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Turnover of soil organic matter and of microbial biomass under C_3–C_4 vegetation change: Consideration of ^{13}C fractionation and preferential substrate utilization

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ABSTRACT

Two processes contribute to changes of the $\delta^{13}C$ signature in soil pools: ^{13}C fractionation *per se* and preferential microbial utilization of various substrates with different $\delta^{13}C$ signature. These two processes were disentangled by simultaneously tracking $\delta^{13}C$ in three pools – soil organic matter (SOM), microbial biomass, dissolved organic carbon (DOC) – and in CO_2 efflux during incubation of 1) soil after C_3–C_4 vegetation change, and 2) the reference C_3 soil.

The study was done on the Ap horizon of a loamy Gleyic Cambisol developed under C_3 vegetation. *Miscanthus giganteus* – a perennial C_4 plant – was grown for 12 years, and the $\delta^{13}C$ signature was used to distinguish between 'old' SOM (>12 years) and 'recent' *Miscanthus*-derived C (<12 years). The differences in $\delta^{13}C$ signature of the three C pools and of CO_2 in the reference C_3 soil were less than 1‰, and only $\delta^{13}C$ of microbial biomass was significantly different compared to other pools. Nontheless, the neglecting of isotopic fractionation can cause up to 10% of errors in calculations. In contrast to the reference soil, the $\delta^{13}C$ of all pools in the soil after C_3–C_4 vegetation change was significantly different. Old C contributed only 20% to the microbial biomass but 60% to CO_2. This indicates that most of the old C was decomposed by microorganisms catabolically, without being utilized for growth. Based on $\delta^{13}C$ changes in DOC, CO_2 and microbial biomass during 54 days of incubation in *Miscanthus* and reference soils, we concluded that the main process contributing to changes of the $\delta^{13}C$ signature in soil pools was preferential utilization of recent versus old C (causing an up to 9.1‰ shift in $\delta^{13}C$ values) and not ^{13}C fractionation *per se*.

Based on the $\delta^{13}C$ changes in SOM, we showed that the estimated turnover time of old SOM increased by two years per year in 9 years after the vegetation change. The relative increase in the turnover rate of recent microbial C was 3 times faster than that of old C indicating preferential utilization of available recent C versus the old C.

Combining long-term field observations with soil incubation reveals that the turnover time of C in microbial biomass was 200 times faster than in total SOM. Our study clearly showed that estimating the residence time of easily degradable microbial compounds and biomarkers should be done at time scales reflecting microbial turnover times (days) and not those of bulk SOM turnover (years and decades). This is necessary because the absence of C reutilization is a prerequisite for correct estimation of SOM turnover. We conclude that comparing the $\delta^{13}C$ signature of linked pools helps calculate the relative turnover of old and recent pools.

1. Introduction

Evaluating the stability of soil carbon (C), which is the major C stock in terrestrial ecosystems (Batjes, 1996; Amundson, 2001), is complicated because soil organic matter (SOM) consists of various heterogeneous pools with different stability and turnover rates. Some recent approaches for evaluating the stability of SOM pools are based on natural differences in $\delta^{13}C$ isotopic signature after C_3–C_4 vegetation change (Balesdent and Mariotti, 1996; Flessa et al., 2000; Werth and Kuzyakov, 2008; Kramer and Gleixner, 2006, 2008; Cheng, 2009) or after long-term experiments with ^{13}C-depleted CO2 enrichment (i.e. FACE, Van Kessel et al., 2000; Glaser et al., 2006). Other approaches examine changes in the stable isotope composition of mixed C_3/C_4 soils during long-term incubation experiments (Wynn and Bird, 2007; Millard et al., 2008; Coyle et al., 2009).

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The application of ^{13}C natural abundance to trace C mineralization pathways assumes that the $\delta^{13}C$ signature of the SOM mineralization products (such as CO_2 and DOC) is similar to those of the substrate. The $\delta^{13}C$ shift, however, can occur during biochemical reactions in soil. This shift may be related to two different processes (Werth and Kuzyakov, 2010): 1) ^{13}C isotopic fractionation *per se*, which is the preferential usage by microorganisms of substances with light ^{12}C versus the identical substances with heavy ^{13}C (Boschker and Middelburg, 2002); and 2) preferential utilization of easily versus low available substrates having different ^{13}C isotopic composition. The second process is considered here as preference for heavy ^{13}C-substrate of high availability versus light ^{12}C of low availability. Such a preference is common in the utilization of plant residues, where easily available compounds (sugars, cellulose) are ^{13}C-enriched compared to less available compounds (lignin, lipids) (Hobbie and Werner, 2004).

Both processes (isotopic fractionation and preferential substrate utilization) occur simultaneously, causing different isotopic compositions of C pools. Both enrichment (Santruckova et al., 2000; Werth and Kuzyakov, 2009) and depletion (Bol et al., 2003) in ^{13}C were observed by natural isotope technique in CO_2 and DOC pools as related to SOM and microbial biomass. Several studies also suggest that ^{13}C discrimination during soil respiration is negligible (Ekblad et al., 2002). This calls for further investigations to evaluate the contribution of isotopic fractionation in ^{13}C enrichment during SOM decomposition. This evaluation is crucial, especially in studies of SOM decomposition induced by the input of available substrate.

