Distribution and turnover of recently fixed photosynthate in ryegrass rhizospheres

Jessica L. Butler\textsuperscript{a,1}, Peter J. Bottomley\textsuperscript{a,b}, Stephen M. Griffith\textsuperscript{c}, David D. Myrold\textsuperscript{a,*}

\textsuperscript{a}Department of Crop and Soil Science, Oregon State University, 3017 Agricultural and Life Science Building, Corvallis, OR 97331-7306, USA
\textsuperscript{b}Department of Microbiology, Oregon State University, 220 Nash Hall, Corvallis, OR 97331-3804, USA
\textsuperscript{c}National Forage Seed Production Research Center, USDA-ARS, 3450 SW Campus Way, Corvallis, OR 97331, USA

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Abstract

The cycling of root-deposited photosynthate (rhizodeposition) through the soil microbial biomass can have profound influences on plant nutrient availability. Currently, our understanding of microbial dynamics associated with rhizosphere carbon (C) flow is limited. We used a \textsuperscript{13}C pulse-chase labeling procedure to examine the flow of photosynthetically fixed \textsuperscript{13}C into the microbial biomass of the bulk and rhizosphere soils of greenhouse-grown annual ryegrass (\textit{Lolium multiflorum} Lam.). To assess the temporal dynamics of rhizosphere C flow through the microbial biomass, plants were labeled either during the transition between active root growth and rapid shoot growth (Labeling Period 1), or nine days later during the rapid shoot growth stage (Labeling Period 2). Although the distribution of \textsuperscript{13}C in the plant/soil system was similar between the two labeling periods, microbial cycling of rhizodeposition differed between labeling periods. Within 24 h of labeling, more than 10\% of the \textsuperscript{13}C retained in the plant/soil system resided in the soil, most of which had already been incorporated into the microbial biomass. From day 1 to day 8, the proportion of \textsuperscript{13}C in soil as microbial biomass declined from about 90 to 35\% in rhizosphere soil and from about 80 to 30\% in bulk soil. Turnover of \textsuperscript{13}C through the microbial biomass was faster in rhizosphere soil than in bulk soil, and faster in Labeling Period 1 than Labeling Period 2. Our results demonstrate the effectiveness of using \textsuperscript{13}C labeling to examine microbial dynamics and fate of C associated with cycling of rhizodeposition from plants at different phenological stages of growth.

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1. Introduction

Because soil is the largest reservoir of organic carbon (C) in the terrestrial biosphere (Cardon \textit{et al.}, 2001), worldwide efforts have focused on trying to understand the dynamics of soil organic matter in hopes of gaining insight into global C cycling and ecosystem functioning. Although the microbial biomass represents a relatively small portion of soil organic C, generally 1–3\% (Anderson and Domsch, 1989), it is essential that more knowledge be obtained about cycling of C and other nutrients through this pool because most primary productivity (plant material) passes through the soil microbial biomass at some point in time (Ryan and Aravena, 1994). Quantifying the flow of root-deposited photosynthate through the soil microbial biomass is of great importance because of its profound influence on the nutrient supply for plant growth; however, our current knowledge is limited. Thus, there is a fundamental need to gain more information on the microbial dynamics associated with C cycling in the rhizosphere.

The rhizosphere, a zone of high microbial activity in the vicinity of growing plant roots, has received considerable attention since Hiltner first coined the term in 1904 (see Hale and Moore, 1979). Through the use of the C isotopes, \textsuperscript{13}C and \textsuperscript{14}C, the flow of C from the above- to below-ground plant parts, and the subsequent release of some of this photosynthate into the rhizosphere, have been widely investigated (e.g., Meharg, 1994; Swinnen \textit{et al.}, 1995). Collectively referred to as rhizepots, these C compounds, which reach the soil from living roots, consist of a number of organic compounds that differ in their mode of arrival and their degree of complexity/degradability (Lynch...
and Whipps, 1990). Rhizodeposits include any component of the plant cell but are often conveniently classified into soluble and insoluble compounds. It has been estimated that as much as 40% of plant primary production may be lost through rhizodeposition (Lynch and Whipps, 1990) and several researchers have demonstrated that the flow of C into the rhizosphere is influenced by a number of physiological (e.g., plant growth stage, defoliation) and environmental (e.g., temperature, CO2 concentration, soil fertility, soil texture, and light intensity) factors (Martin and Kemp, 1980; Merckx et al., 1985; Whipps, 1985; van Ginkel et al., 1997, 2000; Hütsch et al., 2002). Studies examining the influences of plant growth stage on C allocation below-ground have found mixed results. For example, in most plants the amount of C allocated below-ground has been shown to generally decrease with plant age; however, there have also been reports suggesting an increase in C allocation below-ground in some perennials with age (see review by Kuzyakov and Domanski, 2000). Furthermore, researchers have also demonstrated that the quality of rhizodeposits entering the rhizosphere changes with age or developmental stage, plant species, and environmental conditions (Martin, 1977; Krafczyk et al., 1984; Hütsch et al., 2002). For example, the amount of proteins and carbohydrates in rhizodeposition decreases with age (Brimecombe et al., 2001).

