Using natural ^{13}C abundances to differentiate between three CO_2 sources during incubation of a grassland soil amended with slurry and sugar

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Summary—Zusammenfassung

This study describes a novel approach to separate three soil carbon (C) sources by one tracer method (here ^{13}C natural abundance). The approach uses the temporal dynamics of the CO_2 efflux from a C_3 grassland soil amended with added C_3 or C_4 slurry and/or C_3 or C_4 sugar to estimate contributions of three separate C sources (native soil, slurry, and sugar) to CO_2 efflux. Soil with slurry and/or sugar was incubated under controlled conditions, and concentration and $\delta^{13}C$ values of evolved CO_2 were measured over a 2-week period. The main assumption needed for separation of three C sources in CO_2 efflux, *i.e.*, identical decomposition of applied C_3 and C_4 sugars in soil, was investigated and proven. The relative contribution to the CO_2 efflux was higher, but shorter with an increased (microbial) availability of the C source, *i.e.*, sugar > slurry > SOM. The shortcomings and limitations as well as possible future applications of the suggested method are discussed.

Key words: ^{13}C natural abundance / CO_2 sources / separation methods / slurry / sugar / soil organic matter / grassland

Anwendung der natürlichen ^{13}C-Abundanz zur Trennung von drei CO_2-Quellen bei der Inkubation eines mit Gülle und Zucker behandelten Graslandbodens

Die Untersuchung beschreibt ein neues Verfahren zur Trennung von drei C-Quellen im Boden mit Hilfe einer Tracermethode (hier die natürliche ^{13}C-Abundanz). Die zeitliche Dynamik des CO_2-Effluxes aus einem C_3-Graslandboden nach der Applikation von C_3- oder C_4-Gülle und/oder C_3- oder C_4-Zucker wurde verfolgt, um die Anteile der drei unabhängigen C-Quellen (organische Bodensubstanz (OBS), Gülle und Zucker) am CO_2-Efflux zu untersuchen. Boden mit Gülle und/oder Zucker wurde unter kontrollierten Bedingungen inkubiert, und die CO_2-Konzentration und ihre $\delta^{13}C$-Werte wurden im Laufe von zwei Wochen gemessen. Die Hauptvoraussetzung der Methode zur Trennung der drei C-Quellen – identischer Abbau von zugeführten C_3- und C_4-Zuckern im Boden – wurde geprüft. Der relative Beitrag zum CO_2-Efflux wurde höher, die Dauer jedoch kürzer mit steigender (mikrobieller) Verfügbarkeit der C-Quelle: Zucker > Gülle > OBS. Die Mängel, Einschränkungen und mögliche künftige Anwendungen der vorgeschlagenen Methode werden diskutiert.

1 Introduction

The ^{13}C natural abundance method has frequently been used in the last two decades to elucidate the distinct C_3- and C_4-plant-derived sources of C in soil organic matter (SOM). Its principles, advantages, limitations, and shortcomings have been described previously (*Boutton*, 1996; *Balesdent* and *Mariotti*, 1996; *Boutton* et al., 1998). The method has quantified the flows of plant- or slurry-derived C in SOM studies of the bulk soil (*Glaser* et al., 2001), particle-size fractions (*Ludwig* et al., 2003; *Bol* et al., 2004), rhizosphere (*Kuzyakov* and *Cheng*, 2001, 2004), microbial biomass and water-soluble C (*Gregorich* et al., 2000; *Bol* et al., 2003a), and soil CO_2 efflux (*Rochette* et al., 2000; *Bol* et al., 2003b).

A limitation of ^{13}C natural abundance has been, as with any other single C-tracer method (*e.g.*, artificial ^{14}C or ^{13}C labeling), that only two C sources can be separated in one C pool (*e.g.*, SOM) or C flow (*e.g.*, CO_2). However, the coupling of two tracer methods, *e.g.*, ^{13}C natural abundance and ^{14}C pulse labeling, was successfully used to separate three C sources in rhizosphere C flows (*Kuzyakov* and *Cheng*, 2001, 2004). This separation of three C sources was only possible, because three C isotopes, ^{12}C, ^{13}C, and ^{14}C, were used simultaneously. The separation of three C sources by coupling of two C tracer methods remains however very seldom used. The complexity and interdependence of soil C flows and food webs urgently requires concurrent quantification of more than two C sources. To our knowledge, one C-tracer method has never been employed to separate more than two C sources in one C pool or C flow. However, increasingly concurrent separation of more than two C sources will be required. In view of this, we have endeavored to develop an approach to separate three C sources by one tracer method (here using ^{13}C natural abundance). We used the CO_2 efflux evolved from soil by individual and combined incubation with slurry and sugar. The main hypothesis was that multiple combinations of three C sources (soil, slurry, and sugar) with different labeling (C_3 or C_4) would allow an estimation of the three separate C contributions to the CO_2 efflux. In this study, we describe our conceptual approach on how the $\delta^{13}C$-natural-abundance tracer technique could distinguish and quantify the short-term CO_2 release from three C sources: SOM, slurry, and sugar.

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2 Material and methods

2.1 Field site, soil sampling, preparation and amendment details

Soil material (Eutric Cambisol) was collected from a permanent grassland pasture (>10 years) at De Bathe located near North Wyke, Devon, SW England (50°45′ N, 4°53′ W). The dominant vegetation is *Lolium perenne* L. No mineral fertilizers were used at the site since 1995, but cattle and sheep have grazed there. The detailed description of the soil was presented earlier (*Bol* et al., 2003b). Three subsites were randomly selected for sampling, and isolated intact soil cores (20 × 25 cm^2) from the topsoil (0–10 cm) were stored at 5°C. The upper 0–4 cm contained too many roots and were not used in the experiment. The remaining soil (4–10 cm depth) was then field-moist sieved (<7 mm) after the removal of vegetation and bigger roots. We originally weighed 257 g of the soil with a C content of 2.33% in Kilner jars or 6 g of soil C ($\delta^{13}C$ = –26.9‰), to which we added 2 g slurry C. No slurry, C$_3$ slurry ($\delta^{13}C$ = –30.7‰), or C$_4$ slurry ($\delta^{13}C$ = –21.3‰) was applied to the soil on January 26, 2001. The samples were incubated at 27°C and soil moisture of 70% of field capacity (FC) for 40 days. The results of the CO$_2$ fluxes and their partitioning have been published (*Bol* et al., 2003b) and will not be described here. After this incubation, the soil was dried and left for 2 years.