Distinguishing of isotopic fractionation *per se* and preferential substrate utilization is hampered in most soils because they are in or close to *steady state* concerning the C content as well as isotopic composition. The *isotopic steady-state* means that no changes in isotopic composition of individual pools occur, and that the differences between the isotopic compositions of the pools are nearly constant over time despite the presence of fractionation and/or preferential substrate utilization. The effect of these two processes on the isotopic composition of the pools can be determined by adding slightly enriched or depleted ^{13}C-labeled mixed substrates to the soil. However, significant altering the isotopic composition of pools against the natural abundance background requires using high amounts of the substrates. This will outbalance the C steady state, especially of those pools with a fast turnover, such as microbial biomass. Note here that the definitions of steady state for SOM and for microbial biomass differ. A *steady state for SOM* is implied when C input to the soil equals C output as CO_2 at annual or longer basis, i.e. C content does not change during the period of mean residence time (MRT) estimation. The *microbial steady state* means that amount of microbial biomass does not change during the period of estimation of microbial turnover time (MTT), i.e. the amount of newly-formed microbial biomass equals the amount of died-off biomass. According to the definitions, the MRT for SOM can be estimated over long periods (years, decades), whereas MTT are on the order of days (Herman et al., 2006). We therefore chose the combination of two approaches: 1) natural isotope technique based on difference in ^{13}C enrichment in 12 years after C_3 and C_4 vegetation change which allowed differentiating between the "old" and "recent" soil organic matter and 2) laboratory incubation of C_3–C_4 and of C_3 soil to evaluate preferential utilization of recent versus old C by microorganisms as well as isotopic fractionation occurring during 54 days of incubation experiment. This allowed us to estimate the relative C turnover time for both SOM and microbial biomass.

The estimation of turnover by $\delta^{13}C$ natural abundance tracer techniques is based on organic matter changes after the C_3–C_4 vegetation change on a decadal to centennial scale. The MRT values estimated for bulk organic matter in soils after C_3–C_4 crop change usually do not exceed 40–50 years (Glaser, 2005; Marschner et al., 2008). A discrepancy occurs, however, when long-term natural isotope labeling techniques are applied to estimate the MRT of those readily bioavailable SOM compounds usually used as biomarkers. Such estimations often reveal unexpectedly long residence times for specific components of microbial or plant biomass (carbohydrates, proteins, and phospholipid fatty acids – PLFAs) similar to that of bulk SOM (as reviewed by Amelung et al., 2008; Marschner et al., 2008). Thus, the 10–125 year MRT values estimated for PLFA by the $\delta^{13}C$ for the 8–90-year period of vegetation change seem to be incorrect (Amelung et al., 2008) because PLFA are found only in viable cells and thus are characteristic biomarkers for living biomass (Kramer and Gleixner, 2006; Denef et al., 2009). We believe that this discrepancy between long MRT and quick decomposability of microbial constituents can be eliminated if a time scale comparable with microbial turnover is used to estimate MRT for microbial biomass and for easily decomposed microbial compounds.

The comparative estimation of microbial biomass turnover based on the $\delta^{13}C$ shift 1) during long-term vegetation change and 2) during short-term incubation is necessary in the same experiment to explain the discrepancy between MRT's found for microbial biomarkers and for bulk SOM.

The present study evaluates the contribution of both processes: ^{13}C fractionation *per se* and preferential substrate utilization to the shifts in $\delta^{13}C$ during SOM decomposition and microbial turnover in soil after C_3–C_4 vegetation change. This involved comparing the $\delta^{13}C$ signatures of recent and old C pools in soil after C_3–C_4 vegetation change with the reference soil without crop-induced changes in isotopic composition. The relative turnover of recent and old C in SOM and microbial biomass pools was estimated by tracing the $\delta^{13}C$ changes in these pools during incubation.

2. Materials and methods

2.1. Soil sampling and preparation

Soil (loamy Gleyic Cambisol, WRB, 1998) was sampled from the upper layer (0–10 cm) of the fields under *Miscanthus giganteus* ($C_{org} = 2.4 \pm 0.1\%$; $N_{total} = 0.20 \pm 0.01\%$, pH ($CaCl_2$) 5.1) and under adjacent grassland ($C_{org} = 2.1 \pm 0.2\%$; $N_{total} = 0.21 \pm 0.01\%$, pH ($CaCl_2$) 5.1) at the experimental station of the University of Hohenheim, Baden-Württemberg, Germany (48°43′N, 9°13′E). The C_4-plant *Miscanthus giganteus* (Greef et Deu) ($\delta^{13}C = -11.8‰$) was grown for 12 years after grassland, causing a shift in the $\delta^{13}C$ of SOM from −27‰ to −19‰. This difference in $\delta^{13}C$ was used to distinguish between SOM older and younger than 12 years. We use the term 'recent' C for the C_4-C originated from *M. giganteus* because it is not older than 12 years. In contrast, for the C originated from previous C_3 vegetation, which is 12 years old or even much older, we use the term 'old' C in this study.

The soil was stored field-fresh in aerated polyethylene bags at 4 °C for a maximum of 6 weeks after sampling. Prior to the experiment, samples were sieved (<5 mm) and fine roots and other plant debris were carefully removed. Twenty-gram (dry weight) sub-samples were weighed and put into 250-ml Schott-jars. The moisture content was adjusted to 50% of the water holding capacity (WHC), and then the soil was pre-incubated at 22 °C for 24 h.

2.2. Experiment design and soil incubation

Before the experiment, distilled water was added to the soil to reach the final soil moisture content of 60% WHC. Soil under adjacent grassland without C_3–C_4 vegetation change was used as a reference soil to estimate the $\delta^{13}C$ shifts between the pools caused

by isotopic fractionation. After adding distilled water to the soil, 3 mL of 1 M NaOH in small vials was placed in the incubation vessels to trap CO_2. The vessels were then closed air tight and incubated for 54 days at 22 °C at 60% WHC. At 1, 3, 5, and 7 days after soil moistening and thereafter weekly, the vials were removed and replaced by vials with a new 3 ml aliquot of 1 M NaOH. After 3, 7, 14, 33, and 54 days of treatment, three replicate incubation vessels were destructively sampled for chemical and $\delta^{13}C$ analyses and to estimate microbial biomass.