The flow of photosynthate into and through the soil microbial biomass has not received nearly the attention that it deserves. However, the flow of photosynthate into and through the soil microbial biomass is the application of isotopically labeled substrates to soil and subsequently monitoring their decomposition or turnover (Saggar et al., 1996; Aoyama et al., 2000; Kouno et al., 2002). The overall objective of this study was to trace photosynthetically fixed C (13C) through annual ryegrass (Lolium multiflorum Lam. var. Gulf) at two different stages of growth. Our primary focus was on the flow of photosynthate into the microbial biomass in rhizosphere and bulk soil. We hypothesized that any changes in C allocation that take place at the different growth stages will have an influence on both the quality and quantity of rhizodeposition, which will therefore influence both the activity and the turnover of the rhizosphere and bulk soil microbial biomass. To test our hypotheses, we used a 13C pulse-chase labeling procedure to examine the flow of photosynthetically fixed 13C into the rhizosphere and bulk soil microbial biomass.

2. Materials and methods

2.1. Soil

Soil was collected in the summer of 2001 from the top 10 cm of four replicate conventionally tilled, high residue incorporated, grass-seed research plots in Marion county, Oregon (part of the Sustainable Grass Seed Cropping System Research Project established in 1992 by USDA-ARS scientists; see Gohlke et al. (1999)) for a comprehensive site summary. This soil is classified as a Nekia silty-clay loam (clayey, mixed, mesic Xeric Haplohumult) with a pH of 5.1, 35% clay, and 3.7% organic C. Soil samples were air-dried, passed through a 4.75 mm sieve, homogenized, and stored at 4 °C until the start of the experiment.

2.2. Growing conditions

Black plastic containers (600 cm3) were filled with 500 g of air-dried soil and wetted to 25% volumetric water content (approximate bulk density of 1.1 g cm−3). Duct tape was placed over the holes in the bottoms of the containers to keep the soil in and discourage roots from growing out. Prior to planting, soil-filled containers were kept in a greenhouse for 4 weeks in order to allow any weed seeds to germinate. All germinated seeds were removed from the soil.

Seeds of annual ryegrass were germinated in the laboratory on moist filter paper in glass petri dishes. After 5 days, seedlings were transferred to the containers (two seedlings per container) in the greenhouse. Soil water content was adjusted every 2 days by weighing six randomly selected containers and determining the average amount of water necessary to maintain 25% volumetric water content. Light intensity averaged 500 μmol m−2 s−1 with a 16 h photoperiod. Plants were moved at every watering period to reduce any potential bias in growing conditions. Maximum and minimum temperatures were recorded daily and converted into growing degree days (GDD) using the following equation (Griffith, 2000):

\[
GDD = \frac{(T_{\text{max}} + T_{\text{min}})}{2}
\]

where \( T_{\text{max}} \) is the daily maximum temperature and \( T_{\text{min}} \) is the daily minimum temperature measured in °C and is 0 °C as the base temperature. During the experiment, maximum daily temperatures ranged from 21 to 35 °C, daily minimum temperatures ranged from 8 to 18 °C.

2.3. Experimental design

Twenty plants were labeled with 13CO2 during each of two growth stages, and four replicate containers were sampled 1, 2, 3, 5, and 8 days after labeling. One unlabeled container was harvested at each sample period to serve as a control for background δ13C values. To account for any autotrophic activity by soil microorganisms, four unplanted...
containers of soil were included in each labeling period: two that served as unlabeled controls and two that underwent the labeling process (one of each was harvested on days 1 and 8 after labeling). Four extra plants were grown, two of which were used for plant biomass assessment prior to labeling and two were used to determine if plants were mycorrhizal at the time of each labeling period. No evidence of arbuscular mycorrhizae was observed (Butler et al., 2003). All plant parts and soil were analyzed for total C and 13C abundance. Microbial biomass C (MBC) and associated δ13C values were determined on all soil samples.