2.2 Experimental design, incubation, and analyses

We established a two-by-three-factorial experiment (Fig. 1). The first factor was slurry application. Soil + no slurry, Soil + C$_3$ slurry, or Soil + C$_4$ slurry was applied to the soil on January 26, 2001 (*Bol* et al., 2003b). Their $\delta^{13}C$ values were measured again prior to the start of this specific experiment and found to be –27.9‰, –28.7‰, and –25.5‰, respectively. On April 4, 2003, we filled nine 150 ml jars with 15 g soil for each of these three treatments. The soil was moistened to 70% of FC and pre-incubated for 2 weeks. The second factor was sugar application. No sugar, C$_3$ sugar, or C$_4$ sugar was applied to each soil–slurry variant. The $\delta^{13}C$ value of the (sugar beet) C$_3$ sugar was –27.2‰, and that of the (sugar cane) C$_4$ sugar was –11.0‰. The amount of sugar C applied corresponds to 1% of previously applied slurry C. This sugar C (1.167 mg C = 2.92 mg sugar) dissolved in 1 ml distilled water was added to 15 g soil. The control soil did not receive any slurry or sugar. Thus, the final experimental design included nine treatments (three replicates each), different in slurry or sugar application (Fig. 1).

The moisture contents of both soils and slurries were determined by weighing before and after drying in an oven at 85°C. Soil, sugar, and slurry samples were then ground to pass through a 0.6 mm sieve. Total C and N content of soil, sugar, and slurry samples was determined using a CHN autoanalyzer (Carlo Erba NA2000, Milan, Italy), and $\delta^{13}C$ values were analyzed at IGER using a continuous flow ANCA 20/20 SL system (Europa, Crewe, UK). Natural abundances of ^{13}C were expressed as $\delta^{13}C$ (‰) which represents the ratios of $^{13}C : {}^{12}C$ relative to the standard (VPDB). The analytical precision of all the $\delta^{13}C$ measurements was 0.1‰.

Periodically (on day 1, 2, 3, 4, 6, 7, 10, and 11 after sugar application, Tab. 2), the jars were flushed with CO$_2$-free air and sealed with a greased rubber ring and a lid with a septum for the needle. After the time between 2 (at the beginning) and 4 (at the end of incubation) hours after closing the 150 ml jars, air samples (12 ml) were taken from the headspace using evacuated exetainers and analyzed for their CO$_2$ concentration and $\delta^{13}C$ value using a GC-IRMS (Europa, Crewe, UK). The resulting CO$_2$ concentration and isotopic content were corrected for the small amount of ambient air (measured at 130 (±<20) ppm CO$_2$ and $\delta^{13}C$ of –7.5‰) remaining in the jars after flushing. We did not measure CO$_2$ efflux continuously. Therefore, the results are presented as CO$_2$-efflux rate, and not as a cumulative CO$_2$ efflux. The data of ppm CO$_2$ measured by GC were recalculated to mass units according to ideal gas law, and all results of CO$_2$ efflux were presented as μg C (g soil)$^{-1}$ h^{-1}.
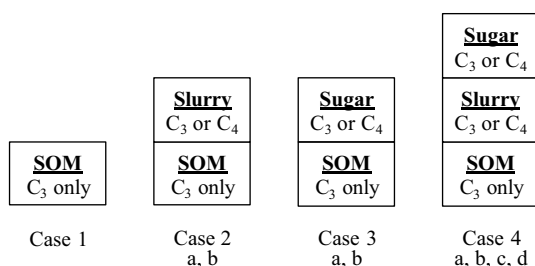

Figure 1: Treatments of the experiment presented as combination of C$_3$ and C$_4$ sources of CO$_2$ efflux from soil.
Abbildung 1: Behandlungen während des Experimentes in Kombination mit den C$_3$- und C$_4$-Quellen zum CO$_2$-Efflux aus dem Boden.

2.3 Calculations of contributions of two and three C sources to the CO$_2$ efflux

Nine different combinations of soil, slurry, and sugar result in maximal three C sources of respired CO$_2$ presented in each jar (Fig. 1): SOM (C$_3$ only), slurry C (C$_3$ or C$_4$), and sugar C (C$_3$ or C$_4$). To calculate the contribution of C$_4$ (slurry- or sugar-derived) carbon from the cases with **two C sources**, the following standard equation was used:

$$C_4{}^* = C_t \times (\delta_t - \delta_3) / (\delta_4 - \delta_3) \tag{1}$$

where $C_t = C_3{}^* + C_4{}^*$ is the total soil-derived CO$_2$, $C_3{}^*$ is the amount of CO$_2$-C derived from the C$_3$ soil, $C_4{}^*$ is the amount of CO$_2$-C derived from C$_4$ slurry or sugar, δ_t is the $\delta^{13}C$ value of the C$_t$ of CO$_2$, δ_4 is the $\delta^{13}C$ of the C$_4$ slurry (= –21.3‰) or C$_4$ sugar (= –11.0‰), and δ_3 is the $\delta^{13}C$ of the C$_3$ soil ($\delta^{13}C$ of soil was –27.9‰).

To calculate the contribution of SOM (C$_3$) carbon from the cases with two C sources, Eq. 2 was used:

$$C_3{}^* = C_t - C_4{}^* \tag{2}$$

In the treatments with **three CO$_2$ sources**, **with either slurry or sugar originating from C$_4$ vegetation** (Fig. 1, case 4), the contribution of the C$_4$ source was calculated according to

Eq. 1, and the contribution of the sum of two C_3 sources was calculated according to Eq. 2. The closeness of the δ^{13}C values of the soil and C_3 sugar suggested that the error of calculation of the contribution of C_4 slurry by this method is rather small. The differences of the δ^{13}C values of the soil and C_3 slurry could mean that the calculated contribution of C_4 sugar can be biased. Therefore, we used the weighted δ^{13}C value of Soil+C_3-slurry mixture of –28.7‰ measured before the incubation (36% slurry C + 64% soil C). Possible shortcomings of our approach are discussed below.