2.3. Microbial biomass and DOC

Soil microbial biomass was determined by the chloroform fumigation extraction (FE) method (modified after Vance et al., 1987). After destructive sampling or at the end of the experiment, the soil was carefully mixed and DOC from 10 g soil was extracted with 0.05 M K_2SO_4 in a 1:4 ratio. Another 10 g of soil were firstly fumigated with chloroform for 24 h and then extracted in the same way. The extracts were frozen until analyses for total C concentrations on a TOC/TIC analyzer (Multi N/C 2100, Analytik Jena, Germany).

The total amount of extractable microbial C (C_{MB}) was determined by the difference between K_2SO_4-extractable C in fumigated and non-fumigated soil. The k_{ec} factor was not used to estimate microbial biomass by the FE technique in order to avoid the uncertainties due to instability of k_{ec} values over time and in different treatments. The Figures and Tables therefore consider only the contribution of different C sources to the extractable part of microbial biomass. To estimate microbial turnover time (MTT) and specific microbial turnover, however, a k_{ec} value of 0.20 was used to correct the obtained MTT for comparative purposes. The k_{ec} value was estimated for the C_3–C_4 soil in a preliminary experiment based on ^{14}C budget according to Blagodatsky et al. (2010).

2.4. Chemical and isotopic analyses

CO_2 trapped in NaOH solution was precipitated with 0.5 M $BaCl_2$ solution. The excess NaOH was then titrated with 0.1 M HCl using the phenolphthalein indicator (Zibilske, 1994).

Since only solid samples could be analyzed by the isotope ratio mass spectrometer (IRMS), the CO_2 and microbial biomass samples had to be specifically prepared. CO_2 trapped as Na_2CO_3 in 3 ml of NaOH was precipitated with 0.5 M $SrCl_2$ aqueous solution. The NaOH solutions containing the $SrCO_3$ precipitants were then centrifuged three times at 1450g for 10 min and washed in between with deionized and degassed water to remove NaOH and to reach a pH of 7. After washing, the remaining water was removed from the vials and the $SrCO_3$ was dried at 105 °C. The $SrCO_3$ was analyzed for $\delta^{13}C$ values on the IRMS (Delta Plus XL IRMS, Thermo Finnigan MAT, Bremen, Germany). For the DOC and for the microbial biomass pools, an aliquot of the K_2SO_4 samples was pipetted directly into tin capsules and dried at 60 °C prior to IRMS analyses (Brant et al., 2006).

2.5. Calculations and statistics

A mass balance equation (Balesdent and Mariotti, 1996) was used to determine the $\delta^{13}C$ value of total microbial biomass ($\delta^{13}C_{MB}$):

$$\delta^{13}C_{MB} = \left(\delta^{13}C_f \cdot C_f - \delta^{13}C_{nf} \cdot C_{nf}\right) \Big/ \left(Cf - C_{nf}\right) \quad (1)$$

where $\delta^{13}C_f$ and $\delta^{13}C_{nf}$ are the $\delta^{13}C$ values of the fumigated and non-fumigated samples, respectively; and C_f and C_{nf} are the amounts of C in the fumigated and non-fumigated K_2SO_4 samples, respectively.

The portion (F) of *Miscanthus*-derived C in total SOC, CO_2, microbial biomass, and in soluble C was calculated according to Amelung et al. (2008):

$$F = \left(\delta^{13}C_t - \delta^{13}C_3\right) \Big/ \left(\delta^{13}C_4 - \delta^{13}C_3\right) \quad (2)$$

where $\delta^{13}C_t$ is the $\delta^{13}C$ value of the C pool under *M. giganteus* and $\delta^{13}C_3$ is the $\delta^{13}C$ value of the corresponding C pool in reference soil with continuous C_3 vegetation. $\delta^{13}C_4$ was calculated based on the $\delta^{13}C$ value of *M. giganteus* (mean of root, shoot, and leaves) and corrected for isotopic fractionation during humification by subtracting the differences between $\delta^{13}C$ of C_3 vegetation and $\delta^{13}C$ of SOC of the C_3 soil. This approach assumes equal isotopic fractionation by humification of C_3 plants and C_4 plants (Schneckenberger and Kuzyakov, 2007).

The $\delta^{13}C$ value of microbial biomass derived from old SOM ($\delta^{13}C_{C3\_MB}$) was calculated according to Eq. (1) for reference soil. The amount of microbial biomass derived from recent SOM (C_{C4_MB}) was calculated as

$$C_{C4\_MB} = C_{MB} \cdot F \quad (3)$$

where C_{MB} is the total amount of SOM-derived C in microbial biomass. The amount of microbial biomass derived from old SOM (C_{C3_MB}) is then:

$$C_{C3\_MB} = C_{MB} - C_{C4\_MB} \quad (4)$$

Since total C content did not change during 12 years of *M. giganteus* growth, its mean residence time (MRT) was calculated based on the fraction of old C (1-F) and assuming steady-state conditions (Amelung et al., 2008):

$$MRT = -T/\ln(1 - F) \quad (5)$$

where T is time.

The turnover time of microbial biomass C (MTT) was calculated according to Cheng (2009) assuming a microbial substrate utilization efficiency $Y = 0.45$ (Qian et al., 1997) and a soil microbial maintenance respiration rate R_m of 0.08% of the biomass day^{-1} (Anderson and Domsch, 1985):

$$MTT = (MB \cdot (1 - Y)/Y)/(R_s - MB \cdot R_m), \quad (6)$$

where MB is microbial biomass C; R_s is the microbial respiration rate.

The experiment was conducted with three replicates for every treatment. Standard errors (SE) were calculated as a variability parameter. The effect of C transformation on $\delta^{13}C$ signature was assessed by one-way ANOVA. The Fischer LSD post hoc test was used to evaluate the significance of differences at $p < 0.05$.

3. Results

3.1. $\delta^{13}C$ in carbon pools in the reference soil before and during incubation

The $\delta^{13}C$ values of three pools – SOM, microbial biomass, DOC – and in the CO_2 efflux in the reference soil varied in rather narrow interval from −25.8 to −27.3‰ (Fig. 1, top). At the beginning of incubation microbial C was significantly ^{13}C enriched (by 0.91‰) compared with SOM-C. At the same time CO_2–C was depleted by 0.56‰ compared with microbial C. The temporal variations in $\delta^{13}C$ signature of C pools were significant only for microbial biomass and amounted up to 1.5‰.

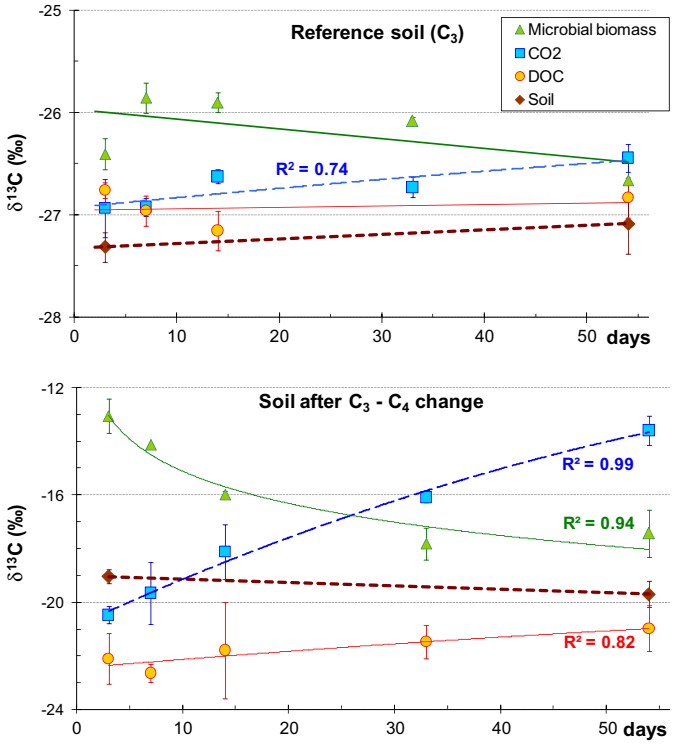

Fig. 1. $\delta^{13}C$ dynamics in C pools and in CO_2 during incubation of the reference C_3 soil (top) and the soil after C_3–C_4 vegetation change (bottom). Bars indicate standard errors, $n = 3$. R^2 are presented only for significant ($p < 0.05$) relationships.

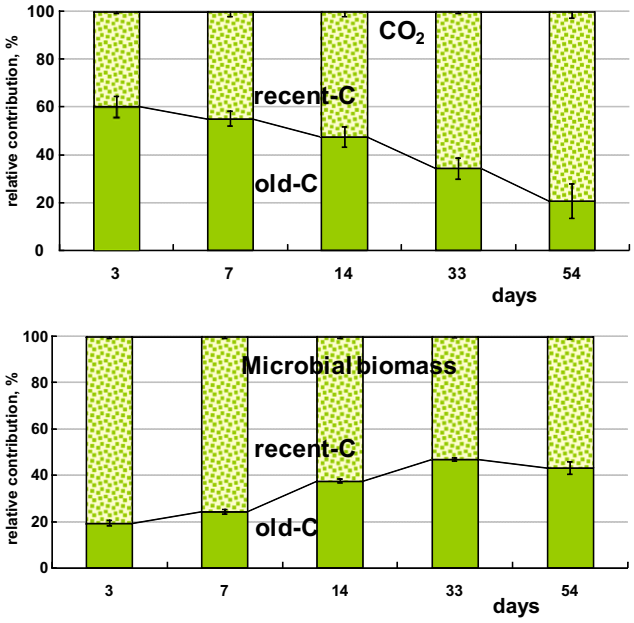

Fig. 2. Relative contribution of recent (C_4) and old C (C_3) to CO_2 (top) and to microbial biomass (bottom) during 54 days of incubation of C_3–C_4 soil. Bars indicate standard errors.

After 54 days of incubation the difference in $\delta^{13}C$ between C pools was very small and varied within 0.6‰. The slight, insignificant increase in ^{13}C–CO_2 during the incubation was observed as a weak trend. Thus, no significant differences in $\delta^{13}C$ signature of C pools were observed between 3rd and 54th day of incubation (Fig. 1, top). Based on the $\delta^{13}C$ values in the reference soil, we conclude that ^{13}C fractionation was <1‰ for the C pools with contrast turnover intensity.

3.2. Dynamics of $\delta^{13}C$ in soil under Miscanthus and contribution of recent and old C to three C pools

The growth of *M. giganteus* after C_3 grassland caused a strong $\delta^{13}C$ increase in all soil C pools (compare top and bottom panels on Fig. 1). This increase was higher in microbial biomass (>13‰) than the increase in total SOM (8.3‰), indicating a higher contribution of recent C to microbial biomass (Fig. 1, bottom). The $\delta^{13}C$ of the CO_2 efflux and of the DOC at the early stage (third day) of incubation was depleted by 1.5 and 3.1‰, respectively, as compared with SOM. The $\delta^{13}C$ values of SOM and DOC were nearly constant during the incubation (Fig. 1, bottom). The $\delta^{13}C$ of CO_2 increased from −20.5 to −13.6‰ during incubation. At the same time, the $\delta^{13}C$ of microbial biomass continuously decreased from −13 to −17.4‰ (Fig. 1, bottom).

Based on these changes of $\delta^{13}C$ values, we calculated the contribution of recent (<12 years) and old C (>12 years) to the C pools (Eq. (3)). According to F-fraction (Eq. (2)), the recent and old C contributed almost equally (51 and 49%, respectively) to SOM under *M. giganteus*. The contribution of recent C to CO_2 efflux increased from 40% at the beginning to 60–80% at the late stage (33–54 days) of incubation (Fig. 2, top). In contrast, microbial biomass consisted to 82% of recent C originated from *M. giganteus* (Fig. 2, bottom) at the beginning of experiment. The increased respiration of recent C corresponds to a 40% decrease in total microbial biomass C (Fig. 3) during the first week of incubation. We therefore conclude that most of the recent C in CO_2 was released by the reutilization of microbial biomass C.

The contribution of recent C_4-C to DOC was 29–32%. The amount of soluble C_4-C was always smaller than the corresponding microbial C_4-C (Table 1). The relatively low contribution of recent C to the DOC indicates that soluble organics originated from C_4-C are preferably decomposed compared to old organics with C_3 signature.

3.3. Mean residence time of C in SOM and turnover time of microbial biomass during incubation

The insignificant differences in C content between C_3–C_4 and reference C_3 soil indicated similar C accumulation under *M. giganteus* and under perennial grasses. After the *M. giganteus* was planted, the SOM content was invariable neither in the *M. giganteus* plots nor under the reference plots, so the steady-state conditions required to correctly estimate MRT were assumed for SOM. Accordingly, the C mean residence time estimated based on

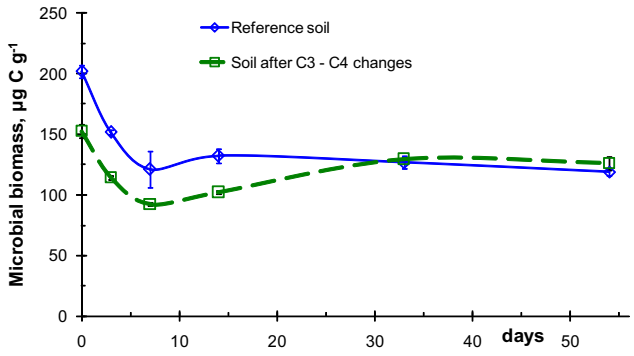

Fig. 3. Dynamics of total microbial biomass during incubation of C_3–C_4 and reference soils. Bars indicate standard errors.

Table 1
Contribution of C_4- and C_3-derived C to CO_2 efflux, microbial biomass (MB), and DOC, along with microbial turnover time (MTT) in soil. MTT was estimated according to Eq. (6) at the time interval from 33 to 54 days of incubation when no changes in the total amount of microbial biomass were observed. Values are means ± standard errors. In each column, values with different letters are significantly different ($P < 0.05$).

C source	MB, μg C g^{-1} soil		CO_2, μg CO_2–C g^{-1} soil day^{-1}		DOC, μg C g^{-1} soil		MTT, days
	Early stage	Late stage	Early stage	Late stage	Early stage	Late stage	Late stage
C_3–C_4 soil							
Total	$114.4^{b} \pm 1$	$126.1^{a} \pm 3.2$	$21.8^{a} \pm 0.8$	$26.6^{a} \pm 1$	$47.4^{a} \pm 2.5$	$38.3^{a} \pm 0.3$	29.5*
C_4	$94.3^{c} \pm 0.2$	$70.4^{b} \pm 1.5$	$8.8^{c} \pm 0.1$	$21.1^{b} \pm 0.8$	$13.6^{c} \pm 0.3$	$12.4^{c} \pm 0.1$	20.7
C_3	$20.1^{d} \pm 1.7$	$55.7^{c} \pm 3.5$	$13.0^{b} \pm 0.8$	$5.5^{c} \pm 1.6$	$33.8^{b} \pm 2.5$	$25.9^{b} \pm 0.3$	64.5
Ratio C_4/C_{total}	0.82	0.56	0.40	0.79	0.29	0.32	
C_4 (late/early stage)	0.68		1.97		1.10		
Ratio C_3/C_{total}	0.18	0.44	0.60	0.21	0.71	0.68	
C_3 (late/early stage)	2.44		0.35		0.96		
Ratio C_4/C_3	0.27 (3.69-inverse**)		5.63		1.15		
Reference C_3 soil							
C_3	$151.7^{a} \pm 1.1$	$119^{a} \pm 1.6$	$25.6^{a} \pm 2.5$	$26.3^{a} \pm 1.5$	$40.8^{a} \pm 0.8$	$39.9^{a} \pm 1.5$	29.1

* Note that the estimations of MTT values were based on data for late stage of incubation as microbial steady state was observed only for the period from 33 to 54 days of incubation experiment (Fig. 3).
** Inverse proportion C_3/C_4 is more indicative here as relative contribution of C_3-C in microbial biomass increased while those of C_4-C decreased during incubation.

the proportions of old and recent C in SOM 12 years after the C_3–C_4 vegetation change (Eq. (5)) was 16.8 years.

As an alternative to this approach, we estimated the microbial turnover time (MTT) based on the amount of CO_2–C released during the incubation compared to the amount of microbial biomass C (Eq. (6)). As steady-state conditions are required to correctly estimate MTT, the whole incubation period (54 days) was not suitable because microbial biomass decreased during the first week of incubation (Fig. 3). We therefore used the period from 33 to 54 days to estimate MTT because during this period both total microbial C and the total CO_2 evolution rate were nearly constant, indicating a steady state for microbial biomass in both soils (Fig. 3, Table 1). This approach yielded a similar MTT for C_3–C_4 (29.5 days) and C_3 soils (29.1 days). The turnover of old microbial C in soil under *M. giganteus* was twice as slow as of total microbial C and three times as slow that as of recent microbial C (Eq. (6), Table 1).

3.4. Relative turnover rates of recent and old C estimated within microbial biomass and by CO_2 efflux

The CO_2 efflux rates from soil at the early and at the late stage of incubation were nearly the same (Table 1). The contribution of recent C to total respired C was smaller than the contribution of old C_3-C at the early stage of incubation (Table 1). The relative contribution of recent C_4-derived C to total CO_2, however, increased from $8.8/(13 + 8.8) = 0.40$ to $21.1/(5.5 + 21.1) = 0.79$ (calculation based on data from Table 1), i.e. almost doubled during the incubation ($0.79/0.40 = 1.97$, Table 1, Fig. 2, top). Analogously, the contribution of old C to total CO_2 decreased from 0.60 to 0.21, i.e. changed by a factor of 0.35. The change in relative contribution of recent C was higher than corresponding change in relative contribution of old C for respired CO_2 by a factor $1.97/0.35 = 5.63$. Similar calculations for microbial biomass (inverse proportion C_3/C_4) give ratio 3.69. We designate this factor as a "relative change in turnover rate" which helps to compare the changes in turnover of microbial biomass, DOC and CO_2 by the data obtained from different studies. Reutilization of microbial biomass and preferential utilization of recent versus old C by soil microorganisms caused strong changes of $\delta^{13}C$ in the CO_2 efflux and in microbial biomass within the 54-day incubation. We therefore conclude that microbial biomass has the faster turnover rate and the shortest MRT when compared with MRT of SOM estimated according to Eq. (5).

4. Discussion

4.1. Contribution of isotope fractionation and preferential substrate utilization to the $\delta^{13}C$ signature

The contribution of isotope fractionation and preferential utilization of recent versus old C was distinguished by comparing the $\delta^{13}C$ values during the incubation of reference (C_3) and *Miscanthus* (C_3–C_4) soil. The differences in $\delta^{13}C$ signature between substrates, microbial biomass and microbial metabolites reflect preferential substrate utilization only if the isotope composition of individual substrates within a mixture is not uniform (Werth and Kuzyakov, 2010). We accept here that the $\delta^{13}C$ differences between the pools in the reference soil are related to ^{13}C fractionation because this soil is under isotopic steady state. In contrast, the soil after the C_3–C_4 vegetation change is not under isotopic steady state, but it does remain under C steady state because the C stock has not changed in the last 12 years (Schneckenberger and Kuzyakov, 2007). Therefore, both processes – ^{13}C fractionation and preferential utilization of recent versus old C – contribute to different $\delta^{13}C$ values between the pools in the C_3–C_4 soil.

The $\delta^{13}C$ of C pools in the reference soil remained within a very narrow range of 1‰ (Fig. 4, left). The ^{13}C enrichment of microbial C (0.9‰) was somewhat lower but still comparable with the 1.4–2‰ ^{13}C enrichment found for microbial biomass in other studies (Santruckova et al., 2000; Potthoff et al., 2003; Coyle et al., 2009). The differences in $\delta^{13}C$ between C pools in the reference soil, however, did not exceed the 5% level of significance (except microbial biomass). In agreement with Ekblad et al. (2002), we conclude that isotopic fractionation *per se* in our study was negligible. Even though isotopic fractionation was minimal, it's neglecting can cause up to 10% errors in caluculations. So, we made corrections for the differences of $\delta^{13}C$ values between the pools when calculating the preferential substrate utilization for C_3–C_4 soil.

The differences in $\delta^{13}C$ between C pools in C_3–C_4 soil were mainly due to preferential utilization of recent (^{13}C-enriched) versus old C (relatively ^{13}C-depleted) sources. This caused a 6‰ ^{13}C enrichment of microbial biomass and a 1.4‰ ^{13}C depletion of CO_2 efflux compared with SOM (Fig. 4, right). The $\delta^{13}C$ increase caused by change to C_4 vegetation was most pronounced in microbial biomass, while it was rather weak for the DOC. This reflects the quick selective microbial uptake of available substrates from DOC

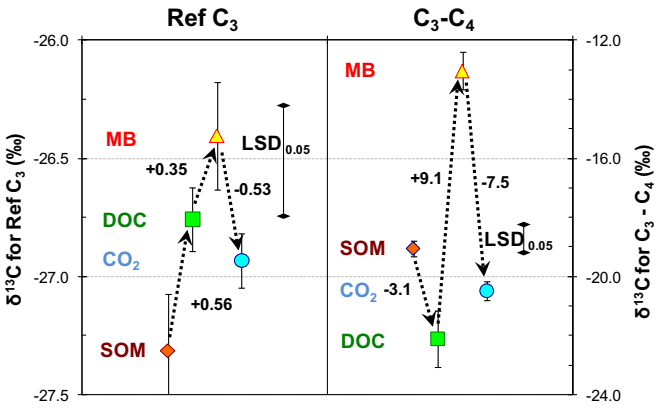

Fig. 4. Contribution of fractionation and preferential substrate utilization to changes in the $\delta^{13}C$ signature of C pools in soil at the third day of incubation experiment. ^{13}C fractionation values are presented based on $\delta^{13}C$ of the reference soil; values for preferential utilization of recent versus old C are presented based on $\delta^{13}C$ of C pools in the soil after C_3–C_4 vegetation change. Note different scales for ^{13}C fractionation (left) and preferential substrate utilization (right). The whiskers present standard errors. We assumed that the transformations go in the following direction: SOM DOC MB CO_2. See text for explanations.

originated from root exudates and plant residues (Esperschütz, 2009). As such substrates are readily available; they are rapidly incorporated into microbial biomass (see the $\delta^{13}C$ shift) and can be observed in the form of carbohydrates and proteins (Bol et al., 2009). A similar higher enrichment of microbial biomass compared to the water-soluble C pool with easily available substrates was previously observed in rhizosphere soil (Yevdokimov et al., 2006; Esperschütz et al., 2009).

^{13}C enrichment of microbial biomass and ^{13}C depletion of soluble C as compared with total soil C agrees with the study of Coyle et al. (2009) on long-term ecosystem development. We explain this difference in ^{13}C enrichment by the selective consumption of C_4-C compounds by microorganisms from DOC. The soluble C was produced mainly from two sources – low available C_3-SOM and the intermediate decomposition products of C_4-plant residues and rhizodeposits. As the organic substrates from the second source are rapidly utilized by microorganisms (Bol et al., 2003; Yevdokimov et al., 2006; Esperschütz et al., 2009), the remaining soluble C originated from SOM and is strongly ^{13}C-depleted compared with microbial biomass. Thus, the old and young DOC pools are not uniformly available for microorganisms (Kalbitz et al., 2003). The more available portion of C_4-derived DOC with a heavier isotopic composition was intensively involved in microbial metabolism (Bird et al., 2002; Pelz et al., 2005; Piao et al., 2006) and therefore amounted to a smaller part (about 30%) of DOC. This confirms our previous observations that most DOC (up to 60%) is less available than readily available substrates such as glucose (Blagodatskaya et al., 2009). The contribution of preferential utilization was about 6 times higher than that of ^{13}C fractionation (cf. left and right schemes on Fig. 4). Therefore, such a utilization of ^{13}C-enriched substrates can counterbalance and even exceed the effect of isotope discrimination *per se*. This is confirmed for example by the fermentation of glucose, leading to strong differences between $\delta^{13}C$ of CO_2 and ethanol (up to 9‰). In this case both products originate from C of different molecule positions, and those C atoms strongly differ in their $\delta^{13}C$ signature (up to 11‰) (reviewed by Hobbie and Werner, 2004). Clearly, the contribution of preferential utilization of recent versus old C to the shift in the isotopic signature of the products strongly depends on the $\delta^{13}C$ difference between individual substances in the mixture. This contribution would be much higher in case of using highly ^{13}C-enriched substances. We used the natural differences, however, because these frequently occur and are commonly used in studies based on C_3–C_4 vegetation change.

The contribution of preferential utilization of recent versus old C to the $\delta^{13}C$ shift in microbial biomass was the highest during microbial metabolism at an early stage of incubation. In the CO_2 efflux, however, this preferential utilization mostly contributed to the $\delta^{13}C$ shift during reutilization of C_4-microbial C at late incubation stage (Fig. 1, bottom). Thus, the ^{13}C discrimination by heterotrophic microorganisms depends on fractionating stages (substrate uptake, microbial metabolism, reutilization) and on the physiological state of the microorganisms (Zyakun, 1996; Coyle et al., 2009). Considering preferential utilization, the $\delta^{13}C$ differences between substrate/SOM and microbial C/CO_2–C found in several studies (Santruckova et al., 2000; Potthoff et al., 2003; Pelz et al., 2005; Murage and Voroney, 2007; McGoldrick et al., 2008; Coyle et al., 2009; Werth and Kuzyakov, 2010; current study) can be explained by two factors: the interactive effects of the isotope discrimination during biosynthesis/reutilization of biomass, and the isotopic composition of organics being preferentially used by soil microorganisms at different metabolism stages (Hobbie and Werner, 2004).

As in other incubation studies (Wynn and Bird, 2007; Bowling et al., 2008), $\delta^{13}C$ of CO_2 in our experiment was initially depleted (relatively to SOM) and became enriched at the end of incubation. The opposite trends observed in various studies can be explained by the $\delta^{13}C$ dynamics of respired CO_2. Thus, the *^{13}C depletion* (Bol et al., 2003; Amelung et al., 2008) of respired versus soil C corresponded in our study to the early stage of incubation (Fig. 1 bottom). The *^{13}C enrichment* in CO_2 as compared with microbial biomass and SOM (Santruckova et al., 2000; Werth and Kuzyakov, 2009) was also confirmed by our study at the end of incubation (Fig. 1 bottom). The *absence of ^{13}C discrimination* during microbial respiration (Ekblad et al., 2002) was observed in reference soil, which is under isotopic steady state (Fig. 1 top).

The decrease in total microbial biomass without permanent input of fresh C_4-C (Fig. 3) was accompanied by a decreasing relative contribution of C_4-C to microbial biomass (Fig. 2, bottom). The increasing contribution of ^{13}C-enriched CO_2 to total respired CO_2 can therefore be related to the reutilization of the C_4-microbial pool. Thus, in the absence of new C input, the microorganisms preferentially utilize recent C already incorporated in microbial cells. So, the absence of C reutilization is a prerequisite for correct estimation of SOM turnover. The ^{13}C-enriched biomass losses are linked with CO_2 production and faster turnover of microbial biomass, which consumes C_4 sources. Accordingly, C from sources with different availability shows different turnover rates within microbial biomass. This is a clear functional confirmation that microbial biomass consists of various groups utilizing C at different rates.

4.2. Turnover of recent and old C estimated by different approaches

We estimated the C turnover in different pools by three approaches: 1) SOM turnover was evaluated based on substituting C_4-C by C_3-C as related to the time elapsed since the vegetation change (Balesdent and Mariotti, 1996); 2) turnover of microbial biomass was assessed by total CO_2 efflux related to the amount of microbial C during the incubation (Cheng, 2009), and 3) relative turnover of old and recent C was compared based on $\delta^{13}C$ changes in microbial biomass, DOC and in CO_2 efflux during incubation.

1) *SOM turnover evaluated based on C_3–C_4 vegetation change*. As the C content of the soil remained unchanged for the last 12 years, the MRT of the SOM calculated by Eq. (4) was 16.8 years. This is 5.8 years longer than the MRT estimated for the same soil three years earlier: 9 years after C_3–C_4 vegetation change,

the MRT was 11 years (Schneckenberger and Kuzyakov, 2007). Thus, during the last three years the MRT of SOM was extended by 5.8 years, i.e. every further year of *M. giganteus* growth the MRT of SOM increased by *ca.* 2 years. This MRT increase reflects the formation of a slow turnover C_4 pool from recent *Miscanthus*-derived C (Amelung et al., 2008) and progressive aging of remaining C_3-SOM due to utilization of remaining available C_3 sources.

2) *Microbial biomass turnover.* The turnover of microbial biomass assessed by CO_2 efflux related to the microbial C was 29–30 days. This MTT very closely resembles the 24-day microbial turnover time reported in Ajtay et al. (1979) and is within the 21–75 day range given in Cheng (2009). If microbial biomass is considered as an inert pool of SOM, then MRT for this pool can be estimated using Eq. (2) and (5) and the data presented in Fig. 1 ($\delta^{13}C$ in microbial biomass C in C_3–C_4, and C_3 soils). The obtained value (6.9 years) cannot be considered reliable, however, because 12 years of microbial steady state cannot be assumed under field conditions: microbial biomass is a very labile pool and changes quickly after any disturbance (such as substrate input, temperature or moisture changes etc.). Improbably large MRT values according to the latter calculation approach indicate that the differences in $\delta^{13}C$ between C_3–C_4 and reference C_3 soil in microbial C cannot be calculated based on the whole time elapsed since the vegetation change. This is because microbial biomass turns over much faster than SOM and thus starts to utilize C sources with a mixed C_3/C_4 signature. The MTT as well as the MRT for microbial biomarkers can be over-estimated by inappropriate time scales chosen for MTT or MRT assessment, because during long periods of assessment microorganisms utilize not only the newly added substrate, but also reutilize their own dead cells and SOM with already changed isotopic composition.

3) *Relative turnover of old and recent C in microbial biomass and DOC, and their contribution to CO_2 efflux.* Microbial turnover time was shorter by a factor of 3 for recent C_4-C (21 days) versus old C_3-C (64 days). This agrees with the observations of Wynn and Bird (2007) that the C_4-derived organics (in our study recent C) are more abundant in the labile components, while the more stable components of SOM are biased toward old materials (C_3-derived). As the recent C cycles faster through the soil pool than old C (Wynn and Bird, 2007), we expect that the MRT of recent C in our study was less than 12 years.

SOM turnover estimates during incubation experiments are usually based on basal rates of heterotrophic respiration (Bowling et al., 2008; Paterson et al., 2009). Our study allows the comparison the relative turnover of labile C based on respired CO_2 and on changes in microbial biomass. According to the changes in the C_4-to-C_3 ratio in respired CO_2 in early and late incubation, the relative change in turnover rate of recent C_4-C was *ca.* 6 times faster than of old C_3-C. In microbial biomass, however, the relative change in turnover rate of recent C was only 3.7 times faster than that of old C. This corresponds with MTT calculated by the second approach, which yielded 3 times faster C_4 versus C_3 turnover.

Summarizing the results of the two first approaches, we conclude that microbial C represents a very small pool with a 200 times faster turnover (MTT = 29 days, Table 1) compared with SOM.

5. Conclusions

Based on the $\delta^{13}C$ signature of C pools with contrasting availability in the soils with and without C_3–C_4 vegetation change, as well as on altered $\delta^{13}C$ signatures during incubation, it is possible to disentangle the contribution of two processes: ^{13}C fractionation and preferential utilization of recent versus old C to microbial turnover. The contribution of isotopic fractionation to the $\delta^{13}C$ shifts between various C pools in soil did not exceed 1‰ but it can cause up to 10% of errors in calculations. Nonetheless, preferential substrate utilization as well as the contrasting turnover intensity of individual pools led to strongly differing $\delta^{13}C$ signatures between the pools (up to 9.1‰).

Considering the strong changes of $\delta^{13}C$ signature in microbial biomass and CO_2 during incubation, we concluded high between- and within-C-pool heterogeneity. This was especially pronounced for microbial biomass, whose $\delta^{13}C$ change during incubation was much higher compared to SOM and DOC. We therefore conclude that microbial turnover rates are affected by the substrate to be metabolized and can vary by a factor of 3–4 depending on its availability. The absence of C reutilization is a prerequisite for correct estimation of SOM turnover.

The effect of C_4-originated C on the $\delta^{13}C$ signature was most pronounced in microbial biomass (13.4‰), but was less in CO_2–C (6.4‰) and in the DOC pool (4.7‰). This, along with $\delta^{13}C$ changes during incubation, showed a 6 and 3 times faster contribution of recent C to CO_2 flux and to microbial biomass, respectively. The enrichment of microbial biomass in recent C coupled with the depletion of the DOC pool in recent C confirmed the rapid incorporation of available plant-derived C into microbial biomass. Comparative assessment of C turnover by different approaches clearly indicated that the appropriate time scale (hours to days for microorganisms and their metabolites, while years to decades for SOM) should be chosen for the correct assessment of C turnover for each C pool.

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