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Three sources of CO_2 efflux from soil partitioned by ^{13}C natural abundance in an incubation study†

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This study describes a novel approach to separate three soil carbon (C) sources by one tracer method (here ^{13}C natural abundance). The approach is based on the combination of C_3 and C_4 sources in different treatments, identical decomposition of C_3 and C_4 substances in soil, and subsequent calculation of their contribution to the total CO_2 efflux. We used 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 organic matter (SOM), 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 increased, but its duration decreased with an increased microbial availability of the C source, i.e. sugar > slurry > SOM. The microorganisms used the C sources according to their availability. The contribution of sugar to the CO_2 efflux was finished after 2–4 days. Separation of three CO_2 sources and comparison of CO_2 from different treatments tracing the changes of SOM and slurry decomposition induced by addition of sugar were investigated. During the sugar decomposition (the first 2–4 days), the SOM decomposition strongly decreased. At the same time the contribution of slurry-C to CO_2 increased. The shortcomings and limitations as well as possible future applications of the suggested method including FACE (Free Air Carbon dioxide Enrichments) and continuous labelling experiments are discussed. Copyright © 2005 John Wiley & Sons, Ltd.

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). The principles, advantages, limitations and shortcomings of the ^{13}C natural abundance have been described previously.[1–3] The method has quantified the flows of plant- or slurry-derived C in SOM studies of the bulk soil,[4] particle size fractions,[5,6] rhizosphere,[7,8] microbial biomass and water-soluble carbon,[9,10] and soil CO_2 efflux.[11,12]

A limitation of ^{13}C natural abundance has been the same as with any other single tracer method (e.g. artificial ^{14}C or ^{13}C labelling), that only two C sources can be separated in one C pool (e.g. SOM) or C flow (e.g. CO_2). However, the complexity and interdependence of soil C flows and food webs urgently requires concurrent quantification of more than two C sources. In view of this we have endeavoured 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 isotopic signature (originated from C_3 or C_4 vegetation) 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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EXPERIMENTAL

Soil sampling and preparation

Soil (Dystric Gleysol, clayey non-calcareous) material was collected from a permanent grassland pasture (>40 years), Rowden Moor, located near North Wyke, Devon, southwest England (50°45′ N, 4°53′ W). The dominant vegetation was *Lolium perenne* L. with varying amounts of *Cynosurus*, *Festuca*, *Agrostis*, *Holcus* and *Dactylis* spp. Application of mineral fertilizers has not occurred at the site since 1995, but cattle and sheep have grazed there. A detailed description of the soil was presented earlier.[12,13] Three subsites were randomly

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sampled and isolated intact soil cores ($20 \times 25\,cm^2$) from the topsoil (0–10 cm) were stored at 5°C. The upper 0–4 cm contained too many roots and was not used in the experiment. The remaining soil (4–10 cm depth) was then field moist sieved (<7 mm) to remove bigger roots and stones. We originally weighed 118 g of the soil with a C content of 5.09% into Kilner jars, corresponding to 6 g of soil C ($\delta^{13}C = -28.6‰$), 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 26 January 2001. The samples had a soil moisture of 70% field capacity (FC) and were incubated at 27°C for 40 days. The results of the CO_2 fluxes and their partitioning into CO_2 derived by decomposition of SOM or slurry have been published[12] and will not be described here. After this incubation, the soil was dried and left for 2 years.

Experiment design and soil incubation

We established a two by three factorial experiment. The first factor was slurry application. Soil + No slurry, Soil + C_3 slurry or Soil + C_4 slurry was applied to the soil on 26 January 2001.[12] The $\delta^{13}C$ values of these samples were measured again prior to the start of this specific experiment and found to be −28.5, −29.0 and −26.6‰, 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 preincubated for 2 weeks. The second factor was sugar application. No sugar, C_3 sugar or C_4 sugar was applied to each soil-slurry treatment. The $\delta^{13}C$ value of the (sugar beet) C_3 sugar was −27.2‰, and the (sugar cane) C_4 sugar was −11.0‰. The amount of sugar-C applied corresponds to 1% of previously applied slurry-C and to 0.3% of SOM. This sugar-C (2.544 mg C = 6.36 mg sugar) dissolved in 1 mL distilled water was added to 15 g soil. The control soil did not receive any slurry or sugar. So, the final experiment design included nine treatments (three replicates each), different in slurry or sugar application.

The moisture contents of the soil and both slurries were determined by weighing before and after drying in an oven at 85°C. Total C and N content of soil, slurry and sugar samples was determined using a CHN auto-analyzer (Carlo Erba NA2000, Milan, Italy). The $\delta^{13}C$ values were analyzed at IGER using a continuous flow ANCA SL 20/20 system (Europa, Crewe, UK). Natural abundances of ^{13}C were expressed as $\delta^{13}C$ (‰) versus VPDB. The analytical precision of all the $\delta^{13}C$ measurements was 0.1‰.

Periodically (on days 1, 2, 3, 4, 6, 7, 10, and 11 after sugar application, Table 1) 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. Between 2 (at the beginning) and 4 (at the end of incubation) h 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 gas chromatography/isotope-ratio mass spectrometry (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 the CO_2 efflux continuously. Therefore, the results are presented as CO_2 efflux rate, and not as a cumulative CO_2 efflux. The ppm CO_2 data measured by GC were recalculated to mass units according to the ideal gas law and all results of CO_2 efflux are presented as $\mu g\,C\,g^{-1}$ soil h^{-1}.

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

C_4 source	Contribution			
Slurry	C_4	—	C_4	C_3
Sugar	—	C_4	C_3	C_4
Sampling hours				
17	46 ± 11	74 ± 11	108 ± 23	60 ± 11
42	46 ± 14	46 ± 33	108 ± 23	45 ± 33
68	35 ± 19	21 ± 54	86 ± 22	27 ± 54
96	42 ± 9	18 ± 44	92 ± 17	23 ± 44
139	34 ± 5	14 ± 73	86 ± 5	18 ± 73
169	34 ± 17	15 ± 95	81 ± 29	17 ± 95
233	23 ± 25	14 ± 96	64 ± 23	11 ± 96
258	24 ± 28	18 ± 129	67 ± 23	9 ± 129

Calculations

Nine different combinations of soil, slurry, and sugar resulted in a maximum of three C sources of respired CO_2 presented in each jar: 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) C from the cases with **two C sources**, the following standard equation was used:

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

where $C_t = C_3^* + C_4^*$ is the total CO_2 efflux from soil, 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 −28.5‰).

To calculate the contribution of SOM (C_3) carbon from the cases with two C sources, the following equation was used:

$$C_3^* = C_t - C_4^* \quad (2)$$

In the treatments with **three CO_2 sources, with either slurry or sugar originating from C_4 vegetation**, the contribution of the C_4 source was calculated according to Eqn. (1), and the contribution of the sum of two C_3 sources was calculated according to Eqn. (2). The closeness of the $\delta^{13}C$ 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 in the $\delta^{13}C$ values of the soil and C_3 slurry (2.2‰) could mean that the calculated contribution of C_4 sugar can be biased. Therefore, we used the $\delta^{13}C$ value of soil + C_3 slurry mixture of −29.0‰ measured before the incubation (21.5% slurry-C + 78.5% soil-C). Possible shortcomings of this approach are discussed below.

In the treatments with **three CO_2 sources, with slurry and sugar both originating from C_4 vegetation,** the contribution of the sum of both C_4 sources to total CO_2 efflux could not be calculated according to Eqn. (1), because C_4 sugar and C_4 slurry have different $\delta^{13}C$ values. We used an indirect calculation based on the following assumptions:

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 concerning the same effect of two slurry types on sugar decomposition could not be fully tested within the current study.

Based on these assumptions, 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 were 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 from the total CO_2 efflux (C_t):

$$C^*_3 = C_t - C^*_{4\ Sugar} - C^*_{4\ Slurry} \quad (3)$$

The experiment was conducted with three replicates. The standard deviation (SD_{pC_4}) of the proportion of C_4 source in CO_2 efflux was calculated from the standard deviations of $\delta^{13}C$ values of CO_2 efflux from sample soil (SD_S) and from reference (SD_R), as well as $\delta^{13}C$ values of CO_2 efflux of sample (δ_S) and reference (δ_R):[6]

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

The standard deviation (SD) of values obtained as differences between total CO_2 efflux and C_4-derived CO_2 was calculated by using the SD of both parameters:

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

where SD_{CO_2} and SD_{C_4} are standard deviations of total and C_4-derived CO_2 efflux, respectively. For the calculation of the SD of C^*_3 values obtained by difference according to Eqn. (3), the following equation was used:

$$SD = \sqrt{(SD_{CO_2})^2 + (SD_{C_4\ Sugar})^2 + (SD_{C_4\ Slurry})^2} \quad (6)$$

Standard errors are presented in the figures.

RESULTS

The CO_2 efflux from the soil and $\delta^{13}C$ of CO_2

Prior to the sugar application (t = 0), the soil CO_2 efflux was in the C_4 slurry treatment ca. 2 and 1.5 times higher than in the unamended and the C_3 slurry treatment, respectively (Fig. 1, top). Different CO_2 efflux rates of C_3 and C_4 slurries were known to occur beyond 10 days after slurry addition[12] and this 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 the C_4 slurry to the 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 based on the values of the contribution from the C_4 slurry as was done for C_3 and C_4 sugars (see below).

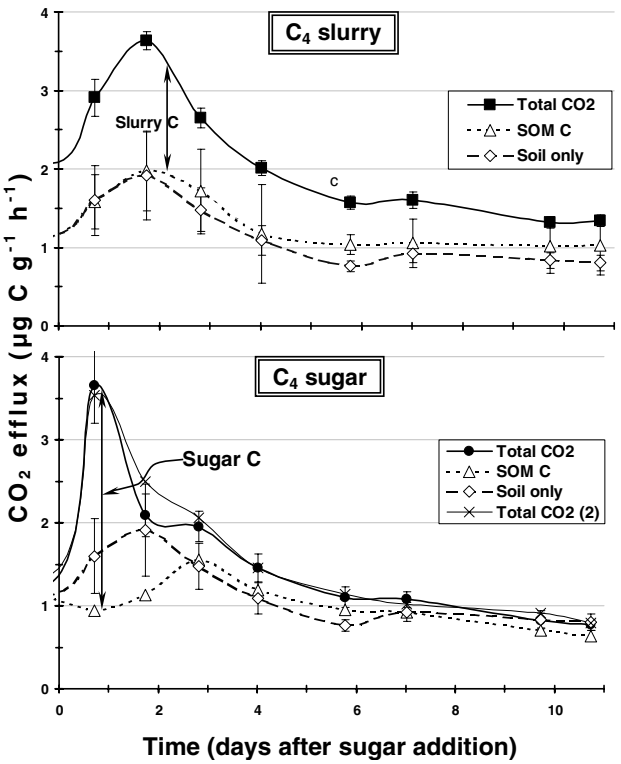

Figure 1. Total CO_2 efflux (±SE) from the soil amended with C_4 slurry (top) or C_4 sugar (bottom) and the contribution of SOM and one of two different C_4 sources (slurry or sugar) to the CO_2 efflux calculated directly by its $\delta^{13}C$ value. ■ or ● total CO_2 efflux; △ CO_2 from C_3 source (=SOM); ◇ total CO_2 efflux from the soil without any addition of C_4 source (=control); ⨯ total CO_2 (2): the total CO_2 efflux from the soil amended with C_3 sugar is shown.

The CO_2 efflux strongly increased in all treatments on day 1 (17 h) after sugar addition (Fig. 1, bottom). The CO_2 efflux then rapidly decreased, but remained at nearly double the level of the flux before the sugar addition between days 2 and 4. Beyond 7 days after sugar addition, the total CO_2 efflux from soil without slurry was no different from that before sugar addition (Fig. 1, bottom). One important assumption is necessary to distinguish between the three C sources in the CO_2 efflux, i.e. that there were no significant differences in decomposition of C_3 and C_4 sugars. Using the chosen experimental approach, we can compare the decomposition of C_3 and C_4 sugars only based on the total CO_2 efflux from soils. Indeed, the curves of total CO_2 efflux from soils treated with C_3 or C_4 sugars do not differ significantly ($p > 0.05$) at any time, and except on day 2 the curves were nearly identical (Fig. 1, bottom). 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**

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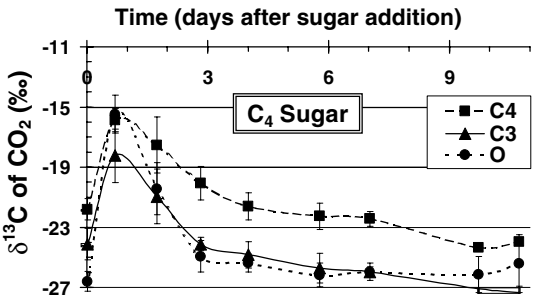

Figure 2. Dynamics of $\delta^{13}C$ values (‰, ±SE) of CO_2 efflux after addition of C_4 sugar to soil previously amended with slurry: ● no slurry; ▲ C_3 slurry; ■ C_4 slurry. The level of X scale shows the $\delta^{13}C$ value of C_4 sugar (11.0‰).

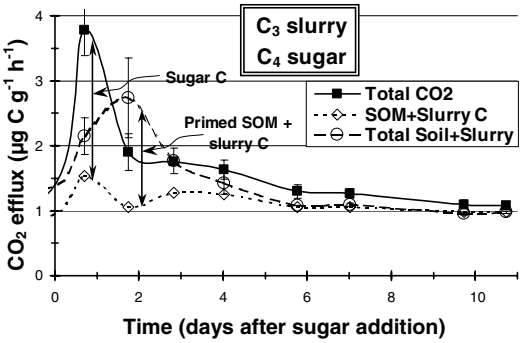

Figure 3. Absolute contribution (±SE) of C_4 sugar to the total CO_2 efflux from the soil previously amended by C_3 slurry calculated directly by $\delta^{13}C$ values of CO_2 and changes in (SOM + slurry)-C mineralization induced by sugar addition (Primed SOM + slurry C). Vertical arrows show the CO_2 efflux originating from C_4 sugar and induced priming effect. ■ total CO_2 efflux; ◇ CO_2 efflux originated from decomposition of SOM + C_3 slurry from the soil after sugar addition; ○ total CO_2 efflux from the soil amended with C_3 without sugar.

calculated contribution of the C_4 sugar to the total CO_2 efflux.

The $\delta^{13}C$ values of CO_2 efflux show the contribution of C_4 sources (sugar or slurry). The addition of C_4 sugar strongly increased the $\delta^{13}C$ value of CO_2 efflux (Fig. 2). The $\delta^{13}C$ maximum coincided with the peak CO_2 concentration on day 1 (17 h) after sugar addition (see Fig. 1). However, the $\delta^{13}C$ value of the CO_2 then did not reach that of the C_4 sugar itself (−11.0‰), thus indicating that the contribution of C_3 sources to the CO_2 efflux was still significant. The maximum increase in the $\delta^{13}C$ value of CO_2 efflux was observed for soil unamended with slurry and the minimum for the soil amended with C_4 slurry. At the same time the rate of decrease of the $\delta^{13}C$ value of CO_2 efflux after the maximum showed the reverse dependence: it was fastest for soil unamended with slurry and slowest for the soil amended with C_4 slurry. This clearly indicates fastest utilization of easily available C source (sugar) in the soil having the smallest total amount of C_{org}.

The $\delta^{13}C$ peak decreased at a slower rate than the total CO_2 efflux (cf. Figs. 1 and 2), indicating that some added C_4 sugar was not immediately decomposed to CO_2, but temporarily 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.

Contribution of two 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 is presented in Fig. 1. 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. 1, top). The C_4 sugar induced a peak in the total CO_2 efflux on days 1 and 2, and the contribution of sugar-C to total CO_2 was significant during the first 4 days. The contribution of C_4 slurry (treatment without sugar) estimated by Eqn. (1) amounted to 23–46% of the total CO_2 efflux (Table 1). It decreased strongly during the incubation period suggesting that slurry decomposition proceeded faster than the decomposition of the native SOM (Fig. 1, top). The estimated contribution of applied sugar (treatment without slurry) to the total CO_2 efflux peaked after 17 h at 74%, but it was effectively finished after 3–4 days (Table 1; Fig. 1, bottom).

The direct estimation of the relative contribution of the C_4 source was more difficult for three CO_2 sources (Table 1, Fig. 3). For example, when C_3 sugar was added to C_4 slurry-amended soil, the contribution of C_4 slurry was between 64–108% (Table 1). The contribution of C_4 slurry to the total CO_2 efflux after sugar addition was much higher than without sugar addition. It implies that sugar addition induced faster slurry decomposition (see below). The addition of C_4 sugar to the C_3 slurry-amended soil was not different from that of the C_4 sugar application to the unamended soil (Table 1). This observation partly confirmed our fourth assumption, that the effect of slurry type (C_3 or C_4) on sugar decomposition was negligible.

The total CO_2 efflux evolved from the soil amended with C_3 slurry + C_4 sugar treatments is presented in Fig. 3. The soil + C_3 slurry (no sugar) was used as the control, in order to estimate the effect of added sugar on soil- and slurry-derived CO_2.

The addition of C_4 sugar to C_3 slurry-amended soil strongly increased total CO_2 efflux through sugar-derived CO_2 (Fig. 3). These effects were similar to the sugar treatment without slurry addition (Fig. 1, bottom). Hence, the total soil CO_2 efflux measured on day 1 was mainly (60%) derived from sugar-C. Note that the maximum variations and therefore uncertainties of calculation of the contribution of the C_4 source were observed (1) at first sampling period when the contribution of sugar (C_4) derived C dominated the total CO_2 efflux (Figs. 1 and 3), and (2) at the last two samplings, because the contribution of sugar was negligible at this time (Table 1).

Estimation of contribution of three C sources to the CO_2 efflux

For estimation of the contributions of three C sources to the total CO_2 efflux from soil, two treatments were evaluated: (i) treatment with C_3 slurry and C_3 sugar, and (ii) treatment with C_4 slurry and C_4 sugar. The principles of the calculations

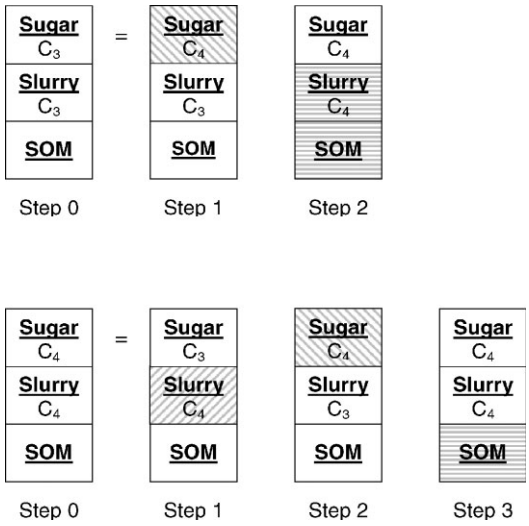

Figure 4. Principles and calculation steps for contributions of three C sources to the CO_2 efflux from soil amended with C_3 slurry and C_3 sugar (top) and amended with C_4 slurry and C_4 sugar (bottom). See text for additional explanations. The calculations were done for each CO_2 sampling time separately.

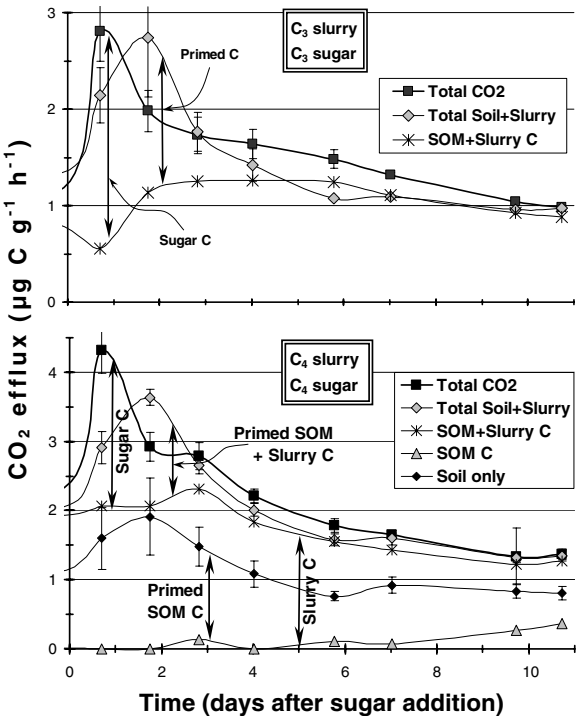

Figure 5. Absolute contribution (±SE) of three C sources to the total CO_2 efflux from the soil calculated indirectly, considering identical decomposition of C_3 and C_4 sugars. The treatments of slurry and sugar are shown in the box in the center of each graph. Vertical arrows show the C_3 or C_4 source. ■ 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; ◆ soil without any additions.

of contributions in both treatments were different and are presented schematically in Fig. 4.

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 that of C_3 sugar (Fig. 4, top; Fig. 5, 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. 4, top). Most of the CO_2 evolved on day 1 comes from C_3 sugar (Fig. 5, top). The sugar contribution then decreased strongly and after day 4 it was about zero. Theoretically, if C_3 and C_4 slurries had identical decomposition, then the decomposition of C_4 slurry + C_3 sugar treatment could have been transposed into the C_3 slurry + C_3 sugar treatment 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. 4, bottom; Fig. 5, bottom). First, we separated the slurry-derived CO_2 by using values from the treatment with C_4 slurry + C_3 sugar addition (based upon the equal decomposition of C_4 and C_3 sugars; see above). Secondly, the contribution of C_4 sugar was taken from the treatment with C_3 slurry + C_4 sugar. Thirdly, the 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. 4, bottom).

Most of the CO_2 emitted during the first 3 days after the sugar addition was derived from the sugar; however, the slurry-C contribution was also important (Fig. 5, bottom). The contribution of SOM was marginal during the first 7 days. However, because of the uncertainty of the CO_2 sources separation during the first 2 days, we cannot exactly estimate the contribution of sugar- and slurry-derived C.

DISCUSSION

Partitioning of two and three C sources

The two C source partitioning of the total CO_2 efflux (Table 1, Fig. 1) was calculated in the same way as in many previous studies.[1,2,7,8,12,17,18] It showed agreement with other results that the contribution to the CO_2 efflux increased, but the duration of the contribution decreased with an increased microbial availability of the C source, i.e. SOM < slur-slurry < sugar.[10,12,19–21] To our knowledge, this is the first time that the contribution of three C sources (SOM, slurry and sugar) of CO_2 efflux has been separated by using only one tracer method (Fig. 5). The separation of three C sources was achieved with a simple experimental design: by comparing the temporal trends in the concentration and $\delta^{13}C$ value of the CO_2 efflux from one soil with three 'past' treatments (no, C_3 and C_4 slurry) and three 'newly' imposed treatments (no, C_3 and C_4 sugar). Based on the existing general assumption regarding similar decomposition of C_3 and C_4 substrates in soil,[1] we successfully separated 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. 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 without C_3 slurry is equal to the contribution of C_3 sugar. The three C source 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.[7,8] Furthermore, there is the tantalizing prospect of using our single tracer three C source 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.

Changes in SOM and slurry decomposition after addition of sugar

The application of organic substances, which are more easily available for microorganisms than SOM (e.g. slurry or sugar), change the soil microbial activity and may induce subsequent associated changes in SOM decomposition. In our experimental design, these two easily available substances (sugar and slurry) were added to soils at different times. The slurry was added to the soil much earlier than the sugar; hence, the main priming effects of SOM induced by slurry would be expected to be (nearly) finished before the application of sugar.[12] Addition of sugar to the soil led in most cases to a short-term (~2 days) decrease in SOM decomposition (e.g. Fig. 1, bottom; Fig. 3). Such a decrease in SOM decomposition is connected with the preferential microbial utilization of an easily available substrate such as sugar, compared with SOM.[14,15] It means that during the first 2 days soil microorganisms switch from the decomposition of recalcitrant SOM to the easily available sugar. Therefore, the decomposition of SOM during the first 2 days was lower than in the sugar unamended control. Such a fast switch to the easily available C source is mostly typical for microorganisms having *r*-strategy.[16]

The use of soil pre-treated with C_4 slurry gives us a unique possibility to differentiate between three different C pools contributing to the total CO_2 efflux, and therefore to evaluate sources of C contributing to priming (Fig. 5, bottom). On day 1 after sugar addition, about 40% of the CO_2 efflux originated from sugar and about 60% originated from slurry-C. The contribution of SOM-derived C to the total CO_2 efflux was marginal during the first 4–5 days. Thereafter, the contribution of SOM-derived C increased gradually and amounted to around 30% on day 11 after sugar addition. These source changes of the total CO_2 efflux after sugar addition can be explained by the following chain of reactions: (1) After sugar addition, the microorganisms mainly switched from the decomposition of heavily available SOM and slurry-derived C to that of sugar. During sugar utilization, microbial biomass activity and probably its *in situ* amount strongly increased. This rapid increase is only possible for microorganisms that have *r*-strategy. (2) After the sugar-C was utilized, the new microorganisms (mainly *r*-strategists) started to utilize the next easily available C source, which is slurry-C. Therefore, the contribution of slurry-derived C strongly dominated after day 3, and the contribution of SOM-derived C was negligible during this period. (3) After about 1 week, the contribution of slurry-derived C steadily decreased and that of SOM-derived C steadily increased, nearly reaching the contribution of SOM-derived C in the treatment without sugar addition at the end of incubation (cf. Fig. 1, top). Such a succession of contributions of different C sources is a clear indicator for the preferential utilization of easily available C not only during decomposition, but also during priming.

Difficulties and limitations of the method

The first limitation of the method is its accuracy. An important part of the separation of C sources is based on the differences between CO_2 efflux rates from different treatments. This difference led to the summation of the variance and resulted in higher errors than in the initial data. Such increase in the variance is especially important for SOM-derived CO_2 efflux calculated by the difference of three variables. So, the calculated SOM-derived CO_2 efflux in the C_4 slurry + C_4 sugar treatment was negative at the first sampling (Fig. 5, bottom). This negative value is the result of estimation of a small value (here SOM-derived CO_2) by its calculation as the difference between two large values (total CO_2 and sugar-derived CO_2) having relatively large errors. Therefore, the SOM-derived CO_2 is not significantly different from zero shortly after sugar addition. In subsequent studies, the use of cumulative systems to trap CO_2 (i.e. sorption on alkali, molecular sieves[22,23] or other techniques to measure soil CO_2 efflux (i.e. at constant CO_2 concentration[24]) will allow a decrease in 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 result in errors of estimation of source contribution to the CO_2 efflux of around 10–15%. By calculation of some values as the difference between two others, this error will be cumulative (see above).

A separate issue is the choice of substrates with appropriate decomposition rates. The substrates we used (partly decomposed slurry and sugar) had decomposition rates that differed by more than one order of magnitude. The rapid decomposition of sugar led to a very strong increase in 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 was not conclusive on this point. The $\delta^{13}C$ of CO_2 efflux evolved by microbial respiration corresponds roughly to $\delta^{13}C$ of microbial biomass.[25] However, by measuring $\delta^{13}C$ of CO_2 respired from 21 Australian soils with C_3 and C_4 vegetation, it was shown that the microbially respired CO_2 is depleted on average by 2.2‰ compared with microbial biomass.[26] At the same time, microbial biomass was enriched by −2.0‰ compared with the $\delta^{13}C$ 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.[26]

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 Eqn. (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 (−28.5‰) 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 $\delta^{13}C$ value of both C_3 sources, i.e. −29.0‰ (78.5% SOM + 21.5% slurry), measured before the experiment, to estimate the C_4 sugar contribution in C_3 slurry-amended soil. The application of such a weighted $\delta^{13}C$ value assumes an equal decomposition rate of both C_3 sources. Because of the last assumption, the calculated contribution of C_4 sugar to 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 the ^{13}C natural abundance method. The use of ^{14}C- and/or ^{13}C-labelled substances would overcome these problems.

CONCLUSIONS AND FUTURE APPLICATIONS

The combination of C_3 and C_4 source treatments (slurry and sugar) applied to a C_3 grassland soil allowed us to distinguish between three CO_2 sources, as well as the changes of C sources contributing to priming. 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 increased, but the period of its contribution decreased with an increased availability of the C source, i.e. sugar $\gg$ slurry > SOM.

The separation of three C sources in the CO_2 efflux by one tracer method showed advantages over the separation of two sources. 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 behaviors in soil. The first possibility is to use uniformly labelled (^{13}C or ^{14}C) substances as analogue for an unlabelled one. The labelling of the substances does not have to be very high: a few hundred 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 completely label in a uniform way. Another easier option would be to obtain uniformly labelled plant residues of Free Air Carbon dioxide Enrichments (FACE) experiments. These experiments[27–29] use CO_2 depleted in ^{13}C (−30‰ to −50‰) compared with atmospheric value ($\delta^{13}C \approx -7.5$‰). After the mixing of 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‰. These ^{13}C-depleted C_3 plant residues can be used as analogues for the same C_3 plants produced under normal air conditions.

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