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Biochemical pathways of amino acids in soil: Assessment by position-specific labeling and ^{13}C-PLFA analysis

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ABSTRACT

Microbial utilization is a key transformation process of soil organic matter (SOM). For the first time, position-specific ^{13}C labeling was combined with compound-specific ^{13}C-PLFA analysis to trace metabolites of two amino acids in microbial groups and to reconstruct detailed biochemical pathways. Short-term transformation was assessed by applying position-specifically ^{13}C labeled alanine and glutamic acid to soil in a field experiment. Microbial utilization of the amino acids' functional groups was quantified by ^{13}C incorporation in total microbial biomass and in distinct microbial groups classified by ^{13}C-PLFA.

Loss from PLFAs was fastest for the highly oxidized carboxyl group of both amino acids, whereas the reduced C positions, e.g. C_{3-5}, were preferentially incorporated into microorganisms and their PLFAs. The incorporation of C from alanines' C_2 position into the cell membrane of gram negative bacteria was higher by more than one order of magnitude than into all other microbial groups. Whereas C_2 of alanine was still bound to C_3 at day 3, the C_2 and C_3 positions were partially split at day 10. In contrast, the C_2 of glutamic acid was lost faster from PLFAs of all microbial groups. The divergence index, which reflects relative incorporation of one position to the incorporation of C from all positions in a molecule, revealed that discrimination between positions is highest in the initial reactions and decreases with time.

Reconstruction of microbial transformation pathways showed that the C_2 position of alanine is lost faster than its C_3 position regardless of whether the molecule is used ana- or catabolically. Glutamic acid C_2 is incorporated into PLFAs only by two out of eight microbial groups (fungi and part of gram positive prokaryotes). Its incorporation in PLFA can only be explained by either the utilization of the glyoxolate bypass or the transformation of glutamic acid into aspartate prior to being fed into the citric acid cycle. During these pathways, no C is lost as CO_2 but neither is energy produced, making them typical C deficiency pathways. Glutamic acid is therefore a promising metabolic tracer in regard to ecophysiology of cells and therefore changing environmental conditions.

Analyzing the fate of individual C atoms by position-specific labeling allows insight into the mechanisms and kinetics of microbial utilization by various microbial groups. This approach will strongly improve our understanding of soil C fluxes.

1. Introduction

Soil organic carbon (SOC) plays a major role in the global carbon (C) cycle. The estimated soil organic C stocks of 1462–1548 Pg (Batjes, 1996) are about twice as high as in the atmosphere and three times as high as in the vegetation (IPCC, 2000). Soils can function both as a sink and a source for C, depending on climate, vegetation and management (Van Miegroet and Jandl, 2007; Vesterdal et al., 2012). Therefore it is important to understand processes that lead to C release from or sequestration in soil.

The main input of C into soils is via plant litter or rhizodeposition (Rasse et al., 2005). Litter is composed of macromolecules such as cellulose, hemicellulose, lignin or proteins (Crawford et al., 1977; Sorensen, 1975); rhizodeposition also contains those macromolecules as well as low molecular weight organic substances (LMWOS) (Farrar et al., 2001). Traditionally, the chemical properties of some of these macromolecules were thought to prevent soil biota from digesting them. This so-called "recalcitrance" should lead to an

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enrichment of those molecules in soil. Newer studies, however, have shown contrary results (Crawford et al., 1977; Grandy and Neff, 2008; Jones and Darrah, 1994; Marschner et al., 2008). In their review, Schmidt et al. (2011) combined data from 20 field experiments with durations of up to 23 years. In such experiments, presumably stable macromolecules have shown turnover rates well below that of bulk soil. In contrast, the supposedly labile products of their decomposition, such as amino acids, sugars and other LMWOS, can persist in soil for years or even decades. As the initial quality of the OM seems to have a minor effect on its persistence in soil, future research is called upon to identify the mechanisms that stabilize SOC, especially from LMWOS.

Growing evidence points to microbial incorporation and transformation as key factors in stabilizing soil organic carbon (SOC) (Koegel-Knabner, 2002). This could explain the lower-than-expected stability of macromolecules: prior to being incorporated into microbial biomass, they are split by exoenzymes and only then are the emerging LMWOS further processed (Cadisch and Giller, 1996; Kuzyakov et al., 2009). After being taken up by microorganisms, LMWOS are partly degraded and transformed to CO_2 and are thus lost from the soil C pool; the other part is transformed into microbial biomass, which is more stable to decomposition even after cell death (Six et al., 2006). Nonetheless, resource utilization by microbial groups differs with respect to uptake preferences and speed (Treonis et al., 2004) and, more importantly, stability of microbial products. Residues from gram positive bacteria can have twice the mean residence time of that from gram negative bacteria (Schmidt et al., 2011). Learning more about the means of SOC degradation and sequestration therefore requires techniques that yield information on: i) microbial groups present in the soil and ii) their metabolic processes.

To achieve the first goal, phospholipid fatty acid (PLFA) analysis has been established. It is based on the production of different PLFAs for cellular membranes by different morphological groups of microorganisms (Zelles, 1999; Zelles et al., 1995). To gain a better understanding of microbial transformations of organic matter by those microorganisms, ^{13}C- or ^{14}C-labeled substances have been applied to soil and traced in various pools (SOM, microbial biomass) and, if possible, in the released CO_2 (Evershed et al., 2006; Kuzyakov, 1997; Treonis et al., 2004). The shortcoming of uniformly labeled substances is that they do not allow distinction of individual positions of a molecule: e.g. if one third of uniformly labeled alanine is incorporated into a pool, one does not know whether the molecule has been split and all of one position was incorporated, while the other two have been mineralized or whether one third of all molecules was incorporated without having been split. By use of uniformly labeled substances only total incorporation respectively degradation of the molecule can be assumed. Nonetheless, in regard to the different oxidation states of C in organic molecules, a preferential incorporation of some positions and degradation of others is conceivable (Dijkstra et al., 2011; Fischer and Kuzyakov, 2010). This hypothesis cannot be investigated with uniformly labeled substances. Instead, position-specific labeling (^{13}C or ^{14}C) should be applied.

We used two model substances in our study: the amino acids alanine and glutamic acid. Amino acids are a source for both C and N and, as such, represent an important link between C and N cycles. The two LMWOS are also very abundant in soil; alanine accounts for about 15% and glutamic acid 10% of the amino acids recovered in DOC (Fischer et al., 2007). They are also important components of root exudates (Fischer et al., 2010).

We tested the following hypotheses:

I. Functional groups in the amino acids are utilized differently:
   a. carboxyl C is lost fastest from soil,
   b. C with lower oxidation states is preferably incorporated in microorganisms.
II. Individual microbial groups incorporate different amounts of amino acid C in their PLFA:
   a. uptake and incorporation is highest for single cell groups,
   b. filamentous groups incorporate more C from lower oxidized positions than prokaryotic, single-cell organisms.
III. The fate of individual C positions can be used for metabolic tracing, i.e. to identify different metabolic pathways among microbial groups:
   a. the ratio of ^{13}C in PLFAs and in total microbial biomass will differ among the microbial groups, reflecting C transfer to fatty acid syntheses pathways,
   b. the preference for the incorporation of C from individual amino acid positions will differ between the microbial groups, reflecting their metabolic pathways.

2. Materials and methods

2.1. Field experiment

Preliminary to this study, a number of short laboratory experiments with position-specifically ^{14}C-labeled LMWOS were conducted (Fischer and Kuzyakov 2010; Dippold and Kuzyakov, 2013). With a field experiment, we tested whether ^{13}C enrichment in various microbial and soil pools would allow metabolic tracing.

2.1.1. Sampling site

The experimental site is located on an agriculturally used loamy Luvisol in northern Bavaria (49°54′ northern latitude; 11°08′ eastern longitude, 500 a.s.l.). The last crop was *Triticale*; before application of the tracers, all above-ground biomass was removed. The mean annual temperature in the region is 7 °C, mean annual precipitation 874 mm. The soil had a pH_{KCl} of 4.88, a pH_{H_2O} of 6.49, TOC content of 1.77% and TN content of 0.19%. CEC was 13 $cmol_C\ kg^{-1}$.

2.1.2. Experiment design

The 12 × 12 m field was divided into four quadrants to allow four replications. PVC-tubes with a diameter of 10 cm and height of 13 cm were installed 10 cm deep in the soil, resulting in a soil sample weight of about 1 kg for each tube. To ease application, the soil inside each column was pierced with five wooden rods 5 days prior to applying the amino acid. A multipette (Eppendorf, Hamburg, Germany) was used to apply 10 ml tracer-solution per column with

Table 1
Concentrations of amino acid solutions for soil labeling.

	Ala–^{13}COOH[a]	Ala–$^{13}CNH_2$[a]	Ala–$^{13}CH_3$[a]	Ala–U[b]	Glu–^{13}COOH[a]	Glu–$^{13}CNH_2$[c]	Glu–U[b]
C concentration (μmol ml^{-1})	65.5	65.3	65.2	65	65.1	68.7	65
C concentration (μg g $soil^{-1}$)	0.59	0.59	0.58	0.58	0.58	0.62	0.58
Atom% ^{13}C	13.2	13.1	13.1	13.9	13.1	7.1	12.8

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concentrations of ^{13}C labeled amino acids according to Table 1. A 7-cm-long needle with lateral holes enabled homogeneous lateral distribution. Leaching was avoided by only injecting solution in the upper 2/3 of the column and blocking rainfall by installing a roof above the plot. In each of the quadrants and per each of both sampling times, alanine and glutamic acid were applied once as 1) non-labeled background (not shown in Table 1), 2) uniformly ^{13}C-labeled and 3) as two and three position-specifically ^{13}C-labeled isotopomeres of alanine and glutamic acid, respectively (see Table 1). The distribution of substances in each block was chosen randomly. The ^{13}C-content and excess ^{13}C atom-% of the residual glutamic acid molecules were calculated by subtracting measured values for the first two positions from results for the whole molecule.

2.1.3. Sampling and sample preparation

Soil was sampled 3 and 10 days after labeling. After 3 days, we expected total uptake of the amino acids (3 days corresponds to approximately 10 mean residence times for the amino acids). After 10 days, we expected incorporation of ^{13}C into PLFAs but yet no degradation of the enriched PLFAs. Both times, complete columns from one set (background, uniformly and position-specifically labeled) of four replications were dug out and the height of the soil inside the column was noted to calculate its volume. Afterward, the soil was transferred into a plastic bag and weighed; a subset was sieved to 2 mm for further analysis and stored at −20 °C for PLFA-extraction and at 5 °C for chloroform-fumigation extraction.

2.2. Analytical methods

2.2.1. Bulk soil measurements

For the analysis of bulk soil C content and $\delta^{13}C$-values, the samples were freeze dried, ground in a ball mill and 5–6 mg per sample were filled into 5 × 12 mm tin capsules (IVA, Meerbusch, Germany). The samples were measured on the Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit with a ConFlo III interface (Thermo-Fischer, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). Uptake of ^{13}C from the applied amino acids into the 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).

$$[C]_{soil} \cdot r_{soil} = [C]_{BG} \cdot r_{C-BG} + [C]_{appAA} \cdot r_{appAA} \quad (1)$$

$$[C]_{soil} = [C]_{BG} + [C]_{appAA} \quad (2)$$

with:

$[C]_{soil/BG/appAA}$ C content of sample/background/applied amino acid (mol g_{soil}^{-1})
$r_{soil/BG/appAA}$ ^{13}C atom%-excess of sample/background/applied amino acid (at%)

2.2.2. Chloroform fumigation extraction

To determine microbial C and its $\delta^{13}C$ values, two subsets of 15 g of soil were taken from each sample. One sample was directly extracted as described below; the other was first fumigated with chloroform for 5 days in an exsiccator to lyse microbial cells.

The samples were extracted twice with 22.5 ml of 0.05 M K_2SO_4. They were shaken on a horizontal shaker, 1 h on the first, 0.5 h on the second extraction. After shaking, the samples were centrifuged (10 min, 2000 rpm) and the supernatant was filtered (Rotilab® round filters, type 15A, cellulose, membrane 70 mm).

The carbon content of the K_2SO_4 extracts was measured on the TOC analyser multi C/N® 2000 (Analytik Jena, Jena, Germany). For $\delta^{13}C$ measurements, all of the remaining extracts (approx. 43 ml) were freeze-dried. A subsample of the freeze-dried crystals was transferred to 5 × 12 mm tin vessels (IVA, Meerbusch, Germany) and then measured on the Euro EA Elemental Analyser (Eurovector, Milan, Italy) unit with a ConFlo III interface (Thermo-Fischer, Bremen, Germany) and the Delta V Advantage IRMS (Thermo Fischer, Bremen, Germany). ^{13}C uptake into the microbial biomass was calculated according to the mixing model (Eqs. (1) and (2)).

2.2.3. PLFA-analysis

Phospholipids were extracted and purified by a modified method of Frostegård et al. (1991). Modifications included using 6 g of soil for extraction and eluting polar lipids four times instead of once with 5 ml of water-free methanol. Before extraction, 25 µl of a 1 M solution of the internal standard 1 (IS 1) phosphatidylcholin–dinonadecanoic acid were added. For measurements on a 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 the internal standard 2 (IS 2) tridecanoic acid methyl ester were added. External standards consisting of the 27 fatty acids given in Supplementary Table 1 and internal standard 1 were prepared with total fatty acid contents of 1, 4.5, 9, 18, 24 and 30 µg, respectively, and derivatized and measured together with the samples.

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 quantified by a linear regression calculated from the six external standards. The recovery rate for every sample was determined based on the area of the initially added 25 µg of IS 1, and applied against the quantified masses of the FAMEs.

$\delta^{13}C$-values were analyzed on a GC–C-IRMS; consisting of the autosampler unit AS 2000, the Trace GC 2000 by ThermoQuest, the combustion unit Combustion Interface III and the isotope-ratio mass spectrometer Delta^{Plus} (Thermo Finnigan, Bremen, Germany). Volumes of 1.5 µl were injected into a liner (Type TQ(CE) 3 mm ID TAPER) at a liner temperature of 250 °C, with a splitless time of 1 min. 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 $\delta^{13}C$ of the second reference gas peak was defined as −40‰ and all other $\delta^{13}C$ values were calculated by comparison. $\delta^{13}C$ of all PLFA samples was measured four times.

The chromatograms were evaluated with ISODAT NT 2.0. The $\delta^{13}C$-value in ‰ was computed from the output in the isotopic ratio $^{13}C/^{12}C$.

To correct for any drift during measurements, linear regressions were calculated from reference gas peaks two and three, and three and four. Eq. (3) was applied to the $\delta^{13}C$ value of FAMEs that were detected before reference gas peak three; Eq. (4) was applied to those that were detected after reference gas peak three.

$$C_{FAME-DK}(at\%) = C_{FAME-0}(at\%) - (m_{RG} \cdot (t_{FAME} - t_{RG})) \quad (3)$$

$$C_{FAME-DK}(at\%) = C_{FAME-0}(at\%) - (m_{RG} \cdot (t_{FAME} - t_{RG})) - \Delta C_{RG}(at\%) \quad (4)$$

with: $C_{FAME-DK}(at\%)$ drift-corrected ^{13}C amount of the FAME (at%)

$C_{FAME\text{-}0}$(at%) measured ^{13}C amount of the FAME (at%)
m_{RG} slope of regression between the reference gas peaks enveloping the FAME (s^{-1})
t_{FAME} retention time of FAME (s)
t_{RG} retention time of reference gas peak prior to FAME (s)
ΔC_{RG}(at%) difference between reference gas peaks (at%) three and two

To correct for amount-dependent ^{13}C isotopic fractionation during measurements (Schmitt et al., 2003) and for the addition of C during derivatization, linear and logarithmic regressions of the external standards $\delta^{13}C$-values to their area were calculated. If both regressions were significant, that with the higher significance was applied. As the $\delta^{13}C$-value for the derivatizating agents was unknown, the correction was performed according to Glaser and Amelung (2002) (Eq. (5)).

$$C_{FS}(at\%) = \frac{N(C)_{FAME}}{N(C)_{FS}} \cdot \left(C_{FAME-DK}(at\%) - \left(m_{lin/ln} \cdot A_{FAME} + t_{lin/ln}\right)\right) + C_{EA-FS}(at\%) \quad (5)$$

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%]

2.3. Divergence index

Discrimination of C from individual positions in one molecule during uptake and/or utilization was assessed. The extent of discrimination between pools, microbial groups and at two sampling times was compared as well. For both of these tasks, the differences in absolute uptake into C pools or microbial groups had to be relativized. Therefore, the divergence index (DI) was defined:

$$DI_i = \frac{n \cdot C_i}{\sum_1^n C_i} \quad (6)$$

with: n number of *C* atoms in molecule

C_i relative incorporation of tracer C [mol mol^{-1}]

As required, the DI can be calculated with relative incorporation of tracer per bulk soil, microbial biomass, single PLFA or ΣPLFA of microbial groups. The DI compares the calculated actual incorporation of C from each position with the mean C incorporation from all positions. This can be understood as the result the experiments would have had if uniformly labeled tracers had been used. A DI of 1 would indicate no discrimination between the positions; values above 1 indicate preferential incorporation, values below 1 show preferential degradation.

2.4. Statistical analysis

For the repetitive measurements of $\delta^{13}C$-values, a Nalimov outlier test with significance levels of 95% (when four repetitions were available) or 99% (when three repetitions were available) was performed. PLFAs were classified into corresponding microbial groups by a factor analysis of C contents of the entire dataset. Fatty acids with a loading of more than 0.5 (absolute value) on the same factor were categorized with regard to previous studies (Zelles, 1999; Zelles et al., 1995). All the data presented in this study were tested with a one-way analysis of variance (ANOVA); significances were determined with the Tukey Honest Significance Difference (Tukey HSD) post-hoc test with a significance level of 99.5%. All positions were tested for significant differences between recoveries in soil, microbial biomass and PLFAs. For every microbial group and soil pool, the difference in DI for the six position-specifically labeled positions was also tested for significance. All statistical tests were accomplished with R version 2.9.0 (17.04.2009).

Table 2
Total C content and ^{13}C incorporation of uniformly labeled amino acids into soil, microbial biomass and sum of PLFA (Σ-PLFA).

		Soil	Microbial biomass	Σ-PLFA
Total C stock (μmol g^{-1})	Day	1229 ± 240	44.0 ± 5.2	0.146 ± 0.058
Incorporation of applied	3	39.5 ± 9.1	31.6 ± 5.0	2.0 ± 0.7
Ala C (%)	10	12.8 ± 1.9	17.5 ± 6.5	3.1 ± 0.4
Incorporation of applied	3	42.4 ± 8.6	35.8 ± 6.0	1.8 ± 0.5
Glu C (%)	10	12.7 ± 4.3	3.9 ± 2.4	1.9 ± 0.6

3. Results

3.1. Incorporation of uniformly labeled amino acids

The C content in the soil was 1230 μmol g^{-1} (Table 2), which corresponds to 15.0 mg C g^{-1} soil. Of this C, 3.5% is contained in microbial biomass, and 0.01% in the sum of PLFA (Σ-PLFA). The incorporation of uniformly ^{13}C-labeled alanine and glutamic acid into soil and microbial biomass decreased between days 3 and 10. The recovery in Σ-PLFA remained stable or even increased. Recovery of applied alanine and glutamic acid C in soil decreased by about half between days 3 and 10. Recovery from applied glutamic acid in microbial biomass decreased by nearly 90% between days 3 and 10 (Table 2).

3.2. Incorporation of position-specifically labeled amino acids

With the tool of position-specific labeling, we were able to trace C from individual positions of alanine and glutamic acid into different soil C pools. On day 3 (Fig. 1, top), a clear discrimination against the carboxyl C of both amino acids and glutamic acids amino-bound position in soil, microbial biomass and Σ-PLFA is evident. On day 10 (Fig. 1, bottom), the recovery of the carboxyl C in soil remained stable, while the recovery of all other positions in soil decreased by up to 60% of applied ^{13}C. This results in an equal recovery of all positions in soil on day 10. In microbial biomass and Σ-PLFA the recovery of ^{13}C from both carboxyl groups and glutamic acids amino bound position on day 10 was still lower than the ^{13}C recovery from other positions of both amino acids. In microbial biomass, the ^{13}C recovery of glutamic acids amino-bound position decreased by 35%. The same amount of ^{13}C from glutamic acid's positions was recovered in Σ-PLFA on both days. Fig. 1 shows that on day 10, all C from the C_2 and C_3 positions of alanine in soil was located in the microbial biomass. The carboxyl C from alanine and glutamic acid, however, was stabilized in soil by other mechanisms.

To identify microbial groups, a PCA was performed on the PLFAs C-content from both sampling times. By comparing classification in the literature (Zelles, 1999; Zelles et al., 1995), the fatty acid groups were matched to microbial groups and through factor loadings they were further subdivided (Supplementary Table 3). Recovery of applied position-specifically labeled C from the two amino acids in

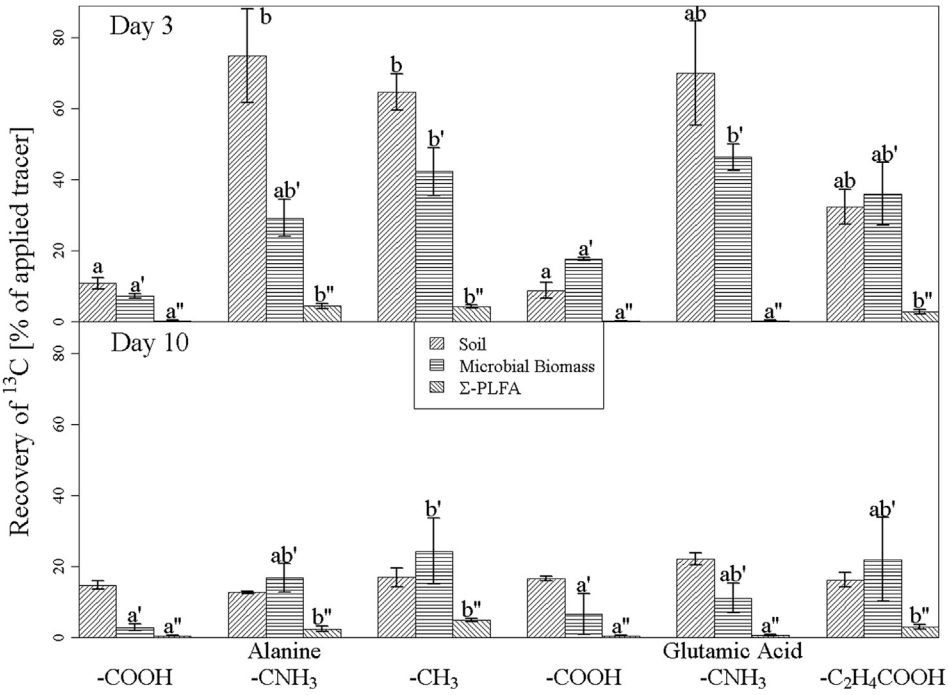

Fig. 1. Recovery of position-specifically ^{13}C labeled Ala and Glu in soil, microbial biomass and Σ-PLFA, 3 (top) and 10 days (bottom) after application. Letters indicate significant differences ($p < 0.05$) between recovery bulk soil (a), microbial biomass (a′) and Σ-PLFA (a″).

most microbial groups (Fig. 2) shows the same pattern as recovery of applied C in Σ-PLFA: The ^{13}C recovery from the carboxyl groups is less than 0.1% of ^{13}C input of both amino acids on both days. On day 3, the recoveries of the amino-bound and the methyl C from alanine were similar. This pattern was different on day 10, when the recovery of alanines amino-bound C was lower than that of its methyl group. In the first days after being taken up by microorganisms and utilized in the cell membrane, only the C_1 position was split from the alanine molecule, while the C_2 and C_3 positions were utilized together. The ^{13}C recovery of glutamic acid's positions reveals that it is transformed differently than alanine. From both the amino-bound and the carboxyl C, less than 0.4% were recovered in Σ-PLFA on both days. In contrast, nearly 4% of the residual amino acid C were recovered in Σ-PLFA. In the microbial pathways both C_1 and the C_2 from glutamic acid were split from the residual molecule, which was then incorporated into PLFAs.

The maximum incorporation of C into PLFAs from all positions of both amino acids was achieved by the group of gram negative I (18:1ω7c, 18:1ω9c) (Fig. 2). This group of gram negative prokaryotes took up 4.5–5.5% of the methyl C from alanine and also of the residual molecules C from glutamic acid. No other microbial group took up more than 2% from any position. Most prokaryotic groups incorporated more C from C_2 and C_3 positions of alanine than the anaerobic bacteria (cy17:0) and the two eukaryotic groups (Fungi (20:1ω9c, 18:2ω6,9) and VA-Mycorrhiza (16:1ω5c)).

3.3. Divergence index

The divergence index (DI) was used to compare the extent of incorporational discrimination of C from different positions between the pools (Fig. 3) and microbial groups (Fig. 4). The DI relativizes differences in absolute uptake. Regarding the DI in soil, microbial biomass and Σ-PLFA (Fig. 3), differences between days 3 and 10 after tracer application can be observed (Fig. 3). The relative incorporation in soil on day 3 shows a clear discrimination against the carboxyl positions; on day 10, there is no significant difference in DI between any position of alanine or glutamic acid. Although only the declined discrimination against alanines carboxyl C between day 3 and 10 is significant ($p < 0.05$), the reduced discrimination between all positions in soil between day 3 and day 10 shows that during the initial reactions, the pathways of C from different positions of the two amino acids differ greatly. However, ten days after application, the source position in the molecules is not determining for fixation in soil. In microbial biomass, a reduction of discrimination between positions may be taking place – the discrimination between positions is not significant anymore – but high standard errors prevent any certain conclusion. In Σ-PLFA, alanine's amino-bound position had a DI equal to that of its methyl position on day 3 but a lower DI on day 10. The incorporation pattern of glutamic acids positions into Σ-PLFA did not change between day 3 and day 10.

Despite the differences in absolute ^{13}C recovery in PLFAs between the microbial groups (Fig. 2), the DI (relative ^{13}C recovery) of most microbial groups PLFAs (Fig. 4) shows a similar pattern, which also generally reflects the pattern described by Σ-PLFA (Fig. 3). In most PLFAs, there was an average to above-average relative incorporation of the amino-bound group of alanine on day 3, which is prominent on day 10 (Fig. 4). The DI for the methyl position of alanine and the residual molecule of glutamic acid was above-average in all microbial groups and on both days. The amino-bound C of glutamic acid was incorporated less than average in all microbial groups and on both days. Exceptions to this pattern were the groups of gram positive II (i15:0, i17:0) and fungi (20:1ω9c, 18:2ω6,9), which both showed no significant discrimination against any position on either days.

4. Discussion

4.1. Incorporation of carbon from amino acids in soil and microbial biomass

On day 3, the ^{13}C recovery from alanine and glutamic acid in soil, microbial biomass and Σ-PLFA shows the same pattern (Fig. 3). C from the carboxyl group is recovered less than that from the amino-bound and methyl positions and the residual molecule of glutamic

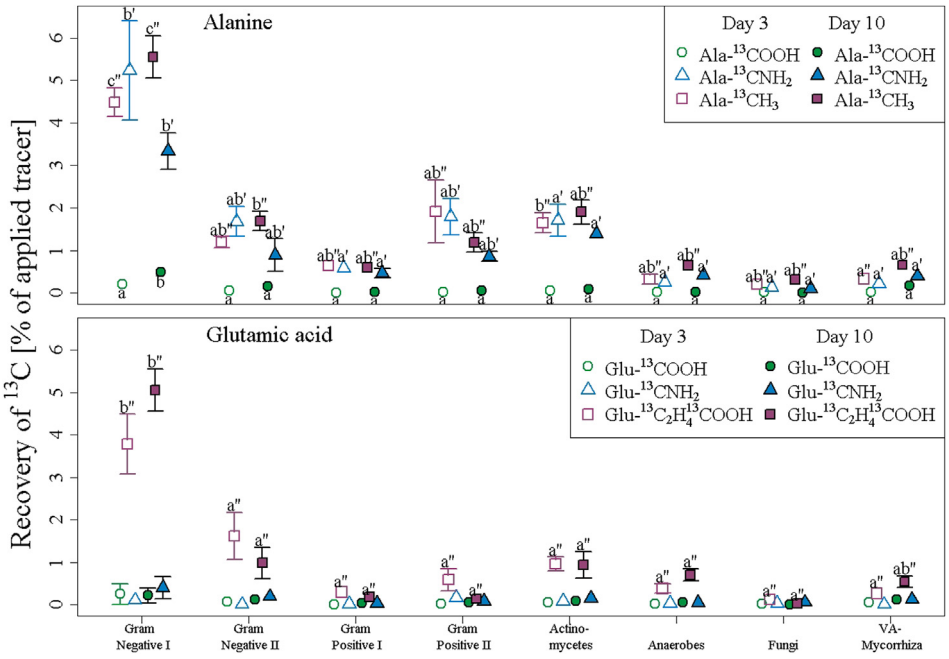

Fig. 2. Recovery of applied ^{13}C from positions of alanine (top) and glutamic acid (bottom) in microbial groups after 3 and 10 days. Letters indicate significant differences ($p < 0.05$) between carboxyl C (a), amino-bound C (a′) and methyl C of alanine or the residual molecule of glutamic acid (a″).

acid in all pools. This was expected as the carboxyl C has the highest oxidation state and is therefore most prone to being removed from the molecules. This is achieved by decarboxylation. Enzymes necessary for decarboxylation of amino acids have been found in soil (Braun et al., 1992; Tena et al., 1986) as well as in prokaryotic and eukaryotic microorganisms (Caspi et al., 2008). On day 10, the amount of carboxyl C from both amino acids in soil remained stable, while the recovery of all other positions decreased. Although it seems contradictory this can also be explained by the high reactivity of the carboxyl C: not only can it be oxidized to CO_2 easily, but it can also react with other soil components and be thus stabilized. This possible stabilization mechanism is supported by results of Kuzyakov (1997), who found position-specifically labeled ^{14}C from the carboxyl position of alanine in humic and fulvic acids. As complex macromolecules, humic and fulvic acids contain a variety of functional groups such as hydroxyl groups, methylenes, ethers and esters in aliphatic chains (Simpson et al., 2002). It is possible for carboxyl C from microbial sources to react with humic macromolecules, e.g. by forming ester-linkages with hydroxy groups. Esters are highly inert, therefore the former carboxyl C will be stabilized from further degradation.

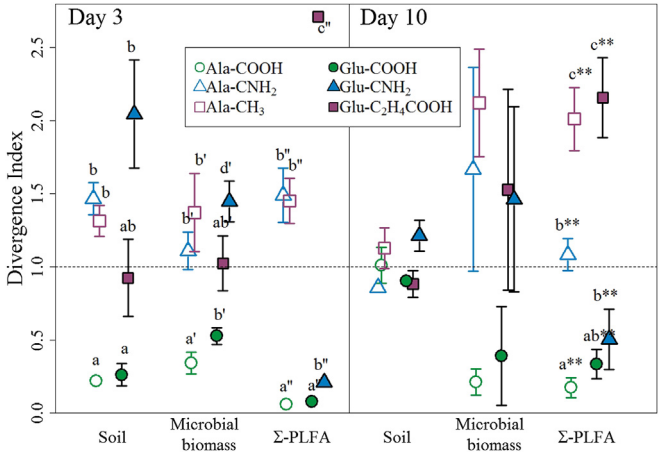

Fig. 3. Divergence index (DI) reflecting incorporational discrimination between C positions into soil, microbial biomass and Σ-PLFA, 3 (left) and 10 (right) days after applying ^{13}C-labeled alanine (Ala) and glutamic acid (Glu). Letters indicate significant differences ($p < 0.05$) in the relative incorporation of the C positions into soil (a), microbial biomass (a′), Σ-PLFA (a″) on day 3, and into Σ-PLFA (a**) on day 10 after tracer application.

In contrast to carboxyl C, the ^{13}C recovery from the amino-bound and methyl group of alanine in soil decreased by up to 60% between days 3 and 10. Compared to the decrease in recovery of these positions in soil, the amount incorporated into microbial biomass is still high on day 10. In microorganisms, alanine can be used catabolically, in the citric acid cycle and anabolically, e.g. to produce sugars or fatty acids (Fig. 5) (Caspi et al., 2008). The first reactions for both pathways are the same. Alanine is first deaminated and then decarboxylated, thereafter the resulting acetyl reacts with coenzyme A to form acetyl-CoA. The acetyl-CoA, which consists of the former amino-bound and methyl C from alanine, is then fed into the citric acid cycle or used for biosynthesis. This explains why C from those two positions is recovered in PLFAs, but C from the carboxyl group is not. After incorporation into PLFAs, C from the former amino-bound position is on the terminal position and thus most prone to being oxidized and decarboxylated (Caspi et al., 2008). This process is hinted at by the slight decrease in relative incorporation of alanines amino-bound position between day 3 and 10. The incorporation of the C from the methyl position of alanine and from the residual molecule of glutamic acid in Σ-PLFAs is still high on day 10.

4.2. Incorporation of tracer into the microbial groups

The ^{13}C incorporation into PLFAs of microbial groups differed by more than one order of magnitude (Fig. 2). As hypothesized, the highest incorporation, with more than 5% ^{13}C uptake, was recorded for a group of gram negative prokaryotes (gram negative I). This fits well with the observations by Griffiths et al. (1999) that gram

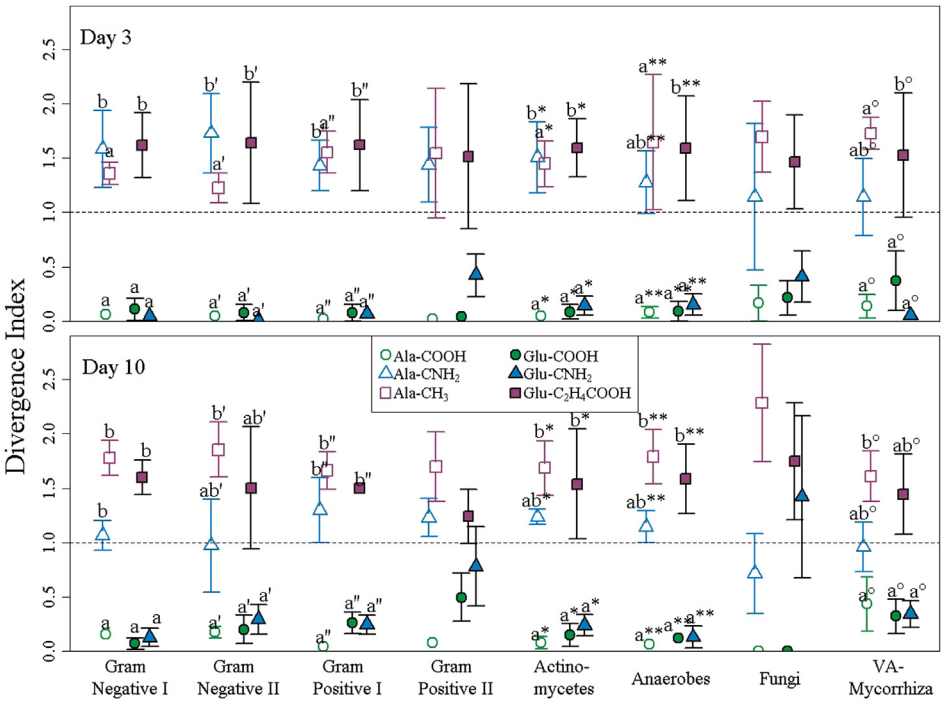

Fig. 4. Divergence Index (DI), reflecting discrimination between C positions of alanine (Ala) and glutamic acid (Glu), 3 (top) and 10 (bottom) days after application. Letters indicate significant differences ($p < 0.05$) between the relative incorporation of the C positions into the microbial group a: gram negative I, a′: gram negative II, a″: gram positive I, a*: actinomycetes, a°: VA-mycorrhiza.

negatives react fastest to addition of LMWOS, which gives them a competitive advantage.

Three other prokaryotic groups (gram negatives II, actinomycetes and gram positive II), also achieved moderate ^{13}C incorporation. The two eukaryotic groups – fungi and VA-mycorrhiza – were unable to take up as much of the applied amino acid C as the prokaryotic group. This is unsurprising because the turnover of the larger, more complex eukaryotes' biomass is slower than that of prokaryotes' (Bååth, 1998; Rousk and Bååth, 2007). Accordingly, enrichment of eukaryotic cell components takes longer (Moore et al., 2005). Apart from a slower turnover, the larger size of eukaryotic cells results in a smaller ratio of surface to volume. As PLFAs are utilized as cell membranes on the surface of the organism and the difference in the ratios of ^{13}C in PLFA to ^{13}C in microbial biomass for various microbial groups is unknown, there is no full comparability between cells of different size. It is also well known that fungi are specialized on more complex substrate than LMWOS.

As in the eukaryotes, the anaerobic bacteria also incorporate only a maximum of 0.7% of the applied C. As the roof we installed prevented excess wetting, the soil was well aerated, so the anaerobic microorganisms could only persist inside anaerobic microhabitats such as microaggregates. Thus, only ^{13}C that permeated into those anaerobic microhabitats could be taken up by anaerobic microorganisms.

4.3. Discrimination of individual carbon positions by microbial utilization differs depending on oxidation state, amino acid and time

As in soil and microbial biomass, discrimination of the individual C positions of both amino acids also took place in the microbial PLFA. As the percent of ^{13}C recovery (Fig. 2) between the microbial groups' PLFA differs greatly, the discrimination between the positions of alanine and glutamic acid is best evaluated with the DI (Fig. 4).

On day 3, there was no difference in relative incorporation of ^{13}C from the methyl and amino-bound C of alanine for most microbial groups. Nearly no ^{13}C from alanine's carboxyl group was recovered in the PLFAs and the incorporation of alanine's ^{13}C into microbial biomass is much lower than that of its amino-bound and methyl position. Accordingly, we can conclude that during the three days after applying the amino acid, the C_1 atom in alanine is split from the molecule quickly, whereas C_2 and C_3 remain bonded. Presumably, the alanine molecule is taken up and then metabolized in the main alanine utilization pathway: deamination to pyruvate and after decarboxylation by pyruvate dehydrogenase, transformation to acetyl-CoA (Fig. 5a). This molecule then either enters the citric acid cycle (de Kok et al., 1998) or fatty acid synthesis (Caspi et al., 2008). On day 10, the DI of the amino-bound C is slightly lower than that of alanine's methyl C in most microbial groups, which can be explained by the further reactions in microorganisms: If the molecule is used catabolically in the citric acid cycle, then the acetyl-CoA condensates with oxalate to citric acid. After this reaction, the former amino-bound C of alanine is one of citric acid's carboxyl groups. Thus, the chance for the amino-bound position to be degraded into CO_2 during the next step – the formation of 2-oxoglutarate (Camacho et al., 1995) – is about 1:3, whereas the methyl position is still incorporated in the non-reactive chain. After every circuit of the citric acid cycle, C from the alanine molecule can either be transferred to a biosynthesis pathway or continue partaking in this cycle (Caspi et al., 2008). We detected ^{13}C in the extracted PLFAs. Thus, the alanine molecules were fed either into the fatty acid biosynthesis pathway or the fatty acid elongation pathway. The initial substance for both these pathways is also acetyl-CoA. As in the citric acid cycle, the former amino-bound C will be the terminal C on the fatty acid molecule and is thus more prone to being degraded than the former methyl C (Caspi et al., 2008). In summary, regardless of whether alanine is used anabolically or catabolically, the former amino-bound C will be degraded before the former methyl C (Fig. 5a).

The DI of glutamic acid shows that it is transformed in different pathways than alanine (Fig. 5b, c and d). We find a discrimination

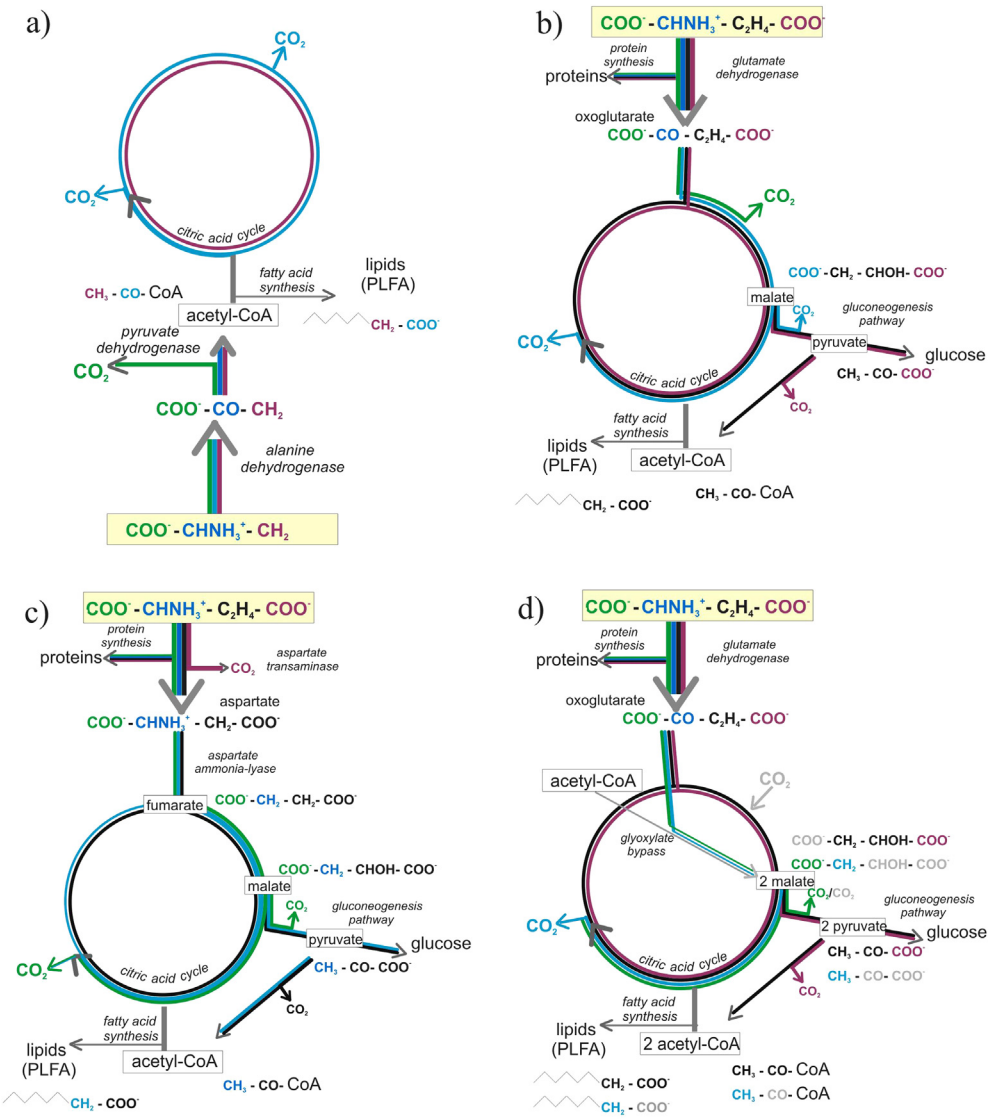

Fig. 5. Microbial transformation pathways of alanine (a) and glutamic acid (b, c, d). As there are different transformation pathways for glutamic acid, it is presented in 3 subfigures. The entrance of alanine (a) occurs from the bottom (in contrast to glutamic acid, b, c, d) of the citric acid cycle because of its initial transformation to acetyl-CoA.

against C from both the carboxyl and the amino-bound position, which means that either C_1 and C_2 were split from the residual molecule together or in short succession. Glutamic acid most commonly enters the citric acid cycle after being transformed into oxoglutarate (Caspi et al., 2008). Oxoglutarate has the original five atoms in its chain and loses the carboxyl group immediately after entering the citric acid cycle. This transformation does not yet explain why the amino-bound C has a DI as low as the carboxyl group. One possible explanation is that the metabolization of the former glutamic acid molecule is so fast that, after three days, the citric acid cycle has already removed most of the formerly amino-bound C. However, the DI of microbial biomass (Fig. 3) does not shows a discrimination against glutamic acid's amino-bound C. If glutamic acids amino-bound positions would be solely lost by repetitive oxidation in the citric acid cycle, we would not only see a discrimination in PLFA but also in overall microbial biomass. Therefore, the explanation for the less than average incorporation of glutamic acids amino-bound position might be found by tracing the anabolic pathway that leads to fatty acid formation in microbial cells. As opposed to alanine, glutamic acid is not transformed into acetyl-CoA (the starting substance for fatty acid synthesis) before it is fed into the citric acid cycle. But in that cycle, glutamate is transformed into malate, which can be fed into the gluconeogenesis pathway, producing sugars and other anabolic products from non-sugar substrates (Caspi et al., 2008; Katz and Tayek, 1999). One of the intermediaries in this pathway is pyruvate, which can be transformed into acetyl-CoA. Following these transformations, the acetyl-CoA molecule will consist of two of glutamic acid residual Cs (Fig. 5b).

In contrast to alanine, the DI for glutamic acid's amino-bound C is not convergent for all microorganisms: the groups gram positives II and fungi show specific incorporation patterns. The difference between glutamic acid's amino-bound position and its methyl position is not significant. Following from the aforementioned transformation pathways of glutamic acid, it is impossible for the amino-bound C to be incorporated into microbial PLFAs, so there should be a significant difference between the amino-bound and methyl C. Detection of this position in the PLFAs can be only be explained by the use of alternative pathways. Two pathways for glutamic acid utilization exist: aspartate production from glutamic acid prior to the citric acid cycle (Fig. 5c) and the glyoxylate bypass (Fig. 5d). Glutamic acid is transformed into aspartate by removing

the C_5 position; thereafter, the aspartate is deaminated and fed into the citric acid cycle. The glyoxylate bypass avoids the exhaustion of CO_2. This yields two instead of one malate molecules, but will produce no energy. The glyoxylate bypass in the citric acid cycle is used by bacteria, and its enzymes have also been found in fungi (Maxwell et al., 1977; Munir et al., 2001). Again, for fatty acid production, pyruvate has to be produced by the gluconeogensis pathway. In contrast to the "common" pathway mentioned above, glutamic acid's former amino-bound position will remain in the molecule. Therefore, both the production of aspartate and the utilization of the glyoxylate bypass can explain why we find no significant difference between the amino-bound and methyl C in the PLFAs of gram positives II and fungi (Fig. 5c and d) (Caspi et al., 2008).

As mentioned above, the bypass produces no energy and is thus only relevant at C deficiency conditions. This indicates that the gram positives II and fungi might be suffering from C deficiency and need to utilize specific pathways to meet their anabolic demands. Such groups might be of special interest when environmental conditions change, especially an altered C input.

5. Conclusions

This study has shown that position-specific ^{13}C labeling and compound-specific ^{13}C-PLFA analysis are a valuable combination to gain new insights into microbiological transformations of amino acids in soil. As hypothesized, the carboxyl C of both amino acids is oxidized rapidly by microorganisms. Methyl C from alanine and glutamic acid residual molecules showed high recoveries in all microbial groups 10 days after the application. While functional group and oxidation state help to predict the incorporational behavior for carboxyl, methyl and residual positions, the amino-bound C from two amino acids is transformed differently. C_2 from alanine is incorporated like its methyl C on day 3, but its recovery decreased slightly on day 10. The DI revealed that, although C_2 from glutamic acid is lost from most microbial groups, gram positives II and fungi incorporate it into their PLFA. This was explained by special microbiological pathways – the glyoxolate bypass and the transformation of glutamic acid into aspartate prior to being fed into the citric acid cycle – used under C deficiency. As glutamic acid has proven to be a sensitive tracer for environmental conditions, it could be applied to observe metabolic changes under environmental gradients.

None of these findings could have been achieved without using position-specifically labeled substances. The method of coupled position-specific ^{13}C labeling and compound-specific isotope analysis can in the future be further expanded to investigate pathways of other microbial or soil constituents, including other amino acids and amino sugars, carboxyl acids, sugars, humic and fulvic acids. This would help to identify further transformation and stabilization processes and improve our knowledge about soil C fluxes.

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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.2013.08.005.

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