2.4. 13C Labeling

A gas-tight labeling chamber (40.5 cm × 40.5 cm × 58.5 cm—length × width × height) was constructed out of plexiglass (6.4 mm thickness). The chamber was placed on a piece of plexiglass that had the dimensions of the chamber lined with weather stripping (1.9 cm wide) to create a gas-tight seal. A hole was drilled in the top of the chamber and fitted with a rubber septum for use in labeling. A shelf was constructed inside the chamber to hold labeling supplies along with two, 6 cm² 12 volt fans to promote air circulation. Temperature and condensation were controlled by flowing water through a copper coil that was attached to a water bath maintained at 10°C. The copper coil was wrapped around the bottom perimeter of the chamber to avoid shading effects. Two holes were drilled on opposite sides of the chamber with ports to fit tubing for a LI-COR 6200 CO2 analyzer (LI-COR Inc. Lincoln, NE). Supplemental lighting was placed approximately 20 cm above the chamber to enhance photosynthesis during labeling.

Prior to each labeling period, 20 plants were randomly divided into two groups of 10, which allowed the plants to be spaced out to reduce shading effects and optimize photosynthetic 13CO2 assimilation. Eleven containers (10 planted and one unplanted) were placed in the chamber and allowed to assimilate CO2 until the concentration fell to 200 ppm (v/v). During this time the overall photosynthetic rate was determined and subsequently used to predict the rate of 13CO2 assimilation. 13CO2 could not be directly monitored with the LI-6200 because the infrared range was set for 12CO2 and only slightly overlapped with the range for 13CO2 (Svejcar et al., 1990).

To initiate 13CO2 labeling, 1 ml of 1.5 M lactic acid was added to a beaker containing 22.4 mg 13C as NaHCO3 (99.9 atom% 13C; Cambridge Isotope Laboratories, Andover, MA), which increased the CO2 concentration to about 600 ppm. Once the CO2 concentration fell below 200 ppm, lactic acid was added to an adjacent beaker containing 22.4 mg C as unlabeled NaHCO3, which increased the CO2 concentration by about 400 ppm. When the CO2 concentration fell below 200 ppm the 11 containers were removed from the labeling chamber and placed in a similar Plexiglas chamber to maximize the assimilation of any respired 13CO2 while the other set of containers was being labeled. Each set of 11 containers underwent this labeling procedure three times, however, the CO2 concentration was only increased by about 200 ppm during the last labeling period (11.2 mg 13C). The amount of 13CO2 added during the labeling scenario was sufficient to label the plants at about 500‰ δ13C, assuming about 50% recovery of the label. Care was taken to minimize any shading effects or labeling biases by rotating the set of containers 180° between the first and second labeling and another 90° between the second and third labeling period. Once each set of plants had undergone all three labeling events, all 20 plants were placed back in the chamber for an additional 1.5 h while sufficient 13CO2 was evolved to increase CO2 concentration by 400 ppm and assimilated three times in an effort to minimize loss of 13CO2. Two days prior to the second labeling period, seed heads were removed from plants to enhance translocation of the 13C label to the roots and associated soil. Once labeling had occurred, however, any new seed heads were left on the plants.

A preliminary plant growth study was conducted to determine when the developmental stages for active root growth and rapid shoot growth occur in annual ryegrass. At weekly intervals throughout a 10-week period, three replicate plants were harvested, dried and weighed to determine root and shoot biomass. Based on the preliminary experiment, we anticipated that active root growth would peak at about 900 GDD to be followed by rapid shoot growth about 100 GDD later. Prior to the first labeling event, two plants were harvested, dried, and weighed (one at 574 GDD and one at 742 GDD) to assess whether the roots were growing at a similar rate as observed in the preliminary experiment. Based on these data, we moved up our labeling schedule to 809 GDD (41 calendar days) for the active root growth phase and to 1014 GDD (50 calendar days; the day after the last set of plants labeled at active root growth had been harvested) for the rapid shoot growth phase.

2.5. Harvesting procedure

On each sampling day (1, 2, 3, 5, and 8 days after labeling), four replicate plants were selected randomly, removed from their containers, and weighed. The root/soil systems were sliced down the middle and shaken in plastic bags until approximately 80% of the initial mass was collected. This portion was considered ‘bulk soil’. The soil remaining attached to the root system was considered ‘rhizosphere soil’. The rhizosphere soil was then carefully removed from the roots with a probe and forceps. Root fragments remaining in the bulk or rhizosphere soil were removed by passing through a 1 mm sieve. Soil samples not used for immediate analysis were stored in plastic bags at −20°C.

Plant and soil samples were dried in an oven at 65°C for 48 h. Dried plant material was weighed, transferred into glass jars, and placed on a roller grinder with 8–10 stainless steel rods until ground to a powder (2–4 days). Soil was ground to a fine powder with a mortar and pestle.
2.6. Microbial biomass carbon

Soil was analyzed for MBC immediately following harvest. The fumigation-extraction procedure of Vance et al. (1987) was used, as modified by Bruulsema and Duxbury (1996) for 13C analysis. Briefly, 15 g samples (wet weight) of fumigated (24 h) and non-fumigated soils were placed on a shaker for 1 h with 30 ml of 0.05 M K2SO4 and subsequently filtered through Whatman #40 filter papers. Aliquots (0.5 ml) of each K2SO4 extract were pipetted onto acetone-rinsed tin squares (37 × 37 mm2) (Environmental Microanalysis, Manchester, MA), dried at 60 °C for 1.5 h prior to adding a second 0.5 ml aliquot of K2SO4 extract, and dried overnight. The tin squares containing the dried extracts were balled up with gloved hands. Samples were analyzed for total C and 13C abundance with a PDZ Europa Roboprep Dumas combustion/reduction apparatus (Cheshire, England) interfaced with a Europa 20/20 isotope ratio mass spectrometer (Cheshire, England). By convention, 13C abundances were expressed relative to Pee Dee Belemnite standard as either 13C abundance. A KC of 0.45 was used to convert chloroform flush C for 1.5 h prior to adding a second 0.5 ml aliquot of K2SO4 extract, and dried overnight. The tin squares containing the dried extracts were balled up with gloved hands. Samples were analyzed for total C and 13C abundance with a PDZ Europa Roboprep Dumas combustion/reduction apparatus (Cheshire, England) interfaced with a Europa 20/20 isotope ratio mass spectrometer (Cheshire, England). By convention, 13C abundances were expressed relative to Pee Dee Belemnite standard as either 13C abundance. A KC of 0.45 was used to convert chloroform flush C into MBC (Vance et al., 1987). The following equation was used to determine the Δ13C value of MBC

\[ \delta^{13}C_{\text{MBC}} = (\delta^{13}C_{\text{fum}} \times C_{\text{fum}} - \delta^{13}C_{\text{con}} \times C_{\text{con}})/(C_{\text{fum}} - C_{\text{con}}) \] 

(2)

where \(C_{\text{fum}}\) and \(C_{\text{con}}\) refer to the mass of C extracted from the fumigated and non-fumigated, respectively, and \(\delta^{13}C_{\text{fum}}\) and \(\delta^{13}C_{\text{con}}\) refer to their corresponding 13C values.

2.7. Isotopic analysis

Plant, soil, and K2SO4 extracts were analyzed for total C and 13C abundance with a PDZ Europa 20/20 isotope ratio mass spectrometer (Cheshire, England) interfaced with a Europa Roboprep Dumas combustion/reduction apparatus (Cheshire, England). By convention, 13C abundances were expressed relative to Pee Dee Belemnite standard as either 13C or atom fraction 13C excess (Boutton, 1999).

2.8. Statistics

Analysis of variance (ANOVA) was used to evaluate all time effects using the SAS statistical software package (SAS Institute, 1996), with each labeling period analyzed separately. For all analyses, rhizosphere and bulk soils were analyzed separately because they were not independent of each other. Differences between rhizosphere and bulk soil properties were evaluated using paired t-tests. Significant differences are reported at the \(p < 0.05\) level. Data are reported as means of four replicates unless otherwise noted.

3. Results

3.1. Plant growth

Shoot and root growth were modeled by a logistic equation (Fig. 1). Based on this model, roots reached their maximal growth rate at about 710 GDD. This was much sooner than in the preliminary growth experiment, perhaps because of a difference in photoperiod: the preliminary experiment took place during the winter months, whereas the labeling experiment took place during the spring months. Although plants were kept in the greenhouse, with a controlled photoperiod, they were still influenced by natural, outside light. Other differences in plant growth conditions in the labeling experiment were the use of two, rather than one, seedlings per container and removal of flower/seed heads in the labeling experiment in order to favor translocation of 13C into the soil.

The faster growth in the labeling experiment resulted in roots being labeled about 5 days after their growth rate had peaked, but still at about 90% of the maximum rate (809 GDD, Labeling Period 1). Root and shoot growth rates were about equal at this time. The second labeling was done at 1014 GDD (Labeling Period 2) when shoots were growing about five times faster than roots. The differences in root and shoot growth at the two labeling times suggested that C allocation between shoots and roots likely varied between the two labeling periods.

3.2. Carbon flow/distribution of 13C

Regardless of the efforts made to alleviate potential 13CO2 assimilation biases (i.e., three labelings, rotating plants in the chamber), the labeling of plants was still quite variable. Fig. 2 shows the amount of 13C retained in the plant/soil system throughout the 8-day chase period within each of the two labeling periods. Enough 13C was added so that each plant would obtain 5.6 mg 13C if 100% were assimilated. On the first day after labeling, the average amount of 13C per plant was 3.1 mg (55% of added) in the first labeling period and 2.7 mg (48% of added) in the second labeling period (Table 1). Because respiration was not accounted for, the difference between what was added and what was retained is the sum of any unassimilated 13C...
and that respired during the first day. There were no statistically significant differences in the amount of $^{13}$C retained at any of the days within or between labeling periods (Fig. 1, Table 1). Although not statistically significant, there was a large difference in the amount of $^{13}$C retained on the second day of each labeling period, much of which is likely due to the inherent plant-to-plant variability. In the first labeling period, the plants that were harvested on the second day were, on average, 25% smaller than the plants harvested 1 day before them and 35% smaller than those harvested 1 day after them (Fig. 1). These plants probably did not have as much leaf area for photosynthesis compared to the rest of the plants, and therefore did not assimilate as much $^{13}$CO$_2$. In comparison, the plants that were harvested on the second day of the second labeling period were, on average, 6% bigger than the plants harvested the day before them and 13% bigger than those plants harvested 1 day after them.

Throughout the two chase periods, the $\delta^{13}$C values in shoots and roots were all significantly higher than the shoots and roots of the unlabeled control plants (shoots: $-28 \pm 0.4\%e$; roots: $-27 \pm 0.3\%e$). In the first labeling period, the average shoot $\delta^{13}$C value was 686%e the day after labeling and declined to 380%e by the eighth day (Fig. 3). Consistent with a lesser amount of total $^{13}$C in the plant/soil system (Fig. 2), plants harvested on day 2 of the first labeling period also had a lower average $\delta^{13}$C value compared to plants harvested a day later. This provides further evidence that the plants harvested on day 2, with lower total plant biomass, were not as photosynthetically active relative to plants harvested on day 3. If these plants had assimilated the same amount as the others, then this would be reflected in their $\delta^{13}$C values, which would be higher, not lower, than those harvested on day 3. Overall, the dynamics of $\delta^{13}$C values in the roots followed a similar trend to the shoots; however, there were no significant differences between the root $\delta^{13}$C values throughout the entire labeling period.

In the second labeling period, shoots had an average $\delta^{13}$C value of 500%e 1 day after labeling. By day 8, the average shoot $\delta^{13}$C value had declined to 350%e; however, there were no significant differences in shoot $\delta^{13}$C values throughout the entire chase period (Fig. 3). There did appear to be some shoot to root translocation between days 1 and 2. Following day 2, root $\delta^{13}$C values generally declined, although there was a subtle increase between days 5 and 8, which was not significant and is likely the result of initial labeling variability.

Throughout each chase period, the $\delta^{13}$C values of both the rhizosphere and bulk soils were significantly higher than the unlabeled, planted control soils (rhizosphere: $-27.2 \pm 0.03\%e$; bulk: $-27.2 \pm 0.04\%e$). Aside from the variability associated with day 2 of the first labeling period, the $\delta^{13}$C value in the rhizosphere soil declined steadily during the 8 days, from an average of $-21.6\%e$ on day 1 to $-25.3\%e$ by day 8 (Fig. 3). Conversely, the $\delta^{13}$C value in the bulk soil did not significantly change during the 8 days. In the second labeling period, the $\delta^{13}$C values in the rhizosphere soil also showed an overall decreasing trend from an average of $-23.8\%e$ on the first day to $-25.1\%e$ on day 8; however, there were no significant differences with

### Table 1

<table>
<thead>
<tr>
<th>Component</th>
<th>Labeling Period 1</th>
<th>Labeling Period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 8</td>
</tr>
<tr>
<td><strong>Percentage of $^{13}$CO$_2$ added</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plants and soil system</td>
<td>55.5 $\pm$ 7.4</td>
<td>39.6 $\pm$ 8.6</td>
</tr>
<tr>
<td><strong>Percentage of $^{13}$C in plant/soil system</strong></td>
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<td></td>
</tr>
<tr>
<td>Shoots</td>
<td>68.8 $\pm$ 1.2</td>
<td>67.8 $\pm$ 3.7</td>
</tr>
<tr>
<td>Roots</td>
<td>18.9 $\pm$ 1.6</td>
<td>23.2 $\pm$ 2.6</td>
</tr>
<tr>
<td>Rhizosphere soil</td>
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<td>3.7 $\pm$ 0.6b</td>
</tr>
<tr>
<td>Bulk soil</td>
<td>5.2 $\pm$ 0.8</td>
<td>5.3 $\pm$ 1.0y</td>
</tr>
<tr>
<td><strong>Percentage of $^{13}$C in soil compartment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizosphere microbial biomass C</td>
<td>97.1 $\pm$ 3.4a</td>
<td>42.1 $\pm$ 4.1bx</td>
</tr>
<tr>
<td>Bulk soil microbial biomass C</td>
<td>88.5 $\pm$ 15.0a</td>
<td>36.4 $\pm$ 6.0b</td>
</tr>
</tbody>
</table>