In the treatments with **three CO_2 sources, with slurry and sugar both originating from C_4 vegetation** (Fig. 1, case 4), the contribution of the sum of both C_4 sources to total CO_2 efflux could not be calculated according to Eq. 1, because C_4 sugar and C_4 slurry have different δ^{13}C values. We used an indirect calculation. The following assumptions are necessary to allow such a calculation: 1) There were no differences between decomposition of C_3 and C_4 sugars and therefore in the contribution of C_3 and C_4 sugars to the CO_2 efflux, 2) C_3 and C_4 sugars have the same effect on SOM decomposition, 3) C_3 and C_4 sugars have the same effect on slurry decomposition, 4) the slurry type (C_3 or C_4) has the same effect (or no effect) on sugar decomposition. The assumptions 1, 2, and 3 are obvious, because chemically the origin of the sugar (very small differences in isotopic composition) should not affect biochemical transformations. Nevertheless, these assumptions were tested with respect to the CO_2 efflux (see below). The last assumption about the same effect of two slurry types on sugar decomposition could not be fully tested within the current study.

The contribution of C_4 sugar ($C_{4\,Sugar}^{*}$) in the treatment with C_4-slurry **and** C_4-sugar addition was then taken from the treatment with C_3-slurry **and** C_4-sugar addition. The contribution of C_4 slurry ($C_{4\,Slurry}^{*}$) in the treatment with C_4-slurry **and** C_4-sugar addition was taken from the treatment with C_4-slurry **and** C_3-sugar addition. The contribution of SOM (C_3^{*}) in the treatment with C_4 slurry **and** C_4 sugar was calculated as the difference to total CO_2 efflux (C_t):

$$C_3^{*} = C_t - C_{4\,Sugar}^{*} - C_{4\,Slurry}^{*} \tag{3}$$

2.4 Statistical analysis

The experiment was conducted with three replicates. The statistical differences between the treatments over time were examined using analysis of variance (ANOVA). The differences significant at $p = 0.05$ level are presented. The standard deviation (SD_{pC_4}) of the proportion of C_4 source in CO_2 efflux was calculated from the standard deviations of δ^{13}C values of CO_2 efflux from sample soil (SD_S) and from reference (SD_R), as well as δ^{13}C values of CO_2 efflux of sample (δ_S) and reference (δ_R) (*Ludwig* et al., 2003):

$$SD_{pC_4} = \sqrt{\left(\frac{SD_S}{\delta_S - \delta_R}\right)^2 + \left(\frac{SD_R}{\delta_S - \delta_R}\right)^2} \tag{4}$$

Standard deviations of values obtained as differences between total CO_2 efflux and C_4-derived CO_2 were calculated by using SD of both parameters:

$$SD = \sqrt{(SD_{CO_2})^2 + (SD_{C_4})^2} \tag{5}$$

where SD_{CO2} and SD_{C4} are standard deviations of total and C_4-derived CO_2 efflux, respectively.

For the calculation of SD of C_3^{*} values obtained by difference according to Eq. 3, the following equation was used:

$$SD = \sqrt{(SD_{CO_2})^2 + (SD_{C_{4Sugar}})^2 + (SD_{C_{4Slurry}})^2} \tag{6}$$

The errors of observed and calculated values are presented as standard errors (SE) calculated as:

$$SE = \frac{SD}{\sqrt{n}}$$

3 Results

3.1 Concentration of CO_2 efflux from the soil

Figure 2 shows the concentrations of the total CO_2 efflux from the soil in all nine treatments, with the zero point at the time-scale set to correspond with the timing of sugar addition. Prior to the sugar application ($t = 0$), the soil CO_2 efflux was in C_4-slurry treatment (Fig. 2, bottom) ca. 2 and 1.5 times higher than in the unamended and C_3-slurry treatment, respectively. Different CO_2-efflux rates of C_3 and C_4 slurries were known to occur beyond 10 days after slurry addition (*Bol* et al., 2003b) and showed that the C_3 and C_4 slurries were not identical. Therefore, the **C_3-slurry contribution was not equal to the calculated contribution of C_4 slurry to total CO_2 efflux**. So, it was not possible to calculate directly the relative contribution of C_3 slurry to the total CO_2 efflux according the contribution of C_4 slurry as it was done for C_3 and C_4 sugars (see below). The CO_2 efflux significantly increased ($p < 0.001$) in all treatments on day 1 (17 hours) after sugar addition. The CO_2 efflux then rapidly decreased, but remained between day 2 and 4 at nearly double the flux before the sugar addition. Beyond 7 days after sugar addition, the total CO_2 efflux from soil without slurry was not different to that before sugar addition (Fig. 2, top). However, there were still significant differences in soils with applied C_3 and C_4 slurry (Fig. 2, middle and bottom). Furthermore, the sequence of the treatments ordered according to CO_2 efflux was also the same as before sugar addition: soil without addition < soil amended with C_3 slurry < soil amended with C_4 slurry.

One important assumption is necessary to distinguish between the three C sources in the CO_2 efflux, *i.e.*, there are no significant differences in decomposition of C_3 and C_4 sugars in the C_3- and C_4-slurry-treated soils. Using the chosen experimental approach, we can compare the decomposition of C_3 and C_4 sugars only according the total CO_2 efflux from soils of variants that were treated with C_3 or C_4 sugar. There are three such combinations (Fig. 2): soil only, soil amended with C_3 slurry, and soil amended with C_4 slurry. Indeed, the curves of total CO_2 efflux from soils treated with C_3 or C_4 sugar do not differ significantly ($p > 0.05$). The CO_2 efflux in C_3-slurry-amended soil (Fig. 2, in the middle) is by 0.43 μg C g^{-1} h^{-1} higher for C_4 sugar than the respective

peak after C_3-sugar addition for one point (after 17 h). This difference was less than 9%, and it was not significant ($p >$ 0.05) within the overall background of 10%–15% variation of CO_2 efflux between replicates. We therefore concluded that the C decomposition of the sugar-beet and sugar-cane sugars was similar and that the **C_3-sugar contribution was equal to the calculated contribution of C_4 sugar to total CO_2 efflux**.
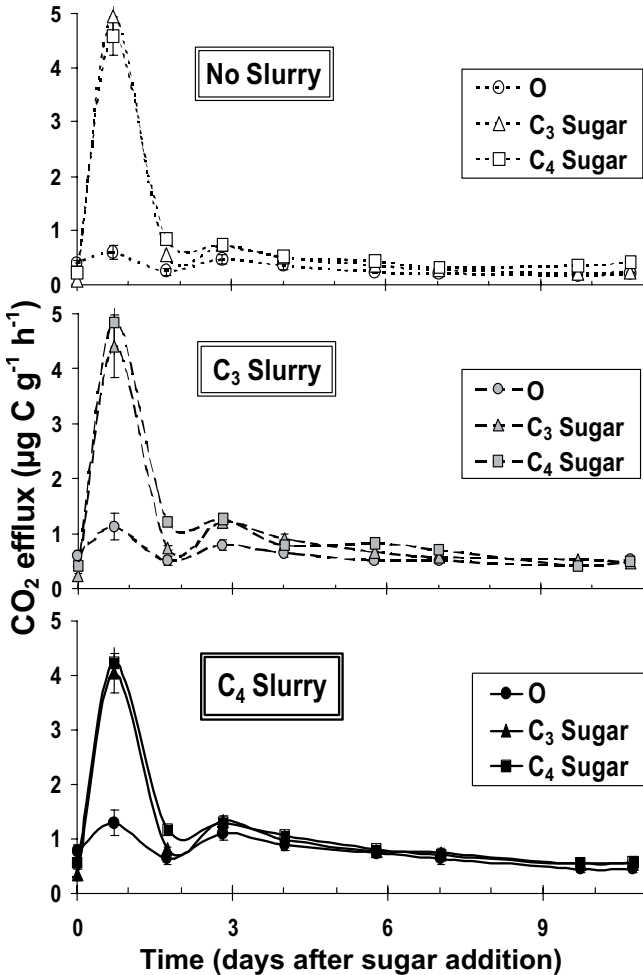
Figure 2: Total CO_2 efflux rate (μg C g^{-1} h^{-1}, ±SE) from the soil after sugar addition. Top: no slurry, middle: C_3 slurry, bottom: C_4 slurry. Sugar addition: ● no sugar, ▲ C_3 sugar, ■ C_4 sugar.
Abbildung 2: Rate des CO_2-Gesamteffluxes (μg C g^{-1} h^{-1}, ±SE) aus dem Boden nach Zuckerzugabe. Oben: keine Gülle, Mitte: C_3-Gülle, unten: C_4-Gülle. Zugabe von Zucker: ● kein Zucker, ▲ C_3-Zucker, ■ C_4-Zucker.

3.2 The $\delta^{13}C$ values of CO_2 efflux from soil

There was a decrease (1‰–2‰) in the $\delta^{13}C$ of emitted CO_2 in the treatments without added sugar during the 2-week incubation (data not shown). This was probably related to an initial rewetting C flush from the decomposition of dead microbial cells (enriched in ^{13}C compared to the soil; *Ryan* and *Aravena*, 1994), which had remained in the soil since wetting had ceased 2 years earlier. The $\delta^{13}C$ of CO_2 efflux from unamended soil and the Soil + C_3 slurry did not change after C_3-sugar addition, because the $\delta^{13}C$ values of the C_3

soil and added C_3 sugar were nearly the same (data not shown). In fact, these treatments followed the same temporal trend in decreasing $\delta^{13}C$ values as observed in the no-sugar treatment.

The addition of C_4 sugar strongly increased the $\delta^{13}C$ value of CO_2 efflux (Fig. 3). The $\delta^{13}C$ maximum coincided with the peak CO_2 concentration on day 1 (17 hours) after sugar addition (see Fig. 2). The $\delta^{13}C$ value of the CO_2 then reached that of the C_4 sugar itself (–11.0‰), thus indicating that most of the CO_2 efflux was derived from the applied sugar C. The $\delta^{13}C$ peak decreased at a slower rate than the total CO_2 efflux (compare Figs. 2 and 3) and indicated that some added C_4 sugar was not immediately decomposed to CO_2, but temporary incorporated into microbial biomass. Furthermore, it indicated that several days were necessary to replace the sugar-derived C_4 in microbial biomass with that of C_3 from the SOM or slurry.
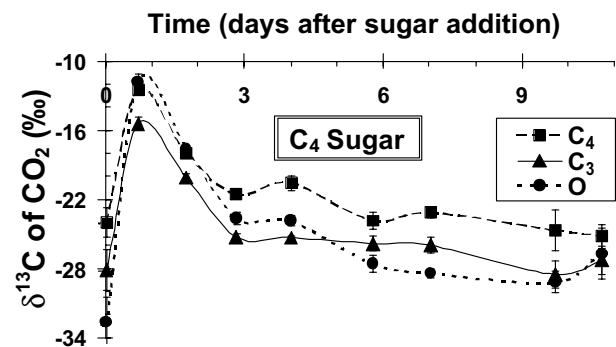
Figure 3: $\delta^{13}C$ values (‰, ±SE) of CO_2 efflux after addition of C_4 sugar to soil previously amended with slurry: ● no slurry, ▲ C_3 slurry, ■ C_4 slurry.
Abbildung 3: $\delta^{13}C$-Werte (‰, ±SE) des CO_2-Effluxes nach der Zugabe von Zucker zum Boden, der vorher mit Gülle gedüngt wurde: ● keine Gülle, ▲ C_3-Gülle, ■ C_4-Gülle.

3.3 Relative contribution of C sources to total soil CO_2 efflux

The contribution of C_4 slurry (treatment without sugar) estimated by Eq. 1 did amount to 16%–78% of the total CO_2 efflux (Tab. 1). It decreased strongly during the latter part of the incubation period (day 10 and 11) suggesting that slurry decomposition proceeded faster than the decomposition of the native SOM (Fig. 4, top). The estimated contribution of applied sugar (treatment without slurry) to the total CO_2 efflux peaked after 17 hours at 95%, but it was effectively finished after 4 days (Tab. 1).

The direct estimation of relative C_4-source contribution was more difficult for three CO_2 sources (Tab. 1; Fig. 5). For example, when C_3 sugar was added to C_4-slurry-amended soil, the contribution of C_4 slurry was between 30% and 80%, hence similar to the treatment with only C_4 slurry (without sugar) (Tab. 1). It implies that sugar addition had no effect on the slurry decomposition (Fig. 2). The addition of C_4 sugar to

Table 1: Relative contribution (percentage $\pm$ SE) of C_4 source to the total CO_2 efflux from the soil calculated by ^{13}C natural abundance before and after substrate additions.

Tabelle 1: Relativer Beitrag (Prozent $\pm$ Standardfehler) der C_4-Quelle zum CO_2-Gesamtefflux aus dem Boden vor und nach Zugabe der Substrate, berechnet mit natürlicher ^{13}C-Abundanz.

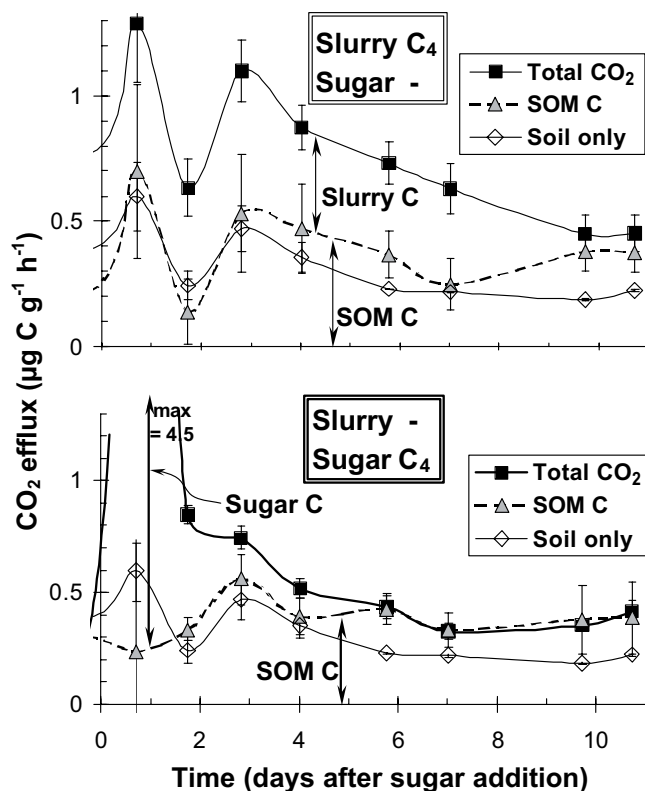| C_4 source	Contribution			
Slurry	C_4	–	C_4	C_3
Sugar	–	C_4	C_3	C_4
Sampling hours				
17	46 ± 20	95 ± 7	41 ± 113	75 ± 6
42	78 ± 10	60 ± 4	80 ± 8	48 ± 5
68	52 ± 18	25 ± 14	51 ± 20	19 ± 6
96	46 ± 18	24 ± 13	56 ± 11	19 ± 5
139	50 ± 5	2 ± 12	51 ± 9	16 ± 22
169	61 ± 4	-3 ± 21	50 ± 24	15 ± 16
233	16 ± 6	-7 ± 43	30 ± 25	1 ± 124
258	18 ± 4	7 ± 37	52 ± 30	8 ± 105

Figure 4: Absolute contribution ($\pm$SE) of different C_4 sources (slurry or sugar) to the total CO_2 efflux from the soil calculated directly with $\delta^{13}C$ values of CO_2. The treatments with slurry or sugar are shown in the box in the center of each graph. ■ total CO_2 efflux, △ CO_2 from C_3 source (= SOM), ◇ total CO_2 efflux from the soil without any addition of C_4 source.

Abbildung 4: Absoluter Beitrag ($\pm$SE) unterschiedlicher C_4-Quellen (Gülle oder Zucker) zum CO_2-Efflux aus dem Boden, berechnet direkt mittels der $\delta^{13}C$-Werte des CO_2. Die Behandlung mit Gülle oder Zucker findet sich eingerahmt im Zentrum der Abbildung. ■ CO_2-Gesamtefflux, △ CO_2 aus der C_3-Quelle (= SOM), ◇ CO_2-Gesamtefflux aus dem Boden ohne Zugabe einer C_4-Quelle.

the C_3-slurry-amended soil was not different from that of the C_4-sugar application to the unamended soil (Tab. 1). This observation partly confirmed our fourth assumption, that the effect of slurry type (C_3 or C_4) on sugar decomposition was negligible.
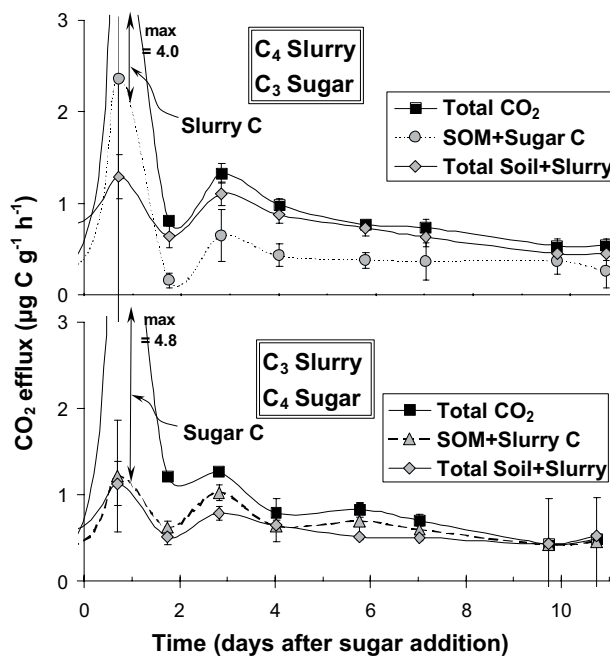

Figure 5: Absolute contribution ($\pm$SE) of C_4 sources (slurry or sugar) to the total CO_2 efflux from the soil calculated directly with $\delta^{13}C$ values of CO_2. The treatments C_3 or C_4 slurry and sugar are shown in the box in the center of each graph. Vertical arrows indicate the C_3 or C_4 source. ■ total CO_2 efflux, △ or ○ CO_2 from the sum of two C_3 sources.

Abbildung 5: Absoluter Beitrag ($\pm$SE) unterschiedlicher C_4-Quellen (Gülle oder Zucker) zum CO_2-Efflux aus dem Boden berechnet direkt mittels der $\delta^{13}C$-Werte des CO_2. Die Behandlung mit Gülle und Zucker findet sich eingerahmt im Zentrum der Abbildung. Vertikale Pfeile zeigen den Beitrag der C_4-Quelle. ■ CO_2-Gesamtefflux, △ oder ○ CO_2 aus der Summe der zwei C_3-Quellen (SOM + Zucker bzw. SOM + Gülle).

3.4 Absolute contribution of C sources to total soil CO_2 efflux

The total CO_2 efflux of the two treatments C_4 slurry + no C_4 sugar and C_4 sugar + no C_4 slurry are presented in Fig. 4. The previous addition of C_4 slurry had nearly doubled the soil CO_2, and its contribution remained significant for the first 9 days of incubation. (Fig. 4, at the top). The C_4 sugar induced on day 1 and 2 a peak in the total CO_2 efflux, and the contribution of sugar C to total CO_2 was significant during first 4 days.

The total soil CO_2 evolved from the treatments C_4 slurry + C_3 sugar and C_3 slurry + C_4 sugar are presented in Fig. 5. The soil + C_4 slurry (no sugar) was used as the control, in order to estimate the added-sugar effect on soil- and slurry-derived CO_2. Sugar-derived and SOM-derived CO_2 efflux could not easily be separated when C_3 sugar was added to the C_4-slurry soil (Fig. 5, top). We concluded, based on the

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CO_2-efflux dynamics, that this peak was mainly caused by sugar-derived CO_2. The contribution of C_4 slurry in the evolved CO_2 increased in the first week after C_3-sugar addition to the soil. The addition of C_4 sugar to C_3-slurry-amended soil strongly increased total CO_2 efflux through sugar-derived CO_2 (Fig. 5, bottom), and these effects were similar to the sugar variant without slurry addition (Fig. 4, bottom). Hence, the total soil CO_2 efflux measured on day 1 was mainly derived from sugar C. Note, that the maximal variations and therefore uncertainty of calculation of contribution of C_4 source was observed 1) at first sampling period when the contribution of sugar (C_4 or C_3)-derived C dominated the total CO_2 efflux (Fig. 4 and 5) and 2) at the last two samplings, because the contribution of sugar was negligible at this time (Tab. 1).

3.5 Indirect calculation of contributions of three C sources to the total CO_2 efflux from soil

For estimation of the contributions of three C sources to the total CO_2 efflux from soil, two variants were evaluated: 1) treatments with C_3 slurry and C_3 sugar and 2) treatments with C_4 slurry and C_4 sugar. The principles of the calculations of the contributions in both treatments were different and are presented schematically on Fig. 6.

Treatments with C_3 slurry and C_3 sugar: The first step in the treatment with C_3-slurry and C_3-sugar addition was the use of earlier calculated contribution of C_4 sugar instead of C_3 sugar (Fig. 6, top; Fig. 7, top). The second step was the
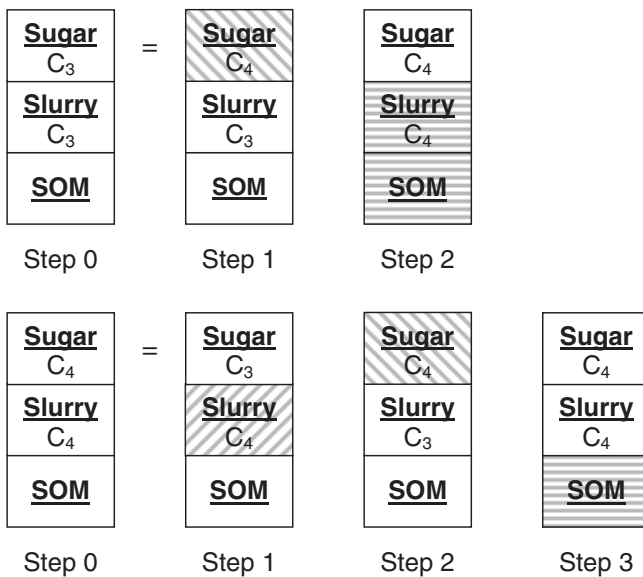
estimation of the summed absolute contribution of SOM C and slurry C as the difference between the total CO_2 efflux and the contribution of C_3 sugar (Fig. 6, top). Most of CO_2 evolved on day 1 comes from C_3 sugar (Fig. 7, top). The sugar contribution then decreased strongly, and after day 2, it amounted to less than 0.2 µg C g^{-1} h^{-1}. Theoretically, if C_3 and C_4 slurries would have identical decomposition, then the decomposition of the treatment C_4 slurry + C_3 sugar could have been transposed into the treatment C_3 slurry + C_3 sugar to separate three CO_2 sources in the treatment with C_3-slurry and C_3-sugar addition.

Treatments with C_4 slurry and C_4 sugar: The estimation of CO_2 efflux from the soil treated with C_4 slurry and C_4 sugar is more complicated and involves three steps (Fig. 6, bottom; Fig. 7, bottom). 1) We separated the slurry-derived CO_2 by using values from the variant with C_4-slurry and C_3-sugar addition (based upon the equal decomposition of C_4 and C_3 sugars; section 3.1). 2) The contribution of C_4 sugar was taken from the treatment with C_3 slurry and C_4 sugar. 3) The

Figure 6: Principles and steps of calculation of contributions of three C sources to the CO_2 efflux from soil amended with C_3 slurry and C_3 sugar (top) and amended with C_4 slurry and C_4 sugar (bottom). See text for additional explanations. The calculations were done for each CO_2 sampling time separately.
Abbildung 6: Berechnungsprinzip und -schritte der Beiträge von drei C-Quellen zum CO_2-Efflux aus dem Boden nach Zugabe von C_3-Gülle und C_3-Zucker (oben) und nach Zugabe von C_4-Gülle und C_4-Zucker (unten). Weitere Erklärungen im Text. Die Berechnung wurde für jede CO_2-Probenahme durchgeführt.
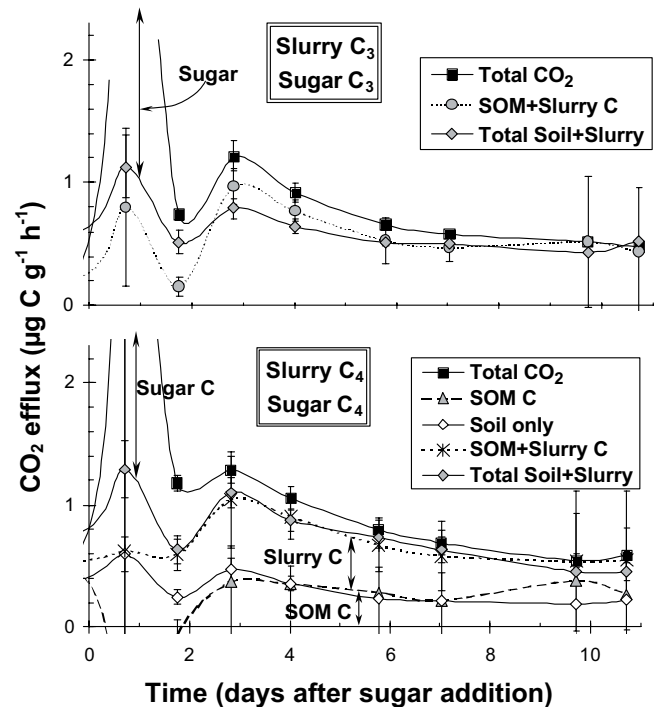
Figure 7: Absolute contribution (±SE) of three C sources to the total CO_2 efflux from the soil calculated indirectly considering identical decomposition of C_3 and C_4 sugars. The treatments slurry and sugar are shown in the box in the center of each graph. Vertical arrows show the C_3 or C_4 source.
■ total CO_2 efflux, △ CO_2 from SOM (C_3 source), × CO_2 from the sum of SOM and slurry, ◇ total CO_2 efflux from the respective treatment with addition of C_4 source.
Abbildung 7: Absoluter Beitrag (±SE) von drei C-Quellen zum CO_2-Efflux aus dem Boden, berechnet indirekt unter Berücksichtigung des identischen Abbaus des C_3- und C_4-Zuckers. Die Behandlung mit Gülle und Zucker findet sich eingerahmt im Zentrum der Abbildung. Vertikale Pfeile zeigen den Beitrag der C_3- oder C_4-Quelle.
■ CO_2-Gesamtefflux, △ CO_2 aus der C_3-Quelle (= SOM), × CO_2 aus der Summe von SOM und Gülle, ◇ CO_2-Gesamtefflux aus dem Boden der entsprechenden Behandlung mit Zugabe der C_4-Quelle.

difference between the total CO_2 efflux and the estimated summed contributions of C_4 slurry + C_4 sugar allows the estimation of SOM-derived CO_2 (Fig. 6, bottom).

Most of the emitted CO_2 during the first 3 days after the sugar addition was derived from the sugar, however, the slurry C contribution was also important (Fig. 7, bottom). The contribution of SOM was marginal during the first 2 days. However, because of the uncertainty of the CO_2-source separation at day 1, we cannot exactly estimate the contribution of sugar- and slurry-derived C. It is interesting that, except on the first day, the absolute amount of CO_2 originated from SOM in this treatment was very similar to SOM-derived CO_2 from soil treated with C_4 slurry without sugar addition (Fig. 4). Similarly, the SOM+slurry-derived CO_2 after sugar addition was nearly the same as in the variant without sugar addition.

4 Discussion

4.1 Separation of two C and three C sources

The various two-C-sources separations of the total CO_2 efflux (Tab. 2; Fig. 4) were calculated in the same way as in many previous studies (*e.g.*, *Balesdent* and *Mariotti*, 1996; *Boutton*, 1996; *Flessa* et al., 2000; *Bol* et al. 2003b; *Kuzyakov* and *Cheng*, 2001, 2004; *John* et al., 2003). It showed in agreement with other observations that the relative contribution the CO_2 efflux was higher, but of a shorter time span with an increased microbial availability of the C source, *i.e.*, SOM < slurry < sugar (*Kuzyakov* et al. 2000; *Bol* et al. 1998, 2000, 2003a, b). To our knowledge, the separation of three C sources (SOM, slurry, and sugar) of CO_2 efflux by one tracer method was conducted for the first time (Fig. 7). The separation of three C sources was achieved with a simple experimental design: by comparing the temporal trends in the concentration and the $\delta^{13}C$ value of the CO_2 efflux from one soil with three 'past' treatments (no, C_3 and C_4 slurry) and three 'newly' imposed (no, C_3 and C_4 sugar) treatments. Based on existing general assumption regarding similar decomposition of C_3 and C_4 substrates in soil (*e.g.*, *Balesdent* and *Mariotti*, 1996), we could successfully separate the contribution of the three C sources to the CO_2 efflux. We did add some additional assumptions, as the CO_2 efflux from the C_3 and C_4 slurry was found to be different. However, as the decomposition of C_3 and C_4 sugars was indeed identical, the contribution of C_4 sugar to the total CO_2 efflux from soil amended with or not amended with C_3 slurry could be used to calculate the contribution of C_3 sugar to the total CO_2 efflux from soil amended with or not amended with C_4 slurry. The three-C-sources separation using one C tracer was achieved by experimental design and not through the tracer method itself. Furthermore, either ^{13}C **or** ^{14}C can be used as the single C tracer. However, if ^{13}C **and** ^{14}C are combined, the necessity of complicated experimental design can be omitted, and no assumptions are necessary (*Kuzyakov* and *Cheng*, 2001, 2004). Furthermore, there is the tantalizing prospect of using our single-tracer three-C-sources design **and** a combination of ^{13}C and ^{14}C tracers to separate four or more C sources simultaneously. This could open new ways for C studies in soils and other complex systems.

4.2 Difficulties and limitations of the method

The first limitation of the method is its accuracy. As presented on Fig. 6 and in Eq. 2 and 3, an important part of the separation of C sources is based on the differences between CO_2-efflux rates from different treatments. This difference leads to the summation of the variance (Eq. 5) and resulted in higher errors compared to the initial data. Such increase of the variance is especially important for SOM-derived CO_2 efflux calculated by difference of three variables (Eq. 6). Thus, the calculated SOM-derived CO_2 efflux in the treatment with C_4 slurry and C_4 sugar was negative at the first sampling (Fig. 7, bottom). We explain this negative value by the errors of estimation of a small value (SOM-derived CO_2) as the difference between two large values (total CO_2 and sugar-derived CO_2). In subsequent studies, the use of cumulative systems to trap CO_2 (*i.e.*, sorption on alkali or molecular sieves; *Bol* and *Harkness*, 1995; *Bol* et al., 2003c) or other techniques to measure soil CO_2 efflux (*i.e.*, at constant CO_2 concentration; *Subke* et al., 2004) will allow to decrease the variation between replicates and associated error of values estimated as differences.

Another point was the background variation of $\delta^{13}C$ values. The variation of $\delta^{13}C$ values in SOM between the replicates is frequently higher than 0.5‰ and that of CO_2 efflux is about 1‰–1.5‰. Such variations lead to the error of about 10%–15%, and this error will be cumulated by estimation of values obtained as difference (see above). A separate issue is the choice of substances with appropriate decomposition rates. We used the substances (slurry and sugar), the decomposition rates of which were different by more than one order of magnitude. The very fast decomposition of sugar led to very strong increase of CO_2 efflux during the first 2 days and induced inaccuracy of estimation of SOM-derived CO_2 efflux. Combination of other substances could improve the accuracy of the method.

We assumed that the isotopic discrimination by CO_2 production from different sources is negligible. The literature provided no univocal conclusive picture. The $\delta^{13}C$ of CO_2 efflux evolved by microbial respiration corresponds roughly to $\delta^{13}C$ of microbial biomass (*Ekblad* and *Högberg*, 2000). However, *Santruckova* et al. (2000) measured $\delta^{13}C$ of CO_2 respired from 21 Australian soils with C_3 and C_4 vegetation which showed that the microbially respired CO_2 is depleted on average by 2.2‰ compared to microbial biomass. However, microbial biomass was enriched by –2.0‰ compared to $\delta^{13}C$ of SOM. Thus, the observed ^{13}C enrichment in microbial biomass is balanced by a corresponding ^{13}C depletion in respired CO_2 resulting in the $\delta^{13}C$ of respired CO_2 being similar to the $\delta^{13}C$ of SOC (*Santruckova* et al., 2000).

A further shortcoming affecting the accuracy is the correspondence of $\delta^{13}C$ values between two C_3 (or C_4) sources to calculate the contribution of the third C_4 source (or C_3 source) by Eq. 1. In an ideal case, the $\delta^{13}C$ values of both C_3 sources should be the same. In our study, despite the close correspondence between the $\delta^{13}C$ values of soil (–27.9‰) and of C_3 sugar (–27.2‰), the $\delta^{13}C$ of C_3 slurry (–30.7‰) was different from that of the soil. We used therefore the weighted $\delta^{13}C$

value of both C_3 sources, *i.e.*, –28.7‰ (64% SOM + 36% slurry) to estimate the C_4-sugar contribution in C_3-slurry-amended soil. The application of the weighted $\delta^{13}C$ value assumes equal decomposition rates of both C_3 sources. Because of the last assumption, the results of the contribution of C_4 sugar in the C_3-slurry treatment could be biased. Clearly, most shortcomings of the approach are associated with the detection level of different C sources by ^{13}C-natural-abundance method. The use of ^{14}C- and/or ^{13}C-labeled substances would overcome these problems.

5 Conclusions and future applications

The combination of C_3- and C_4-sources variants (slurry and sugar) applied to a C_3 grassland soil allowed for the first time to distinguish between three CO_2 sources. This possibility is extremely useful for the investigation of complex natural systems (*e.g.*, soil), which generally contain more than two C sources. However, methods reducing the initial variance of CO_2 efflux as well as its $\delta^{13}C$ values are necessary to increase the significance of the observed separation of the CO_2 sources. The relative contribution to the CO_2 efflux was higher but of a shorter time span with an increased availability of the C source, *i.e.*, sugar >> slurry > SOM.

The results of separation of three C sources by one tracer method showed advantages above two-sources separation (see above). Furthermore, if the C_3 and C_4 slurries had been more identical, more results would have been obtained with the same experimental effort. At this point, it makes sense to consider future possibilities, other than C_3 and C_4 sugars, of substrate or compounds with identical behavior in soil. The first possibility is to use uniformly labeled (^{13}C or ^{14}C) substances as analogue for an unlabeled one. The labeling of the substances does not have to be very high: few hundreds delta units would be enough to obtain significant differences. However, nearly all commercially available substances are pure chemicals. In soil and environmental studies, we mostly deal with complex substances, *e.g.*, like plant residues, microbial biomass, etc., which are difficult to label completely uniform. Another easier option would be to obtain uniformly labeled plant residues of Free Air Carbon dioxide Enrichments (FACE) experiments. These experiments (*Ineson* et al., 1996; *van Kessel* at al., 2000; *Leavitt* et al., 2001) use CO_2 depleted in ^{13}C (–30‰ to –50‰) compared to atmospheric value ($\delta^{13}C \approx - 7.5$‰). After mixing the 'artificial' CO_2 with atmosphere CO_2, the $\delta^{13}C$ of the mixed supplied CO_2 is about –20‰ to –30‰. Therefore, C_3 plants produced under FACE will have $\delta^{13}C$ values of around –40‰ to –50‰. Therefore, these ^{13}C-depleted C_3-plant residues can be used analogous to the same C_3 plants produced under normal air conditions. Small differences in C : N ratio between normal and FACE plant residues can be neglected in the most studies.

Acknowledgments

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