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Review

^{13}C fractionation at the root–microorganisms–soil interface: A review and outlook for partitioning studies

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ABSTRACT

Natural variations of the $^{13}C/^{12}C$ ratio have been frequently used over the last three decades to trace C sources and fluxes between plants, microorganisms, and soil. Many of these studies have used the natural-^{13}C-labelling approach, i.e. natural $\delta^{13}C$ variation after C_3–C_4 vegetation changes. In this review, we focus on ^{13}C fractionation in main processes at the interface between roots, microorganisms, and soil: root respiration, microbial respiration, formation of dissolved organic carbon, as well as microbial uptake and utilization of soil organic matter (SOM). Based on literature data and our own studies, we estimated that, on average, the roots of C_3 and C_4 plants are ^{13}C enriched compared to shoots by $+1.2 \pm 0.6‰$ and $+0.3 \pm 0.4‰$, respectively. The CO_2 released by root respiration was ^{13}C depleted by about $-2.1 \pm 2.2‰$ for C_3 plants and $-1.3 \pm 2.4‰$ for C_4 plants compared to root tissue. However, only a very few studies investigated ^{13}C fractionation by root respiration. This urgently calls for further research. In soils developed under C_3 vegetation, the microbial biomass was ^{13}C enriched by $+1.2 \pm 2.6‰$ and microbial CO_2 was also ^{13}C enriched by $+0.7 \pm 2.8‰$ compared to SOM. This discrimination pattern suggests preferential utilization of ^{13}C-enriched substances by microorganisms, but a respiration of lighter compounds from this fraction. The $\delta^{13}C$ signature of the microbial pool is composed of metabolically active and dormant microorganisms; the respired CO_2, however, derives mainly from active organisms. This discrepancy and the preferential substrate utilization explain the $\delta^{13}C$ differences between microorganisms and CO_2 by an 'apparent' ^{13}C discrimination. Preferential consumption of easily decomposable substrates and less negative $\delta^{13}C$ values were common for substances with low C/N ratios. Preferential substrate utilization was more important for C_3 soils because, in C_4 soils, microbial respiration strictly followed kinetics, i.e. microorganisms incorporated heavier C ($\Delta = +1.1‰$) and respired lighter C ($\Delta = -1.1‰$) than SOM. Temperature and precipitation had no significant effect on the ^{13}C fractionation in these processes in C_3 soils. Increasing temperature and decreasing precipitation led, however, to increasing $\delta^{13}C$ of soil C pools.

Based on these ^{13}C fractionations we developed a number of consequences for C partitioning studies using ^{13}C natural abundance. In the framework of standard isotope mixing models, we calculated CO_2 partitioning using the natural-^{13}C-labelling approach at a vegetation change from C_3 to C_4 plants assuming a root-derived fraction between 0% and 100% to total soil CO_2. Disregarding any ^{13}C fractionation processes, the calculated results deviated by up to 10% from the assumed fractions. Accounting for ^{13}C fractionation in the standard deviations of the C_4 source and the mixing pool did not improve the exactness of the partitioning results; rather, it doubled the standard errors of the CO_2 pools. Including ^{13}C fractionations directly into the mass balance equations reproduced the assumed CO_2 partitioning exactly. At the end, we therefore give recommendations on how to consider ^{13}C fractionations in research on carbon flows between plants, microorganisms, and soil.

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1. Introduction: the relevance of ^{13}C fractionation to root–microorganisms–soil interfaces

In the last three decades, a strong research interest has arisen to trace soil carbon (C) inputs and outputs. Besides artificial ^{14}C and ^{13}C labelling, the natural variation of the $^{13}C/^{12}C$ ratio in various terrestrial pools has often been used in C budgeting and C flow studies as

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well as in investigations into tracing C sources (see Meharg, 1994; Hanson et al., 2000; Ehleringer et al., 2000; Dawson et al., 2002; Hobbie and Werner, 2004; Kuzyakov and Larionova, 2005; Glaser, 2005; Subke et al., 2006; Bowling et al., 2008; Morgun et al., 2008; Amelung et al., 2008; Paterson et al., 2009 and others for further review). Those studies include C_3- to C_4-vegetation change or *vice versa*, decomposition studies with C_4-plant residues, incubation of naturally labelled compounds (i.e. sucrose or glucose originating from sugar cane or sugar beet), and maize-slurry applications, but also Free Air Carbon dioxide Enrichment (FACE), tree canopy labelling, continuous labelling by slightly enriched or depleted $^{13}CO_2$, etc. The most important processes involved in such studies are: root respiration, rhizodeposition, microbial uptake of plant compounds, microbial respiration, humification or stabilization of organic compounds, and some other indirectly relevant processes such as assimilate transport from shoots to roots.

Most chemical and biochemical processes favour the initial incorporation of the lighter isotope in the product, leaving the substrate enriched in the heavy isotope. This preference of one isotope in reactions is called 'isotopic effect'. It leads to differences between the isotopic composition of substrates and products (Högberg, 1997). The intensity of the isotopic effect is termed 'isotopic fractionation'. The magnitude of isotopic fractionation differs for various processes and depends on the specific reaction mechanism. In biological systems, isotope fractionation is also called 'discrimination' because specific enzymes discriminate against the heavier and favour the lighter isotope (Dawson et al., 2002). Thus, in studies based on ^{13}C natural abundance isotopic fractionation should be considered when calculating C partitioning ratios, C fluxes, and C budgeting.

Our review is focussed on ^{13}C fractionation by biotic processes during C flow from plant roots or plant residues to soil microorganisms and from soil organic matter to CO_2. Here, we do not review ^{13}C fractionation by photosynthesis and post-photosynthetic metabolic processes or by abiotic processes such as CO_2 diffusion through soil profiles, dissolution of CO_2 in soil water, carbonate precipitation, etc. These processes have been excellently reviewed by O'Leary (1981), Dawson et al. (2002), Hobbie and Werner (2004), and Morgun et al. (2008).

Our aim is to evaluate the most important fractionation processes at the interface between roots, microorganisms, and soil and to work out the consequences for studies based on small variations of the $^{13}C/^{12}C$ ratio (i.e. ^{13}C natural abundance), especially carbon partitioning studies. In this compilation we only review ^{13}C discrimination in processes under oxic conditions. ^{13}C discrimination under O_2 limitation contributing e.g. to the ^{13}C depletion in methane production was described by Conrad (2005).

2. Background

2.1. Definitions

Carbon has three naturally occurring isotopes (^{12}C, ^{13}C, and ^{14}C). ^{12}C and ^{13}C are stable C isotopes, whereas ^{14}C is radioactive. Their natural abundances are ca. 98.89% for ^{12}C, 1.11% for ^{13}C (Boutton, 1991a), and $<10^{-10}$% for ^{14}C (Goh, 1991) of the total carbon content in natural pools (air, plants, soil, etc.). Since the absolute variation in the natural stable carbon-isotope ratio R ($=^{13}C/^{12}C$) is small, sample C isotope ratios R_{sample} are expressed relative to the international PDB limestone standard as $\delta^{13}C$:

$$\delta^{13}C = \frac{R_{sample} - R_{PDB}}{R_{PDB}} 1000‰, \quad (1)$$

where R_{PDB} is the isotope ratio of the limestone fossil *Belemnitella americana* from the Cretaceous PeeDee Formation in South Carolina, which is set to $\delta^{13}C = 0‰$ as zero point reference. It has an absolute $^{13}C/^{12}C$ ratio of 0.0112372 (Craig, 1953).

Due to isotope effects during chemical reactions, isotopic fractionation occurs between a substrate ($R_{substrate}$) and a product ($R_{product}$) pool. This isotopic fractionation α is defined as:

$$\alpha = \frac{R_{substrate}}{R_{product}}. \quad (2)$$

For convenience, isotopic fractionations are more commonly reported as discrimination values Δ in ‰. α is related to Δ by:

$$\Delta = \alpha - 1. \quad (3)$$

These fractionations between a substrate and a product can be related to isotopic compositions through the following equation:

$$\Delta = \frac{\delta_{substrate} - \delta_{product}}{1 + \delta_{product}}, \quad (4)$$

where $\delta_{substrate}$ is the $\delta^{13}C$ value of the source and $\delta_{product}$ is the $\delta^{13}C$ value of the product (Lajtha and Michener, 1994). Since the denominator is mostly very close to 1, the simplified equation

$$\Delta \approx \delta_{substrate} - \delta_{product} \quad (5)$$

can also be used. Exact ^{13}C fractionations have to be determined in a single chemical reaction considering the $\delta^{13}C$ values of the substrates and products (Hobbie and Werner, 2004). In root respiration, for example, this consideration would include $\delta^{13}C$ values of the sugars involved in respiration for $\delta_{substrate}$ and of the respired CO_2 for $\delta_{product}$.

Most processes in the rhizosphere involve numerous individual reactions for which the determination of $\delta_{substrate}$ and $\delta_{product}$ of single compounds is hardly possible. Rhizosphere-related studies therefore tend to consider $\delta^{13}C$ values of bulk roots, soil organic matter (SOM), and/or microbial biomass instead of single compounds. It has to be noticed, however, that differences in $\delta^{13}C$ values between these bulk materials and the emitted CO_2 reflect various transformation processes. They involve their unique isotopic fractionations caused by biologically preferred utilization of ^{13}C-enriched (or -depleted) compounds and chemically faster or more slowly reacting isotopes (kinetic isotope effect). Hence, another measure often used is simply the isotopic difference between two pools, e.g. bulk roots and root respiration, defined as:

$$\Delta = \delta_{pool\ 1} - \delta_{pool\ 2} \quad (6)$$

According to Eq. (6), we will refer to positive Δ values (e.g. $\Delta = +3‰$) as ^{13}C enrichment of the considered pool (pool 1 for example is CO_2) compared to the source pool (pool 2 for example is roots), and to negative Δ values as ^{13}C depletion. This is in contrast to Eq. (5), where the source pool would be expressed by $\delta_{substrate}$ (i.e. by $\delta_{pool\ 1}$ − and not by $\delta_{pool\ 2}$ − as equivalent in Eq. (6)), but makes the fractionation processes in their description clearer.

2.2. Discrimination within the plants

Discriminations by the three photosynthesis pathways have been described in detail in many reviews (e.g. O'Leary, 1981; Farquhar et al., 1989) and books and are out of scope of this review. As the ^{13}C fractionation by C_3, C_4, and CAM photosynthesis provides the background for fractionation in further processes at the root–microorganisms–soil interface, we shortly repeat it here.

The $^{13}C/^{12}C$ ratio of organic carbon in terrestrial ecosystems is mainly influenced by the C isotope fractionation occurring during

photosynthesis (Wolf et al., 1994). Thus, when considering photosynthesis processes, Eq. (4) becomes:

$$\Delta = \frac{\delta_{CO_2} - \delta_{leaf}}{1 + \delta_{leaf}}. \quad (7)$$

Plants with the C_3 photosynthetic (Calvin–Benson) pathway have $\delta^{13}C$ values between −22 and −32‰ with an average of −27‰ (Boutton, 1996). C_4 plants discriminate less against $^{13}CO_2$ due to the pre-fixation of CO_2 in the Hatch–Slack pathway. Thus, $\delta^{13}C$ values of C_4 plants range from −9 to −17‰ with an average of −13‰ (Boutton, 1996). Some plants with the Crassulacean acid metabolism (CAM) are able to switch between those two photosynthetic pathways and consequently their $\delta^{13}C$ values range from −10 to −28‰ (Boutton, 1996). C_3-plant species dominate most temperate zone and all forest communities. C_4-plant species as well as CAM plants are more common in climates and locations where transpiration is reduced: arid, semiarid, or salty environments, where water availability limits photosynthesis (Ehleringer, 1991; Boutton, 1991b).

Once C_3 or C_4 plants have assimilated carbon with their typical discrimination against ^{13}C, further fractionation processes take place. Within plants, the $\delta^{13}C$ values of different compounds vary. It has been observed that lignin and lipids are usually ^{13}C depleted compared to the bulk plant material, while sugars, amino acids, and hemicelluloses are ^{13}C enriched (Boutton, 1996; Hobbie and Werner, 2004; Wiesenberg et al., 2004, 2008; Bowling et al., 2008). The specific enrichment of ^{13}C in transport compounds like sucrose leads to an enrichment of ^{13}C in the roots (Hobbie and Werner, 2004). Therefore, the discrimination Δ between shoots and roots is mostly positive (Table 1). On average, roots are enriched by +1.2‰ compared to shoots for C_3 plants and by +0.3‰ for C_4 plants. However, Table 1 presents only those studies that measured not only the $\delta^{13}C$ values of shoots and roots, but also the $\delta^{13}C$ of CO_2 respired by roots. A much larger number of studies on $\delta^{13}C$ values of C_3 plants,

Table 1
^{13}C fractionation between shoots and roots and between roots and CO_2 from root respiration.

Plant	Vegetation type	Age	Δ (roots–shoots)[a] ‰	Δ (CO_2-roots)[a] ‰	CO_2 sampling[b]	Reference (experimental set-up for CO_2 sampling)
Eucalyptus delegatensis	C_3	<90-yr	n.d.	+0.7 to +3.1	DGS	Gessler et al., 2007 (excavated roots)
Sunflower						
Low density stand	C_3	3 to 5-wk	+0.8 ± 0.2	−0.5 ± 0.4	DGS	Klumpp et al., 2005
High density stand	C_3	4 to 5-wk	+0.9 ± 0.1	−2.0 ± 0.3	DGS	(roots in quartz sand with nutrient solution)
Alfalfa						
Low light pretreatment	C_3	2 to 4-wk	+1.6 ± 0.3	−3.0 ± 0.9	DGS	
High light pretreatment	C_3	after plot assembling	+1.0 ± 0.2	−1.5 ± 0.3	DGS	
High light/high nitrogen	C_3	8-wk	+1.0 ± 0.2	−3.7 ± 0.4	DGS	
High light/low nitrogen	C_3	8-wk	+1.0 ± 0.4	−2.8 ± 0.4	DGS	
Low light/high nitrogen	C_3	8-wk	+1.4 ± 0.1	−2.4 ± 1.2	DGS	
Low light/low nitrogen	C_3	8-wk	+1.2 ± 0.1	−2.7 ± 0.6	DGS	
Perennial ryegrass	C_3	9-wk	+1.5 ± 0.5	−5.4 ± 0.2	DGS	
Wheat	C_3	27-d	+2.3	n.d.	none	Larsen et al., 2007 [c]
Wheat	C_3	56-d	+2.1	n.d.	none	
Wheat	C_3	84-d	+1.8	n.d.	none	
Lolium perenne						
25 °C/23 °C day/night	C_3	8 to 10-wk	+2.2	−3.2	DGS	Schnyder and Lattanzi, 2005 [c]
15 °C/14 °C day/night	C_3	8 to 10-wk	+0.5	−5.6	DGS	(roots in quartz sand with nutrient solution)
Paspalum dilatatum						
25 °C/23 °C day/night	C_4	8 to 10-wk	−0.2	−0.8	DGS	
15 °C/14 °C day/night	C_4	8 to 10-wk	+0.9	−5.5	DGS	
Agropyron repens	C_3	n.d.	+1.2	n.d.	none	Wedin et al., 1995
Poa pratensis	C_3	n.d.	+0.7	n.d.	none	
Agrostis scabra	C_3	n.d.	−0.1	n.d.	none	
Schizachyrium scoparium	C_4	n.d.	+0.4	n.d.	none	
Halimium halimifolium	C3	n.d.	n.d.	-2.4	DGS	Wegener et al. 2010
Melissa officinalis	C3	n.d.	n.d.	-0.2	DGS	(roots in nutrient solution)
Maize high nutrients	C_4	29-d	+0.3 ± 0.1	−0.3 ± 0.2	NaOH	Werth and Kuzyakov, 2006
Maize low nutrients	C_4	29-d	+0.3 ± 0.1	+0.2 ± 0.5	NaOH	(roots in nutrient solution)
Maize no nutrients	C_4	29-d	+0.6 ± 0.2	−0.2 ± 0.5	NaOH	
Maize	C_4	16-d	+0.2 ± 0.2	n.d.	none	Werth et al., 2006
Maize	C_4	22-d	+0.1 ± 0.4	n.d.	none	
Maize	C_4	28-d	+0.2 ± 0.1	n.d.	none	
Maize	C_4	34-d	−0.1 ± 0.1	n.d.	none	
Maize	C_4	40-d	0 ± 0.1	n.d.	none	
Maize	C_4	mean	0 ± 0.1	n.d.	none	
Maize	C_4	124-d	+0.9 ± 0.1	n.d.	none	Werth and Kuzyakov, 2009
Means						
C_3			+1.2 ± 0.6	−2.1 ± 2.2		
C_4			+0.3 ± 0.4	−1.3 ± 2.0		

[a] Discrimination was calculated by the equations: $\Delta = \delta(\text{roots}) - \delta(\text{shoots})$ and $\Delta = \delta(CO_2) - (\text{roots})$.
[b] Gas sampling method: DGS: direct gas sampling, NaOH: CO_2 in NaOH.
[c] Data obtained from diagrams.

reviewed by Bowling et al. (2008), showed an average enrichment between bulk leaves and roots of about +2.3‰. However, this higher enrichment compared to our review partly reflects the fact that we presented discrimination between whole shoots and roots of mainly gramineous plants (Table 1), whereas Bowling et al. (2008) reviewed discrimination between leaves and roots mainly of trees. This variation in ^{13}C fractionation for various plants and environmental conditions (Table 1) clearly shows that, for rhizosphere and at least some SOM studies, it is insufficient to analyse $\delta^{13}C$ solely of shoots or leaves and to equalise it to the $\delta^{13}C$ of roots. This is especially important when SOC is mainly C derived from roots (Rasse et al., 2005) and rhizodeposits (Kuzyakov and Domanski, 2000) and not from the aboveground plant parts.

Despite small differences between $\delta^{13}C$ of shoots and roots (about 1–2‰), the isotopic composition of soil organic matter largely reflects the photosynthetic pathway type of the vegetation growing on a certain soil for a long period. Changes from an initial C_3 vegetation to a C_4 vegetation or *vice versa* can hence be used as a 'natural-^{13}C-labelling technique' (Balesdent and Mariotti, 1996). In this technique, the isotopic composition of the new source vegetation acts as a continuous carbon tracer when introduced to an SOM pool or to a belowground CO_2 flux derived from the old vegetation with a different isotopic signature. In the following, we will refer to soils originally developed under C_3 or C_4 plants as 'C_3 soils' or 'C_4 soils', respectively.

2.3. Preferential substrate utilization and preferential decomposition

Inputs of organic substances into the soil occur mainly in the form of a broad mixture of very complex substances. Such mixtures are common on different levels: (1) on the ecosystem level, as various plants may contribute to the C input (common in natural ecosystems and uncommon in intensive agriculture), (2) on the whole plant level, as various plant organs have different chemical compositions, (3) on the plant organs level, as cells with different functions are composed of different substances, (4) on the cell level, as chemical compositions of cell organelles differ, (5) on the cell organelles level, as they consist of different chemical substances, and (6) on the molecular level, as individual C atoms in one molecule differ in their isotopic signature. This very complex nature of plant C input has consequences for the application of ^{13}C natural abundance techniques, especially for partitioning of C pools or fluxes if the utilization of individual pools (or substances) is different.

We will use the terms 'preferential substrate utilization' or 'preferential decomposition' if individual substances in plant residues (or any other complex substrates, e.g. rhizodeposits) with a specific isotopic signature are preferred by microorganisms and decomposed to CO_2. This means that, after the input, some substances will be utilized and decomposed earlier and/or faster than others. The term 'preferential substrate utilization' has been frequently used in studies on rhizosphere priming effects (Kuzyakov, 2002; Kuzyakov and Bol, 2006), where it has been applied to easily decomposable substances like glucose or sucrose. Since these are ^{13}C enriched in contrast to lignin or lipids, we use this term here in a sense of 'preferential degradation of substrates enriched in ^{13}C' (Cotrufo et al., 2005). Other terms such as 'selective use of organic compounds' (Šantrůčková et al., 2000a) or 'differential decomposition' (Feng, 2002) have also been suggested but will not be further used here.

This review shows that the $\delta^{13}C$ values between above and belowground plant biomass differ by +1.2‰ (from +2.3 to +0.5‰) for C_3 plants and by +0.3‰ (from +0.9 to −0.2‰) for C_4 plants (Table 1). Hobbie and Werner (2004) reported that, within a single plant, differences in isotopic signatures of individual substances and substance classes can reach up to 9‰ for C_3 plants and up to 10.3‰ for C_4 plants. After microbial uptake, these differences in isotopic signatures between individual plant organs (Table 1) and between individual substances (Hobbie and Werner, 2004) may strongly affect the $\delta^{13}C$ of rhizosphere microorganisms and of respired CO_2.

The input of plant residues and other substances into the soil occurs not continuously, but as pulses related to vegetation period and plant development. Growing roots occupy alternating parts of the soil volume and hence induce pulse inputs by root exudates to alternating locations (Pausch and Kuzyakov, in press). Moreover, the decomposition rates of various substances in soil may range from a few minutes and hours (Jones et al., 2005; Fischer et al., in press) up to months and years and more (Kuzyakov et al., 2009). Accordingly, if the decomposition rates of individual components added simultaneously to the soil differ from each other, their contribution to any mixing pool such as CO_2 or microbial biomass will change during the decomposition period. At the initial stages the substances with fast decomposition rates will dominate the mixing pool; later, when these substances are already decomposed, the main (or even sole) contribution will come from the substances with slow decomposition rates. This means that during decomposition of a complex substrate such as plant residues, the isotopic composition of any mixing pool will be changed according to the contribution of individual substances and their $\delta^{13}C$. This effect is commonly not considered by soil carbon and CO_2-partitioning studies.

3. Fractionation during individual processes at the root–microorganisms–soil interface

3.1. Root respiration

Assimilates, like sucrose, are transported to the roots and are respired in the mitochondria to gain energy for the cells. A significant fractionation between the root tissue and the respired CO_2 has been frequently discussed, but was confirmed only in a few studies. Because of lacking experimental data, most rhizosphere-CO_2 studies have assumed equal $\delta^{13}C$ values of roots and root-derived CO_2 (Cerling et al., 1991; Cheng, 1996; Lin and Ehleringer, 1997; Amundson et al., 1998; Ekblad and Högberg, 2000; Fu and Cheng, 2002). ^{13}C-depleted CO_2 from root respiration compared to the root biomass, i.e. negative discrimination Δ, has been reported in some studies (Table 1), but this fractionation was not always significant. Such a ^{13}C depletion in root-respired CO_2 could be related to re-assimilation of respiratory CO_2 in roots by phosphoenolpyruvate carboxylase (PEPc) (Badeck et al., 2005; Klumpp et al., 2005; Gessler et al., 2009): The substrate for PEPc (HCO_3^-) is ^{13}C enriched relative to the CO_2 pool from which it is formed. Thus, the remaining respiratory CO_2 escaping from the roots would be ^{13}C depleted relative to the respiratory substrate.

In contrast, Cheng (1996) reports the absence of fractionation during root respiration when growing winter wheat on C-free vermiculite and on a vermiculite–sand mixture. In a study with *Zea mays* grown in nutrient solution, Werth and Kuzyakov (2005) found varying fractionations between roots and CO_2 from −0.7‰ for nutrient-rich solutions to +0.3‰ for nutrient-poor solutions. In another study, Bathellier et al. (2008) found a ^{13}C depletion (−1‰) between whole *Phaseolus vulgaris* plants and CO_2 for the first 8 days of plant growth, followed by an enrichment (up to +3.08‰). Those lacking ^{13}C fractionations or even enrichments in root respiration could be related to a change of PEPc activity due to soil nitrogen type (NO_3^- vs. NH_4^+), availability of other nutrients in the soil solution, or soil CO_2 partial pressure (Badeck et al., 2005).

We conclude that ^{13}C fractionation by root respiration is insufficiently studied and may vary depending on plant species and nutrient supply. Hence, further research is needed to clarify whether and when ^{13}C fractionation during root respiration occurs. In such studies, $\delta^{13}C$ of CO_2 should be compared not only to that of the bulk roots, but also to

$\delta^{13}C$ of young roots (which are much more active than older roots) and to $\delta^{13}C$ of sucrose in the roots, an approach which has commonly been used for leaves (Badeck et al., 2005; Gessler et al., 2009).

3.2. Microbial utilization

Uptake and utilization of organic substances by soil microorganisms may alter the isotopic composition of the products (microbial biomass, respired CO_2) compared to the substrates (soil organic matter, DOC, rhizodeposition, shoot and root residues). Many factors can cause ^{13}C fractionation during uptake of organic substances by microorganisms. These differ in nature and include (1) a very broad range of organic substances with different availability and accessibility for microorganisms, (2) enzymes involved in splitting of polymers to monomers and producing important parts of DOC, (3) transport of monomers into the cells, as well as (4) the methods used for microbial biomass C estimation. Here, we mainly evaluate the ^{13}C fractionation of organic substances having contrasting availability for microorganisms and then discuss methodological problems.

3.2.1. Microbial utilization of soil organic matter

Heavy carbon (^{13}C) tends to accumulate in soil trophic chains by about +0.5 to +1‰ per trophic level (Tiunov, 2007). Compared to SOM, microbial biomass was ^{13}C enriched by an average Δ of +1.2‰ for both C_3 and C_4 soils (Supplement 1). This ^{13}C enrichment in microbial biomass can be explained by (1) isotope discrimination during biosynthesis of new microbial biomass and (2) the heavier isotopic composition of organic compounds preferentially used by soil microorganisms – at least for C_3 soils (Potthoff et al., 2003). Preferential substrate utilization, however, seems to be of minor importance for C_4 soils (for more details see Section 3.2.2).

Supplement 1 is a compilation of the effects of the source vegetation, the microbial biomass analysis method, and the K_2SO_4 molar concentration on $\delta^{13}C$ of microbial biomass or DOC and the resulting discriminations Δ. There was no difference in the influences of either C_3 or C_4 vegetation on discrimination between SOM and microbial biomass or DOC (Table 2). The method of microbial biomass analysis, however, highly significantly affects the measured discrimination (Table 2). Chloroform fumigation extraction (CFE) (Vance et al., 1987) yielded Δ(MB-SOM) between +4.1 and −1.6‰ (Supplement 1). Microbial biomass determined by chloroform fumigation incubation (CFI) (Jenkinson and Powlson, 1976) in comparison to SOM is ^{13}C depleted by −0.1 to −5.3‰. These differences between microbial biomass estimated by CFE or CFI reflect methodological differences (i.e. sampling of DOC versus CO_2), which lead to a different apparent isotopic fractionation between SOM and microbial biomass (i.e. ^{13}C enrichment for CFE versus ^{13}C depletion for CFI). Furthermore, these differences could be explained by differential utilization of substrates for respiration, on which the CFI is based, compared to the extraction by CFE. Besides the method of microbial biomass analysis, the molar concentration of the extraction solution also significantly influences the resulting $\delta^{13}C$ values and discriminations Δ – at least of C_4 soils (Table 2). The higher the K_2SO_4 concentration, the more positive is usually the discrimination (Supplement 1). Possibly, an increasing desorption of stronger bound ^{13}C-enriched compounds occurs with increasing K_2SO_4 concentration. These methodological constraints call for an urgent standardization of microbial biomass analyses, especially in the case of isotope measurements. Usage of lower salt concentrations (about 0.05 M) is favourable, since the long established 0.5 M K_2SO_4 concentration in the CFE procedure destroys the IRMS unit on the long run and might influence the precision of $\delta^{13}C$ measurements.

3.2.2. Microbial respiration

In processes connected with microbial respiration, the fractionation should be considered compared to two C sources: microbial biomass cells and DOC. Instead of DOC, however, most of the studies related ^{13}C fractionation to SOM. According to Šantrůčková et al. (2000b), $\delta^{13}C$ values of CO_2 respired from 21 Australian soils with C_3 and C_4 vegetation were depleted on average by −2.2‰ compared to microbial biomass (Supplement 2). For individual soils, the $\delta^{13}C$ difference between microbial biomass and respired CO_2 varied between −0.1‰ and −5.7‰. Other studies, however, have found a ^{13}C enrichment of CO_2 between +4.3 and +0.6‰ compared to microbial biomass (Qian et al., 1997; Werth et al., 2006; Werth and Kuzyakov, 2009). While in all studies Δ (CO_2-MB) ranged from +4.3‰ (enrichment) to −3.2‰ (depletion) for C_3 soils (Supplement 2), it was significantly more depleted – up to −5.7‰ – for C_4 soils (Table 3).

The fractionation between SOM as a substrate and microbial CO_2 as a product is the sum of microbial uptake and respiration. Usually, CO_2 from microbial respiration is ^{13}C depleted compared to the feeding substrate (Blair et al., 1985; Mary et al., 1992; Potthoff et al., 2003). In a further study by Šantrůčková et al. (2000a), the difference between $\delta^{13}C$ of litter and that of respired CO_2 varied between a depletion of −0.5‰ and an enrichment of +1.6‰. Formánek and Ambus (2004) reported a ^{13}C enrichment of respired CO_2 compared to SOM with a Δ between +3.6‰ and +5‰. These results imply a ^{13}C enrichment of CO_2 compared to the bulk substrate in most cases for C_3 soils (Figs. 1 and 2). Such positive Δ indicate that these mineralisation processes mainly used a ^{13}C-enriched SOM

Table 2
Differences in ^{13}C fractionations Δ(MB-SOM) or Δ(DOC-SOM) from Supplement 1 between C_3 and C_4 plants, between the microbial biomass methods chloroform fumigation–extraction (CFE) and chloroform fumigation–incubation (CFI), and between different K_2SO_4-extraction concentrations for CFE. Influences of other factors (microbial biomass method, c(K_2SO_4) or vegetation type) were excluded when examining one particular factor. Differences were tested by one-way ANOVA.

	Microbial biomass method and c(K_2SO_4)				
Vegetation type (C_3 plants vs C_4 plants)	CFE 0.5 M	CFE 0.25–0.5 M	CFE 0.125 M	CFE 0.05 M	CFI
Δ(MB-SOM)	n.s.	n.s.	C_3 only	C_3 only	C_3 only
Δ(DOC-SOM)	C_3 only	n.s.	C_3 only	C_3 only	C_3 only
	Vegetation type and c(K_2SO_4)				
Microbial biomass method (CFE vs CFI)	C_3 0.5 M	C_3 0.25–0.5 M	C_3 0.125 M	C_3 0.05 M	C_4
Δ(MB-SOM)	$P < 0.001$	$P = 0.05$	$P < 0.001$	$P < 0.001$	CFE only
	Vegetation type and microbial biomass method				
c(K_2SO_4) (0.5 M vs 0.25–0.5 M vs 0.125 M vs 0.05 M)	C_3 CFE	C_3 CFI	C_4 CFE	C_4 CFI	
Δ(MB-SOM)	n.s.	no data	$P < 0.05$	no data	

Table 3
Differences in ^{13}C fractionations $\Delta(CO_2\text{-MB})$ or $\Delta(CO_2\text{-SOM})$ from Supplement 2 between C_3 and C_4 plants, and between CO_2 sampling in NaOH solution and direct gas sampling. Differences in $\Delta(CO_2\text{-MB})$ were not tested for CO_2 sampling since all studies used NaOH sampling. Influences of other factors (CO_2 sampling or vegetation type) were excluded when examining one particular factor. Differences were tested by one-way ANOVA.

Vegetation type	CO_2 sampling		
(C_3 plants vs C_4 plants)	CO_2 in NaOH	Direct gas sampling	CO_2 in KOH
$\Delta(CO_2\text{-MB})$	$P < 0.05$	no data	C_3 only
$\Delta(CO_2\text{-SOM})$	$P < 0.05$	C_3 only	C_3 only
CO_2 sampling (NaOH sampling vs direct gas sampling)	Vegetation type C_3	C_4	
$\Delta(CO_2\text{-SOM})$	n.s.	NaOH sampling only	

fraction (including sugars, starch, cellulose etc.) of the total organic C (Cotrufo et al., 2005). This isotope effect associated with the preferential use of organic compounds in C_3 soils is more pronounced than the ^{13}C-depletion effect of metabolism itself (Šantrůčková et al., 2000a). The preferential use of this ^{13}C-enriched SOM fraction leads to a more rapid loss of ^{13}C than ^{12}C during decomposition and therefore depletes the ^{13}C in the remaining SOM (Benner et al., 1987; Ågren et al., 1996). Hence, by preferential substrate utilization in C_3 soils microbial biomass gets enriched in ^{13}C (Figs. 1 and 2), but respires CO_2 depleted in ^{13}C to itself (but still enriched compared to SOM). This effect therefore additionally enriches soil microorganisms with ^{13}C.

Methodological differences due to the CO_2 sampling method (direct gas sampling versus trapping in NaOH) can be excluded for the ^{13}C discrimination $\Delta(CO_2\text{-SOM})$ of C_3 soils (Table 3). Trapping of CO_2 in NaOH was the only sampling method used for the other discriminations considered in Table 3 ($\Delta(CO_2\text{-SOM})$ of C_4 soils and $\Delta(CO_2\text{-MB})$ of both – C_3 and C_4 soils), hence, there is also no difference related to methodology. Differences in ^{13}C discrimination due to CO_2 sampling method might, however, occur when further sampling methods are employed. In Supplement 2 we have pooled all direct gas sampling methods together, although Crow et al. (2006) used a flow-through method, while the other three studies used a closed-chamber method (Formánek and Ambus, 2004; Stevenson et al., 2005; Boström et al., 2007). While discriminations $\Delta(CO_2\text{-SOM})$ were always positive for the latter, they were both – positive and negative – for the former, which implies differences in $\delta^{13}C$ of CO_2

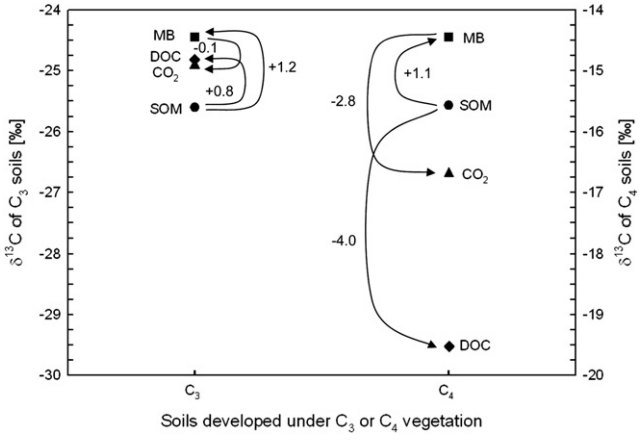

Fig. 1. ^{13}C discrimination processes between soil organic matter (SOM, ●) and the soil carbon pools: dissolved organic carbon (DOC, ◆), microbial biomass (MB, ■), and SOM-derived CO_2 (▲) for C_3 and C_4 soils. Mean $\delta^{13}C$ and discrimination values are obtained from the studies in Supplements 1 and 2.

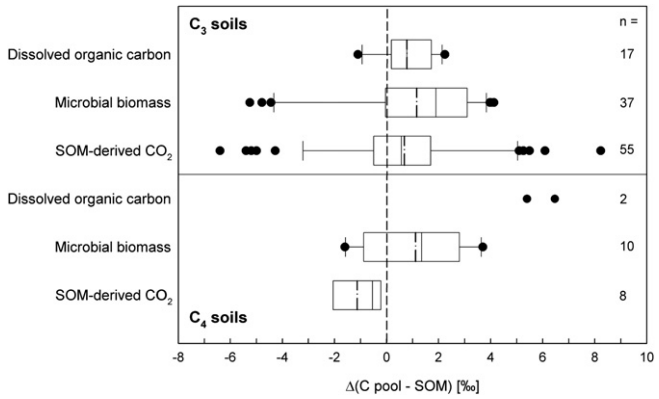

Fig. 2. ^{13}C discrimination Δ between soil organic matter and the soil carbon pools: dissolved organic carbon, microbial biomass, and SOM-derived CO_2 for C_3 and C_4 soils. The boxes encompass the upper and lower quartiles of the data, the solid line shows the median, the dash-dot line shows the arithmetic mean, the error bars show the upper and lower 10th percentiles of the data, and the dots show single data within these percentiles. The dashed line represents SOM as a reference, indicating ^{13}C depletion to the left and enrichment to the right. Data are obtained from Supplements 1 and 2.

caused by usage of flow-through or closed-chamber methods. These differences could, however, also derive from the different soil fractions (light/heavy SOM) and sampling times (1 or 65 days incubation) analysed by Crow et al. (2006) in contrast to the non-recurring sampling of CO_2 of bulk SOM in the other three studies. Hence, a bias caused by different sampling methods might lead to different discriminations, but this could not be detected for the studies mentioned in this publication.

Besides preferential substrate utilization, the activity of the microorganisms in soil is very important for ^{13}C fractionation. Only a minor part of microorganisms is metabolically active in soil (Stenström et al., 2001). Some studies have shown that only 2–14% of the total microbial biomass are active and the remaining part is in a dormant state (Qian and Doran, 1996; Rochette et al., 1999; Werth and Kuzyakov, 2008, 2009). Only these active organisms respire CO_2 and thus only the ^{13}C fractionation of these active organisms can be determined in CO_2. Most of the studies have related, however, the complete microbial biomass fraction determined by chloroform fumigation extraction – and thus including active and dormant microorganisms – to the $\delta^{13}C$ signature of CO_2 respired by active organisms only. That is why ^{13}C discrimination in microbial respiration should be considered only as 'apparent' fractionation composed of the three effects: (1) kinetic ^{13}C fractionation, (2) preferential substrate utilization, and (3) heterogeneity and activity of microorganisms. A similar problem arises if we consider root respiration once again: at multiple scales a root is composed of various functional tissues, which again contain various cells, which in turn contain organelles. Altogether a root is composed of various heterogeneous substances (lignin, lipids, sugars, etc.) but the whole root – not a single source level (tissues, cells, organelles etc.) or substrate – is considered as a pool for root respiration when looking at ^{13}C fractionation. The CO_2, however, only derives from the sugars respired in mitochondria, hence the real ^{13}C fractionation should be considered between sugars and CO_2. Up to now, studies have got along with or concealed these discrepancies, but in future experiments ^{13}C fractionations should be used more carefully.

Preferential substrate utilization or microbial heterogeneity is apparently less important in C_4 than in C_3 soils. C_4 soils, typical of arid and semiarid climates, contain generally significantly less SOM than C_3 soils (compare Šantrůčková et al. (2000b) in Supplement 1) and thus microorganisms consume the SOM more completely than in C_3 soils once the environmental conditions are at optimum. Consequently, microorganisms become ^{13}C enriched by about +1.1‰, while respiring

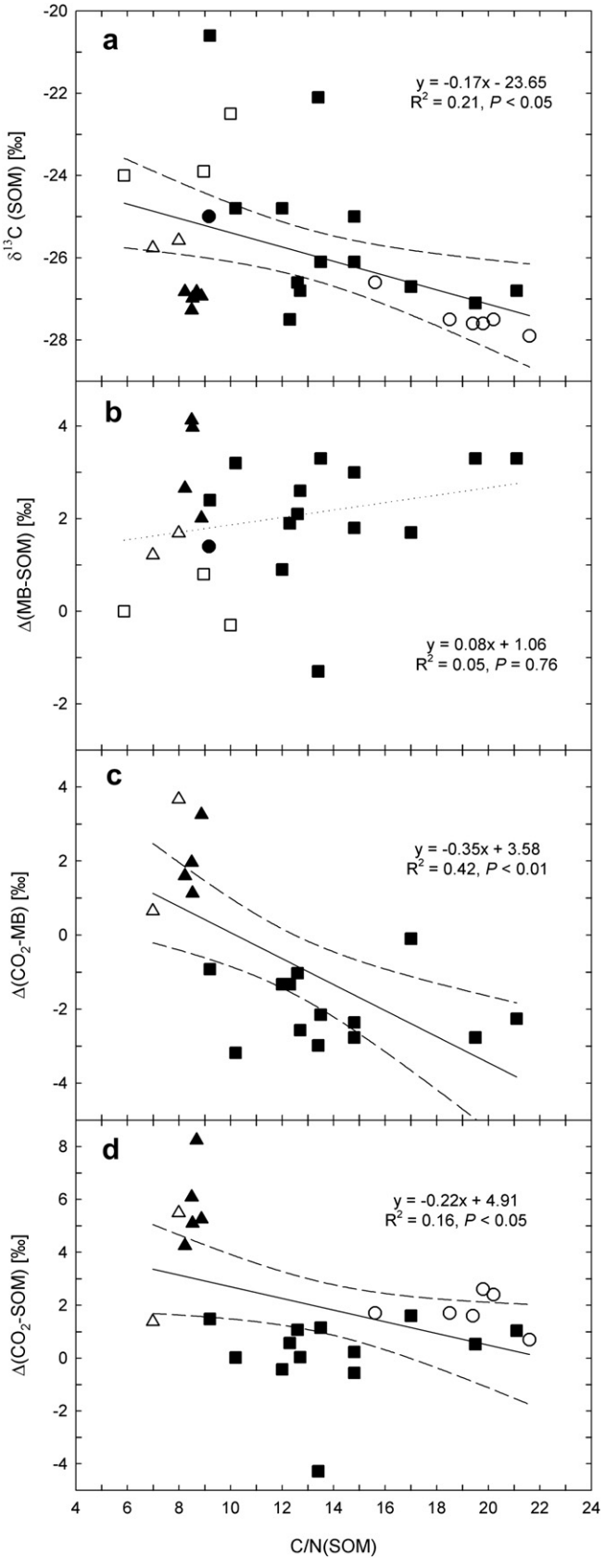

Fig. 3. Soil organic matter $\delta^{13}C$ (a), ^{13}C fractionations Δ between SOM and soil microbial biomass (b), soil microbial biomass and SOM-derived CO_2 (c), and SOM and SOM-derived CO_2 (d) vs. SOM C/N ratio from sites with C_3 vegetation (compare Supplements 1 and 2). The solid line shows the regression line (dotted if not significant), the dashed lines show the 95% confidence interval of the regression. The symbols represent the following studies: ○ (Boström et al., 2007), ● (Dijkstra et al., 2006), □ (Piao et al., 2006), ■ (Šantrůčková et al., 2000b), △ (Werth and Kuzyakov, 2009), ▲ (Werth et al., 2006).

CO_2 depleted by −1.1‰ compared to SOM (Fig. 1 and Supplement 2). Hence, under climate conditions in arid zones, soil microorganisms do not preferentially decompose a certain SOM fraction – due to brief periods with high temperature and sufficient soil moisture they consume every kind of SOM. In humid-zone C_3 soils with generally higher SOM contents than in C_4 soils (compare Šantrůčková et al. (2000b) in Supplement 1), on the contrary, microorganisms can "afford" to select only easily available SOM because temperature and moisture conditions are not limiting and more organic substances are available in dissolved form. Consequently, different mechanisms concerning the microbial consumption and respiration of soil organic matter have apparently developed due to different climate conditions (temperature, moisture, etc.) between C_3 and C_4 soils (Table 3).

An additional factor that can explain the smaller variance of discrimination of respired CO_2 compared to SOM in C_4 soils is the smaller variation of the $\delta^{13}C$ values of C_4 plants (−9‰ to −17‰) compared to C_3 plants (−22‰ to −32‰) (Boutton, 1996). This is connected with much stronger effects of environmental conditions on ^{13}C discrimination by C_3 photosynthesis compared to C_4 photosynthesis. Therefore, the isotopic variation of plant residues contributing to SOM is lower in C_4 versus C_3 soils. With this smaller variation of $\delta^{13}C$ values in C_4 soils, the preferential substrate utilization contributes less to the ^{13}C fractionation between SOM and respired CO_2 compared to C_3 soils.

The soil organic matter C/N ratio is one important factor related to carbon-isotope discrimination in microbial processes – including SOM decomposition and respiration. High C/N ratios imply strong ^{13}C discrimination by SOM formation (Fig. 3a), which can be explained by the presence of hardly decomposable compounds that are already ^{13}C depleted by synthesis in plants. Such high amounts of stable compounds – like ^{13}C-depleted lignin and lipids (Wiesenberg et al., 2004, 2008; Bowling et al., 2008) and their humification products – can lead to low $\delta^{13}C$ values in SOM in soils with high C/N ratios. Since the SOM C/N ratio has no significant influence on the ^{13}C fractionation between SOM and microbial biomass (Fig. 3b), it can be concluded that ^{13}C fractionation during microbial uptake is constant through a variety of different C_3 soil types (Fig. 3b: discrimination is parallel to the *X* axis). Under steady-state conditions, soil microorganisms can be viewed as an intermediate pool in which they are changing the $\delta^{13}C$ of SOM and SOM-derived CO_2, but keeping their own $\delta^{13}C$ constant. At low C/N ratios, SOM-derived CO_2 is ^{13}C enriched compared to microbial biomass (Fig. 3c) or SOM (Fig. 3d). An explanation is the preferred consumption by soil microorganisms of easily decomposable SOM with enriched $\delta^{13}C$, i.e. sugars, starch, cellulose, proteins, or organic acids (Bowling et al., 2008). This leads to an increasing enrichment of the CO_2 towards the substrate (i.e. increasingly positive discrimination Δ). At high C/N ratios, parts of humified lignin or lipids also become decomposed, leading to low positive discrimination $\Delta(CO_2\text{-SOM})$ or even negative $\Delta(CO_2\text{-MB})$. Thus, at high C/N ratios there is a tendency to preferred respiration of ^{12}C. We assume that the mechanisms of this C/N effect can be explained by different microbial communities in soils with different C/N ratios. Fungi play a more important role in soils with high than with low C/N ratios. Fungi have a higher substrate use efficiency than bacteria (Payne, 1970), a slower metabolism, and the ability to decompose more recalcitrant ^{13}C-depleted substrates (Neely et al., 1991; Paterson et al., 2008). Accordingly, they directly affect

discriminations $\Delta(CO_2\text{-SOM})$ or $\Delta(CO_2\text{-MB})$ as well as the preferential substrate utilization, which are also indirectly affected by the C/N ratio of the soil. Note that $\delta^{13}C$ of SOM and $\Delta(CO_2\text{-MB})$ were not correlated, and that $\delta^{13}C$ of SOM and $\Delta(CO_2\text{-SOM})$ were only weakly correlated ($R^2 = 0.16$, $P < 0.05$; data from Fig. 3). This means that high (slightly negative) $\delta^{13}C$ does not automatically imply higher (more positive) discrimination Δ, as could be presumed from Fig. 3a, c and d.

Several investigators have observed the above-mentioned ^{13}C fractionations during microbial respiration: CO_2 evolved during the mineralisation of organic substrates (plant residues, leaf litter, roots, root mucilage, or glucose) was either significantly ^{13}C enriched or depleted compared to the substrate (Mary et al., 1992; Schweizer et al., 1999; Šantrůčková et al., 2000a; Fernandez et al., 2003; Kristiansen et al., 2004). In other studies, however, this isotopic fractionation did not occur or was considered to be negligible (Cheng, 1996; Ekblad and Högberg, 2000; Nyberg et al., 2000; Ekblad et al., 2002). Hence, it is still uncertain which factors control the magnitude of isotopic ^{13}C fractionation. According to Fernandez and Cadisch (2003), carbon-isotope discrimination by heterotrophic microorganisms seems to depend on many factors: temperature, molecule isotopic distribution, chemical nature of the substrate, metabolic pathways of carbon, and physiological conditions of microbial growth. The soil organic matter C/N ratio – a variable related to the chemical nature of the soil substrate – has been shown to be one of these factors.

3.3. Effects of precipitation and temperature on $\delta^{13}C$ of soil C pools

Leaf $\delta^{13}C$ values vary across broad gradients of precipitation, with the general pattern being that C_3 plants in wetter ecosystems tend to have more depleted $\delta^{13}C$ in their leaves than those in drier regions (Read and Farquhar, 1991; Stewart et al., 1995; Schulze et al., 1998). Under water stress, C_3 plants close their stomata, improving water use efficiency and decreasing the p_i/p_a ratio. These factors result in lower ^{13}C discrimination during carbon assimilation. Such a negative correlation between leaf $\delta^{13}C$ and mean annual precipitation (MAP) has also been found between soil organic matter $\delta^{13}C$ and MAP and between soil microbial biomass $\delta^{13}C$ and MAP (Fig. 4a and b), which both derive from the plant litter. These relationships, however, were

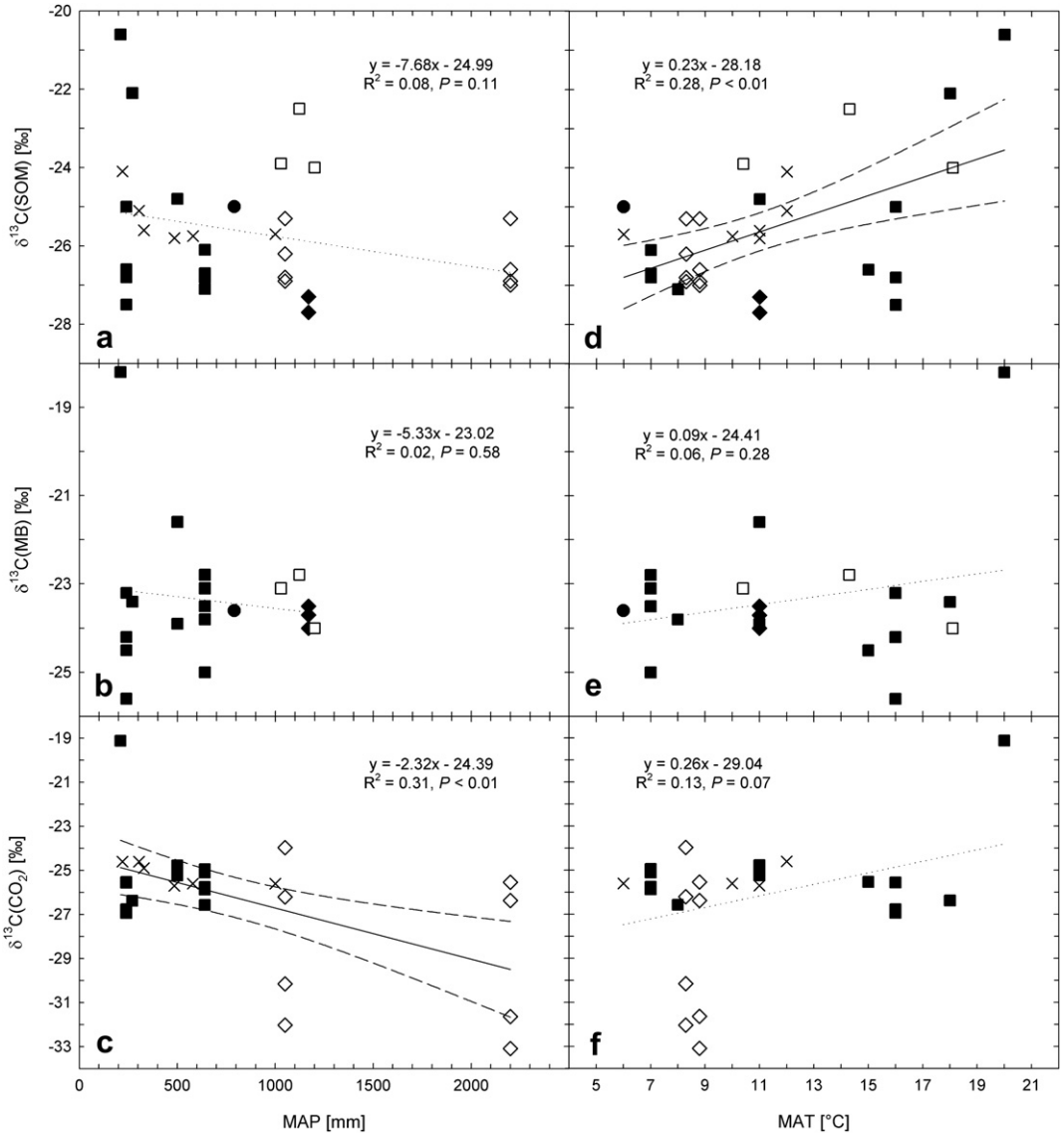

Fig. 4. Soil organic matter (a, b), soil microbial biomass (c, d), and soil-derived CO_2 (e, f) $\delta^{13}C$ values vs. mean annual precipitation (MAP) and mean annual temperature (MAT) from sites with C_3 vegetation (compare Supplements 1 and 2). The solid line shows the regression line (dotted if not significant), the dashed lines show the 95% confidence interval of the regression. The symbols represent the following studies: ◇ (Crow et al., 2006), ● (Dijkstra et al., 2006), ◆ (Pelz et al., 2005), □ (Piao et al., 2006), ■ (Šantrůčková et al., 2000b), × (Stevenson et al., 2005).

not significant. A pattern similar to that in leaves has been observed in the $\delta^{13}C$ of respiration across a wide range of biomes: for sites whose MAP ranged from 200 to 2300 mm, the $\delta^{13}C$ of ecosystem respiration ranged from −24 to −30‰ (Pataki et al., 2003; Bowling et al., 2008). Again, this relationship was confirmed by SOM-derived CO_2 (Fig. 4c).

The relationships between $\delta^{13}C$ and mean annual temperature (MAT) were reverse to the ones with MAP (Fig. 4d, e and f). This effect can be primarily attributed to the ^{13}C discrimination of C_3 plants: in cooler and wetter climates, stomata can open widely, leading to high discrimination, i.e. low $\delta^{13}C$ values. This plant isotopic signal proceeds particularly in SOM, but also as a trend in microbial biomass and SOM-derived CO_2 with their inherent post-photosynthetic fractionations. Relationships between discriminations Δ(MB-SOM), Δ(CO_2-MB) or Δ(CO_2-SOM) and MAP or MAT were not significant, indicating that even though MAP and MAT do influence the $\delta^{13}C$ of different pools, they do not affect the Δ of microbial processes.

In contrast to SOM and CO_2, no significant relationships between MAP or MAT and $\delta^{13}C$ were found for the microbial biomass. This indicates that MAP and MAT influence the substrate SOM and the final product CO_2, but not the dominating cell compounds of soil microorganisms themselves. This underlines again the functionality of soil microbial biomass as an intermediate decomposition pool, as stated in Section 3.2.2 for the SOM C/N ratio.

In conclusion, the two most important environmental factors – precipitation and temperature – affect the $\delta^{13}C$ of assimilates, which influences the $\delta^{13}C$ of plant tissues, SOM, and CO_2 from SOM decomposition. However, for the here reviewed studies with a broad range of environmental conditions, these factors had no significant effect on discrimination Δ in processes between the tested C pools in soils. Note that the overall variation of the observed discrimination from various studies may mask possible effects of MAP and MAT on discrimination Δ.

4. Consequences of ^{13}C fractionations for natural abundance studies at the root–microorganisms–soil interface

4.1. Accounting for ^{13}C fractionation when calculating C-source contributions

Natural ^{13}C labelling (see Section 2.2) is commonly used to address the following issues: (1) Calculating contributions of two (or seldom three) sources to a mixing pool or flux; (2) Tracing the origin of substances.

As both topics are closely related, we present here the calculations using examples of the first topic only.

Mass balance equations are used to calculate the fraction of the labelled material in a particular pool. The master equation (Hayes, 1983) uses the fractional abundance of ^{13}C F ($=^{13}C/(^{12}C + {}^{13}C)$) and the molar quantities n of the pool components:

$$F_T n_T = F_1 n_1 + F_2 n_2 + \ldots + F_k n_k. \quad (8)$$

The subscript T refers to the total sample derived by the sum of subpools 1, 2,…, k. The same equation is often used in rhizosphere studies in approximate form by replacing F values with $\delta^{13}C$ values. The magnitude of the error introduced by this approximation will be less than 0.02‰ for most calculations involving only materials with natural ^{13}C variation (Hayes, 1983) and can therefore be neglected in most studies. In studies with highly enriched or depleted materials, however, the exact form of the equation with F values instead of $\delta^{13}C$ values has to be used, because $\delta^{13}C$ values are based on R ($=^{13}C/^{12}C$) instead of F ($=^{13}C/(^{12}C + {}^{13}C)$) and thus, simply replacing F by $\delta^{13}C$ would introduce a high error.

In the following calculations, we will consider studies using the ^{13}C natural abundance between an original carbon source A (C_3 or C_4 plants) and a substitute carbon source B (C_4 or C_3 plants) after a vegetation shift from C_3 to C_4 plants or *vice versa*. Using $\delta^{13}C$ values of these isotopically different sources and replacing the molar quantities by absolute carbon amounts C in a pool, Eq. (8) becomes (Hayes, 1983; Balesdent and Mariotti, 1996):

$$\delta_T C_T = \delta_A C_A + \delta_B C_B \quad (9)$$

with

$$C_T = C_A + C_B. \quad (10)$$

C_A stands for the amount of carbon derived from the original vegetation A, δ_A for the isotopic composition of that carbon, C_B for the amount of carbon derived from vegetation B used in the natural labelling study, δ_B for its isotopic composition, C_T for the amount of carbon from both sources, and δ_T for the isotopic composition of this pool. Replacing C_A in equation (9) by $C_T - C_B$ and rearrangement will give the fraction f of carbon from source B in this pool:

$$f = \frac{C_B}{C_T} = \frac{\delta_T - \delta_A}{\delta_B - \delta_A}. \quad (11)$$

Eq. (11) is the strict mixing equation relating f to δ that is used in most natural-^{13}C-labelling studies. In a plant–soil-system, however, the values of δ_A and δ_B cannot be measured directly and must be estimated. Considering soil, CO_2, or microbial biomass samples, most investigators using the natural-^{13}C-labelling technique assume δ_B to be equivalent to the $\delta^{13}C$ of vegetation B or its litter (i.e. δ_{vegB}), and δ_A to be equivalent to the initial $\delta^{13}C$ of the sample or, more frequently, to the composition of a corresponding sample at reference site kept under the initial vegetation A (i.e. δ_{refA}). On these assumptions, Eq. (11) becomes:

$$f = \frac{\delta_T - \delta_{refA}}{\delta_{vegB} - \delta_{refA}}. \quad (12)$$

Isotopic fractionations have to be considered when calculating the contribution of one source to a mixing pool, e.g. the contribution of root or microbial respiration to total soil respiration. For this purpose, we rewrite Eq. (9) by replacing the hypothetical samples from the mixed pool with reference samples and then dividing by C_T:

$$\delta_T = \delta_{refA}\frac{C_{refA}}{C_T} + \delta_{refB}\frac{C_{refB}}{C_T} \quad (13)$$

with $f_{refA} = C_{refA}/C_T$ and $f_{refB} = C_{refB}/C_T$ and $f_{refA} + f_{refB} = 1$, we can write:

$$\delta_T = \delta_{refA}(1 - f_{refB}) + \delta_{refB} f_{refB} \quad (14)$$

The shift of $\delta^{13}C$ between the substrates and the next trophic level (e.g. microorganisms) has to be considered for both sources:

$$\delta_{refA} = \delta_{vegA} - \varepsilon_{vegA} \Leftrightarrow \varepsilon_{vegA} = \delta_{vegA} - \delta_{refA} \quad (15)$$

$$\delta_{refB} = \delta_{vegB} - \varepsilon_{vegB} \Leftrightarrow \varepsilon_{vegB} = \delta_{vegB} - \delta_{refB} \quad (16)$$

If we combine Eq. (14)–(16), we get:

$$\delta_T = (\delta_{vegA} - \varepsilon_{vegA})(1 - f_{refB}) + (\delta_{vegB} - \varepsilon_{vegB}) f_{refB} \quad (17)$$

Mostly, only the fractionation ε_{vegA} on a control plot under the original vegetation A can be determined. Thus, as an approximation, equalling fractionation ε_{vegB} to ε_{vegA} and rearranging Eq. (17) with Eq. (15) will lead to the final term:

$$f_{refB} = \frac{\delta_T - (\delta_{vegA} - \varepsilon_{vegA})}{(\delta_{vegB} - \varepsilon_{vegA}) - (\delta_{vegA} - \varepsilon_{vegA})} = \frac{\delta_T - \delta_{refA}}{\delta_{vegB} - \delta_{vegA}} \quad (18)$$

Alternatively, if we cannot assume equal ^{13}C fractionations for source-*A*- and source-*B*-derived substrates, we have to write:

$$f_{refB} = \frac{\delta_T - (\delta_{vegA} - \varepsilon_{vegA})}{(\delta_{vegB} - \varepsilon_{vegB}) - (\delta_{vegA} - \varepsilon_{vegA})} = \frac{\delta_T - \delta_{refA}}{\delta_{refB} - \delta_{refA}} \quad (19)$$

While Eq. (12) only accounts for ^{13}C fractionation in substrate *A*, Eq. (18) also accounts for an equal fractionation in substrate *B*. Consequently, preference should be given to Eq. (18) if possible. If the fractionation ε is no longer equal for C_3- and C_4-derived substrates, then Eq. (19) will be the more correct calculation. It is difficult to be applied, however, because it is almost impossible to find a reference soil pool relying only on the new organic matter (e.g. a pure C_4 soil as a reference next to the sample soil with C_4 vegetation on C_3 soil).

4.2. Possible uncertainties of results obtained by ^{13}C natural abundance with and without considering ^{13}C fractionation

For this review we tried to estimate statistical uncertainties connected with various problems of ^{13}C natural labelling used to partition C fluxes and mixing pools. These uncertainties reflect the variability of $\delta^{13}C$ in carbon pools and the variability of ^{13}C fractionation. For all further estimations of uncertainties, we used the partitioning equations suggested by Balesdent and Mariotti (1996) (i.e. Eqs. (12) and (19)) and statistical estimations of uncertainties for two source partitioning by using stable isotopes described by Phillips and Gregg (2001) (The MS-Excel sheet with visualizations of uncertainties by 2-source and 3-source partitioning according to Phillips and Gregg (2001) can be downloaded from: www.aec.uni-bayreuth.de/isotope-error.xls).

As an example we used the $\delta^{13}C$ values typical for C_3–C_4-vegetation-change studies: the $\delta^{13}C$ of the first 'endmember' was $-27 \pm 1‰$ (mean ± SD) and the $\delta^{13}C$ of the second 'endmember' was $-13 \pm 1‰$ (Supplement 3). We assumed a contribution of the C_4 source (i.e. root-derived CO_2) amounting to between 0% and 100% and calculated the standard errors of contributions of both sources to a mixing pool (i.e. soil-derived CO_2). For all calculations, mixing pool $\delta^{13}C$ values (Supplement 3) were taken from Eq. (19) because, there, all $\delta^{13}C$ values are based on the gas phase and ^{13}C-fractionation effects are already included. The standard deviation (SD) of $\delta^{13}C$ of the 'endmembers' and of the mixing pool was set to $\pm 1‰$, which is an adequate estimate for many natural materials measured with four replications. We assumed that analytical errors are smaller than the natural $\delta^{13}C$ variation of the 'endmembers' and of the mixing pool. In the first calculation with Eq. (12) (which does not consider any ^{13}C fractionation of the C_4 source) the assumed values for CO_2 partitioning were underestimated by between 0% and 10% with increasing C_4-source contribution (Fig. 5). The standard errors (SE) of the partitioning mean were at 4.4% for 50% C_4-source contribution and increased towards both 'endmembers'– the C_3 and the C_4 source – up to a maximum of 5.5% for 0% C_4-source contribution.

In a second calculation, we consider the fractionation-related uncertainties of the CO_2 partitioning when a C_4 plant is grown on a C_3 soil. Prior to calculating the partitioning according to Eq. (12), the SD of the $\delta^{13}C$ in the 'endmembers' or the mixing pool should be considered. For these calculations we kept the SD of $\delta^{13}C$ in the C_3-source 'endmember' at $\pm 1‰$ as before (Supplement 3). We then used an SD of $\pm 2.4‰$ for the ^{13}C discrimination of C_4-derived root respiration based on the literature review (Table 1). For the C_3 pool, we only consider the natural $\delta^{13}C$ variation (i.e. SD = 1‰) and initially neglect any uncertainties related to ^{13}C fractionations for rhizomicrobial and SOM-derived respiration (Supplement 2).

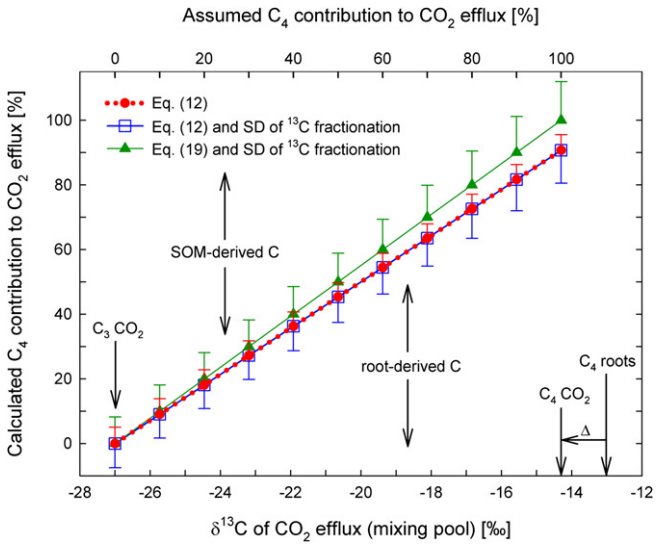

Fig. 5. Partitioning of soil CO_2 efflux into root-derived and SOM-derived carbon. Natural ^{13}C labelling was used by planting a C_4 plant on soil developed under C_3 vegetation. Root-derived respiration from the C_4 plant (i.e. root respiration and rhizomicrobial respiration) was assumed to contribute between 0% and 100% to the CO_2 efflux (top *x*-axis). In the partitioning calculations (*y*-axis), ^{13}C fractionation between root-derived sources and CO_2 was either disregarded (Eq. (12), positive standard errors are shown only), accounted for in the SD of the C_4 roots and the total CO_2 efflux (Eq. (12) and SD of ^{13}C fractionation, negative standard errors are shown only), or accounted for in the partitioning equation and in the SD of the C_4 roots and the total CO_2 efflux (Eq. (19) and SD of ^{13}C fractionation, positive standard errors are shown only). The $\delta^{13}C$ values of the three 'endmembers' used are shown towards the bottom *x*-axis, the $-1.3‰$ ^{13}C discrimination between roots and root-derived CO_2 used in Eq. (19) is indicated by Δ.

The standard deviation of the mixing pool $SD_{mixing\ pool}$ (i.e. total CO_2) was then calculated by the following equations:

$$SD_{mixing\ pool} = \sqrt{\frac{(n_{C_3} - 1)SD_{C_3}^2 + (n_{C_4} - 1)SD_{C_4}^2}{n_{C_3} + n_{C_4} - 2}} \quad (20)$$

where n_{C_3} and SD_{C_3} are the number of replications and the standard deviation of the C_3 source $\delta^{13}C$, respectively, and n_{C_4} and SD_{C_4} are the number of replications and the standard deviation of the C_4 source $\delta^{13}C$, respectively. For equal sample sizes (i.e. $n_{C_3} = n_{C_4}$), Eq. (20) can be simplified to:

$$SD_{mixing\ pool} = \sqrt{\frac{SD_{C_3}^2 + SD_{C_4}^2}{2}}. \quad (21)$$

Based on Eq. (21), the resulting SD of the mixing pool was $\pm 1.8‰$. Consequently, the uncertainty of CO_2 partitioning increases to standard errors between 7.5% and 10.2% with increasing C_4-source contribution (Fig. 5). The assumed CO_2 contributions were missed on the same scale as in the first calculation omitting ^{13}C fractionation in the standard deviations of C_4-derived root respiration and the mixing pool.

In the third case, by using Eq. (19), we repeated the calculations with the same increased standard deviations for root respiration and mixing pool as for Eq. (12). Furthermore, we accounted for ^{13}C fractionation by root respiration by using the mean Δ of -1.3 from Table 1 (Supplement 3). The assumed contributions of C_3 and C_4 sources to CO_2 partitioning were hit exactly but, compared to Eq. (12), the standard errors slightly increased to values from 8.2% to 11.9% with increasing C_4-source contribution (Fig. 5). Consequently, this approach yields exact mean CO_2 partitioning with a slightly higher standard error like without accounting for ^{13}C fractionation.

These estimations of 'endmember' contributions to a mixing pool (CO_2, microbial biomass, DOC, SOM, …) based on the natural-^{13}C-labelling approach clearly showed very high uncertainties, and even deviations up to 10% from the assumed contributions. The uncertainties and deviations were particularly high at contributions close to the new source (i.e. the C_4 source in our case). This calls for very cautious interpretation of the results of partitioning studies obtained based on the natural-^{13}C-labelling approach.

4.3. Possible uncertainties connected with preferential substrate utilization

A next step in our evaluation is to estimate possible uncertainties connected with changing contributions of individual plant components to a mixing pool. We assume that the 'complex substrate' representing plant residues consists only of two components: cellulose (50%) and lignin (50%). The isotopic difference between lignin and cellulose in plant residues varies between +2.5‰ and +4.6‰ (Hobbie and Werner, 2004). We use the mean difference of +3.5‰ between lignin and cellulose and the $\delta^{13}C$ of the whole C_4 plant residues of −13.0‰, i.e. we assume the $\delta^{13}C$ of lignin to be −14.75‰ and the $\delta^{13}C$ of cellulose to be −11.25‰. The decomposition rate of cellulose can be accepted as 0.03 d^{-1} ($T_{½} \approx 25$ days) and that of lignin as 0.004 d^{-1} ($T_{½} \approx$ half a year) (Tilston et al., 2004). Accordingly, in the first weeks to months of litter decomposition the released CO_2 will be enriched up to +1.75‰ compared to that of the plant residues; after 2–3 months it will be increasingly depleted up to −1.75‰. Such uncertainty (±1.75‰) would lead to a possible range of estimated contributions of the C_4 source to CO_2 between 40.3% and 51.8%. For ^{13}C natural abundance studies, this effect underlines the necessity of carefully documenting the sampling time after major litter deposition in an ecosystem. Hamer (unpublished observations), for example, investigated the decomposition of forest floor and maize residues as well as DOC extracted from these residues in C-free quartz sand. Her results clearly showed a $\delta^{13}C$ decrease of CO_2 during decomposition of the forest floor. The trend was, however, inverse for CO_2 from maize residues and from its DOC. This is because a less homogeneous C source concerning individual substances (compared to forest plant residues) was decomposed.

At first glance, the uncertainties connected with preferential utilization/decomposition may be neglected for studies with individual substances such as glucose, cellulose, lignin, etc. However, as reviewed by Hobbie and Werner (2004), the $\delta^{13}C$ of individual atoms in the glucose molecule differ up to 10‰. This means that the contribution of individual C atoms from a molecule to CO_2 or microbial biomass (Haider and Martin, 1975; Haider and Trojanowski, 1975; Kuzyakov, 1997; Kuzyakov and Demin, 1998; Fischer and Kuzyakov, 2009) is not identical. The errors connected with preferential utilization of different C atoms from one molecule will be at least as high as for the plant residues consisting of a mixture of individual substances.

Note that if the input and decomposition of complex substrates (or even individual substances) occurs continuously rather than in pulses (i.e. decomposition of humified soil organic matter; input of litter in tropical forest), then the uncertainties connected with preferential utilization and decomposition can be neglected. This is because of a constant contribution of individual components (even with different decomposition rates) to any mixing pool.

4.4. Possibilities to reduce the uncertainties of partitioning estimations based on ^{13}C natural abundance

In the above calculations, even a small variation with a ±1.0‰ $\delta^{13}C$ SD of 'endmembers' led to strong uncertainties in estimated contributions to a mixing pool without fractionation. If any significant fractionation is present, these uncertainties increase further, leading to very rough estimations. Which solutions could reduce uncertainties by working on the level of natural abundance labelling? We propose various approaches.

The simplest approach is to use 'endmembers' with the most different $\delta^{13}C$ values. So, if the Δ between both 'endmembers' increases from 14‰ to 20‰ (the SD of $\delta^{13}C$ of the 'endmembers' remains ±1.0‰), then the SE of the CO_2-partitioning estimations by Eq. (12) with 50% root-derived CO_2 decreases from 4.4% to 3.1%. An excellent example for the application of 'endmembers' with the most different $\delta^{13}C$ values at the level of ^{13}C natural abundance was in a study by Ineson et al. (1995). They used C_4 soil ($\delta^{13}C = -21.3$‰) from a maize field and grew on it birch seedlings (*Betula pendula* with an original $\delta^{13}C = -28.9$‰) which were continuously labelled in a FACE experiment with depleted $^{13}CO_2$ ($\delta^{13}C = -48.6$‰). This resulted in a difference between the two 'endmembers' – soil and plants – of 24.4‰.

Further increasing the difference between the endmembers, therefore, leads to the switch from natural labelling to artificial labelling (commonly having several-orders-of-magnitude differences between $\delta^{13}C$ of the 'endmembers'). Artificial labelling with strongly enriched ^{13}C completely excludes the errors connected with variations of the natural $^{13}C/^{12}C$ ratio. Even strong fractionation will be negligible when using artificial labelling. In short: studies based on labelling with high ^{13}C enrichment are much more precise than using small natural $\delta^{13}C$ variation (Paterson et al., 2009). Note however, that for high-enrichment labelling the homogeneous distribution of the label within individual substances is a prerequisite. In most tracer studies only pulse labelling with highly enriched substances was applied and a homogeneous label distribution could not always be assumed.

The second approach is to estimate the fractionation for individual processes based on the conditions of the specific study. As fractionations strongly vary in individual studies (Table 1, Supplements 1 and 2) the application of mean values lead to very high uncertainties and even to erroneous estimations. Thus, any reduction of fractionation uncertainties (which can be achieved by considering fractionation in the specific study) improves the partitioning estimates.

This review shows that the complexity of substrates, making up plant residues, rhizodeposits, DOC and SOM, contributes to partitioning estimation uncertainties because of preferential substrate utilization. Analysing $\delta^{13}C$ values of individual substance groups or even substances – i.e. compound-specific isotopic analysis (Glaser, 2005; Amelung et al., 2008) – can strongly reduce the uncertainties of estimations of contributions to any mixing pools. However, preparing samples for compound-specific analyses is laborious and cannot be done to such extents as in bulk $\delta^{13}C$ analyses. Additionally, derivatisation is necessary for most of the substances for GC separation prior to $\delta^{13}C$ analysis on IRMS; this could create additional uncertainties depending on the isotopic composition of products and on the derivatisation efficiency, decreasing the precision of results compared to bulk $\delta^{13}C$ analyses (Rieley, 1994; Gross and Glaser, 2004).

We conclude that any specification of the estimations of $\delta^{13}C$ values of the 'endmembers', specification of the fractionation values or even specification of individual substances as 'endmembers' would decrease the uncertainties of partitioning studies based on ^{13}C natural abundance.

5. Conclusions

Most C transformations at the root–microorganisms–soil interface such as root respiration, formation of DOC, microbial utilization of DOC and SOM as well as microbial respiration result in significant changes of C isotopic signatures of the product pool compared to the source pool. The ^{13}C fractionation within individual steps of C transformation is highly variable and variability is in some cases

(e.g. for $\Delta(CO_2\text{-SOM})$) almost as high (up to 14‰) as the difference between the $\delta^{13}C$ values of C_3 and C_4 derived 'endmembers' commonly used in natural-^{13}C-labelling studies. This makes it inappropriate to accept literature data about possible changes of $\delta^{13}C$ within the processes. Rather, the discrimination should be measured for the specific conditions of the experiment.

Simple calculations of statistical errors in partitioning studies based on the natural-^{13}C-labelling approach showed high uncertainties of the results. This reflects small differences of $\delta^{13}C$ values between the 'endmembers', high natural variation of $\delta^{13}C$ values within the 'endmembers' and the mixed pool, uncertainties of ^{13}C fractionation, heterogeneity of the soil microorganisms, and preferential substrate utilization. This calls for caution in interpreting the results obtained using the natural-^{13}C-labelling approach.

Certain experimental possibilities can help reduce the uncertainties in natural-^{13}C-labelling studies. Increasing the difference of the $\delta^{13}C$ values of the 'endmembers' is the most promising approach. This can be easily achieved by artificial labelling with highly enriched substances.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.soilbio.2010.04.009.

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