Within a Labeling Period and row, dissimilar letters ‘a’ and ‘b’ indicate significant differences between day 1 and day 8. Within a row, significant differences between Labeling Period 1 and 2 for that day are indicated by ‘x’ and ‘y’.
time. Similar to the first labeling period, the $\delta^{13}$C values in the bulk soil in the second labeling period did not significantly change with time.

Overall, the total amount of $^{13}$C retained in each labeling period was similar (Table 1), as was the distribution of the label within each of the four compartments (shoots, roots, rhizosphere soil, and bulk soil). Including the amount of $^{13}$C residing in each of the four compartments on each day throughout the entire 8-day chase period, an average of 46% of the added $^{13}$C was retained in the first labeling period and 48% was retained in the second labeling period. Labeling period had a slight, but not significant influence on C allocation from the shoots to the roots, with more C being allocated to the roots in the first labeling period (Table 1). Overall, in each labeling period, plants transferred about 12% of their photosynthate to the soil, with slightly higher amounts transferred to the rhizosphere than the bulk soils in Labeling Period 1 and vice versa in Labeling Period 2. It is possible that more $^{13}$C was found in the bulk soil in Labeling Period 2 than in Labeling Period 1 because roots occupied a greater portion of the container in the second labeling period.

3.3. $^{13}$C incorporation into microbial biomass

Soluble organic C (SOC: unfumigated K$_2$SO$_4$ extracts) in both the rhizosphere and bulk soils of the first labeling period showed a downward trend throughout the 8-day chase period; whereas in the second labeling period, a slight upward trend was evident (Fig. 4). In the first labeling period, SOC was higher in the rhizosphere than in the bulk soil throughout the 8 days; however, these differences were only significant on days 5 and 8. In the second labeling period, SOC was significantly higher in the rhizosphere compared to the bulk soil on days 1, 2, and 8, but lower on day 3. Throughout the experiment, rhizosphere soil had about 5 mg C kg$^{-1}$ more SOC than bulk soil.

Microbial biomass C varied throughout the experiment (Fig. 5). In the first labeling period there was a significant decline in the rhizosphere MBC between the first and second sampling days, followed by a steady increase through day 8. The bulk MBC followed a similar trend but appeared to lag behind the rhizosphere MBC by 1 day. In the second labeling period, the rhizosphere MBC also declined from days 1 to 2, along with the bulk MBC declining a day later. These differences were not significant, however. There was a significant difference between MBC in the rhizosphere and bulk soils throughout the experiment with the exception of day 2 of each labeling period, with rhizosphere MBC about 50 mg C kg$^{-1}$ higher than the MBC of bulk soil.

Except for the rhizosphere on days 1 and 2 of Labeling Period 1, the $\delta^{13}$C values of SOC pools did not significantly change throughout the entire chase periods (Fig. 4). Soluble organic C in one of the four containers sampled on the first day of the first labeling period had a $\delta^{15}$C value of 220‰ in
the rhizosphere compared to an average of 22‰ for the other three plants sampled that day. This point was therefore removed from the data set prior to data analysis. The significance of this point is not known, but is likely the result of plant-to-plant variability. In each labeling period SOC in the rhizosphere had significantly higher $\delta^{13}$C values relative to the unlabeled, planted and unplanted control soils ($\pm 26.5 \pm 0.1‰$). In the first labeling period, the bulk soil $\delta^{13}$C value of SOC was only significantly higher than the control from days 3 through 8, whereas in the second labeling period, the bulk $\delta^{13}$C value of SOC was significantly higher throughout the entire chase period (Fig. 4). Soluble organic C had higher $\delta^{13}$C in the rhizosphere soil relative to the bulk soil throughout the entire chase periods; in the first labeling period this difference was significant on every sampling day, whereas it was only significant on days 2 and 8 of the second labeling period.

Fig. 5 shows the $^{13}$C incorporation into the MBC pool. Throughout each of the chase periods, the $\delta^{13}$C values of the rhizosphere and bulk MBC were significantly higher than the $\delta^{13}$C values of the unlabeled, planted control MBC ($\pm 24.7 \pm 0.04‰$). Furthermore, there was no evidence of autotrophic activity, as revealed by the $\delta^{13}$C values of the microbial biomass in the unplanted control soils that underwent the labeling treatment ($\pm 24.5 \pm 0.1‰$). Initial incorporation of rhizodeposited $^{13}$C into the MBC occurred within the first 24 h of labeling, as is illustrated by the high $\delta^{13}$C values 1 day after labeling of each labeling period (Fig. 5). In the first labeling period, rhizosphere MBC $\delta^{13}$C values had a downward trend throughout the 8-day chase period (from $455‰$ on day 1 to $50‰$ on day 8). The bulk soil MBC also declined from $64‰$ on day 1 to $1.0‰$ on day 8. Similar trends were evident in the second labeling period: the $\delta^{13}$C values of the MBC declined in the rhizosphere soil from $220‰$ on day 1 to $40‰$ on day 8 and in the bulk soil from $60‰$ on day 1 to $10‰$ on day 8. Rhizosphere microbial biomass was significantly more labeled than the bulk
microbial biomass on days 1, 3, and 8 of the first labeling period and days 1, 2, 5, and 8 of the second labeling period.

There was a strong correlation between the $\delta^{13}C$ values of the rhizosphere and bulk microbial biomass in each labeling period (Labeling Period 1: $R^2 = 0.77$; Labeling Period 2: $R^2 = 0.82$). The rhizosphere MBC was more than six times as highly labeled as the bulk MBC in the first labeling period, whereas in the second labeling period, the rhizosphere MBC was only, on average, three times more highly labeled than the bulk MBC.

The proportion of the $^{13}C$ in the soil that resided in the microbial biomass pool on days 1 and 8 is shown in Table 1. On day 1, 97% of the $^{13}C$ in the rhizosphere soil resided in the microbial biomass pool in the first labeling period, compared to 85% in Labeling Period 2. In the bulk soil, 88% of the $^{13}C$ label resided in the MBC pool in Labeling Period 1, whereas 68% resided in this pool in the second labeling period. By the end of the 8-day chase period in Labeling Period 1, 42% of the rhizosphere soil $^{13}C$ resided in the rhizosphere MBC, whereas 36% of the bulk soil $^{13}C$ resided in the bulk MBC pool. In contrast, in the second labeling period only 27% of the rhizosphere soil $^{13}C$ resided in the rhizosphere MBC and 23% of the bulk soil $^{13}C$ resided in the bulk MBC pool. There were no significant differences between the percentages of soil $^{13}C$ in the rhizosphere MBC compared to the bulk MBC in either chase period.

4. Discussion

Results from this study add to previous studies demonstrating that $^{13}C$ pulse-chase labeling serves as a useful tool for obtaining information on the cycling of rhizodeposition (Kuzyakov and Domanski, 2000).
Although there was a slight, but not significant, shift from more $^{13}$C allocated to the roots in Labeling Period 1 to more $^{13}$C allocated to shoots in the second labeling period, in general, the $^{13}$C distribution was similar between the two labeling periods. This similarity is likely because we labeled during a stage of transition into active shoot growth and then 10 days later during a more active shoot growth stage (Fig. 1) rather than during two distinct growth stages (i.e., active root growth and rapid shoot growth). Nevertheless, several fundamental differences associated with the microbial cycling of $^{13}$C-labeled rhizodeposition were apparent, which suggests that some ecological/physiological changes had occurred within the plant/soil soil system between the first and second labeling periods.

### 4.1. Carbon flow

Several studies have documented that maximum shoot and root respiration of labeled C occur within the first 24 h after pulse-labeling (Gregory and Atwell, 1991; Swinnen et al., 1994; Domanski et al., 2001; Kuzyakov et al., 2001; Hüttsch et al., 2002; Ostle et al., 2003). For example, in a $^{14}$CO$_2$ pulse-chase labeling experiment with 50-day-old wheat and barley, Gregory and Atwell (1991) observed that 15–25% of the assimilated $^{13}$CO$_2$ was respired within the first 24 h, the majority of which occurred in the first 7.5 h. Others have reported shoot respiration rates as high as 50% shortly after assimilation (Warembourg and Morall, 1978). These findings, along with the possibility that our plants did not assimilate all of the $^{13}$CO$_2$ on the day of labeling could explain why only 50% of the added $^{13}$C was retained in the plant/soil system 1 day after labeling. This trend was consistent between the two labeling periods, suggesting that they responded similarly.

Kuzyakov and Domanski (2000) put together an excellent review on above- and below-ground C distribution in pulse-chase labeling experiments. From this review, it appears that on average, 50% (and as high as 80%) of plant-assimilated C is transferred below-ground in pasture plants. Of this 50%, half remains in roots (25% of total assimilated) and the other half is incorporated into microbial biomass and soil organic matter or respired by roots and microorganisms. It is difficult to compare our C allocation results with other studies given that we are not certain what percentage of the $^{13}$CO$_2$ was initially assimilated or how much was respired. Nevertheless, we found an average of 20% retained in roots and an average of 10% retained in soil (Table 1), suggesting that the below-ground distribution of $^{13}$C in our study was similar to previous reports.

The fact that in each of the two labeling periods there were no significant changes in the total amount of $^{13}$C retained in the plant/soil system with time (although there was a decreasing trend; Fig. 2) and that the only significant change in the distribution of $^{13}$C occurred within the rhizosphere (Table 1) suggests that, for the most part, the label had stabilized in the plant system within the first 24 h. Although there might have been some subtle shifts in C allocation within the plant, the most dynamic flow of $^{13}$C throughout the rest of the chase period was clearly evident within the microbial biomass pool.

Through use of mathematical models, Kuzyakov et al. (2001) demonstrated that maximum $^{14}$CO$_2$ efflux from the soil after pulse-labeling occurs in two main phases: the first of which is root respiration, occurring during the first day after labeling, followed by the second phase, occurring between the second and fifth days, which is dominated by microbial respiration of rhizodeposits. Although we can only speculate about root respiration in our study, our findings are consistent with a second phase of soil CO$_2$ efflux as shown by the declining proportion of soil $^{13}$C in the microbial biomass throughout the 8-day chase periods (Table 1).

After pulse labeling 3-week-old wheat plants with $^{14}$CO$_2$, Cheng et al. (1993) found evidence of $^{14}$C in the soil in less than 1 h, suggesting that recently assimilated C moved through the system very rapidly. Additionally, Rattray et al. (1995) found maximum incorporation of $^{14}$C into the microbial biomass in the rhizosphere of perennial ryegrass within 3 h of pulse labeling. By the seventh day of the chase period, they could no longer detect $^{14}$C in the microbial biomass. In a flooded rice system, Lu et al. (2002) found $^{13}$C in SOC and MBC immediately following a 6 h labeling period, with maximum $^{13}$C incorporation into MBC 3 days later. Although we did not sample until 24 h after labeling, translocation of $^{13}$C into the soil and the subsequent incorporation into the microbial biomass occurred rapidly (within 24 h) in our system as well. This rapid flux is also shown by the low $\delta^{13}$C values of the soluble C pools the first day after labeling (Fig. 5), which is similar to the data of Lu et al. (2002).

Although $^{13}$C-labeled rhizodeposition appeared in similar proportions in each of the two labeling periods (Table 1), differences in both the incorporation of $^{13}$C-labeled rhizodeposits into the MBC pool and the subsequent turnover of $^{13}$C-labeled microbial biomass were evident. The fact that only 85% of the rhizosphere soil and 68% of the bulk soil $^{13}$C were incorporated into the MBC on day 1 of the second labeling period, whereas 97% of rhizosphere $^{13}$C and just under 88% of the bulk soil $^{13}$C were incorporated into the MBC in the first labeling period (Table 1), suggests that the substrate quality of exudates might have been lower in Labeling Period 2 than in Labeling Period 1. It has been documented that the amounts of easily metabolized proteins and carbohydrates in rhizodeposition generally decrease with age (Brimecombe et al., 2001). Thus there could have been more complex, less soluble forms of
rhizodeposition entering the soil, such as sloughed-off root cap cells. Alternatively, it is possible that rhizodeposits had lower C:N ratios in Labeling Period 1 and that nitrogen (N) availability was influencing the amount of rhizodeposition that got incorporated into the microbial biomass, which has been documented in other studies (Merckx et al., 1987; Liljeroth et al., 1990). We did not measure soil N availability, however.

Bokhari et al. (1979) found 4.5 times more soluble sugar in the rhizosphere of blue grama, whereas 1.3 times more insoluble sugar was found in the non-rhizosphere soil. Additionally, decreasing ratios of soluble-to-insoluble rhizodepositions with increasing distance from the roots has also been observed in wheat, barley, maize, tomato and pea plants (Whipps, 1984, 1985, 1987). