of KU1184/19-1 and DI2136/1-1, Stefanie Boesel from the Martin-Luther University of Halle-Wittenberg for performing the bulk isotope measurements and Michael Zech and Bjoern Buggle for their helpful discussion about fatty acid transformations in soils.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2015.10.032.

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Associate editor: Jochen J. Brocks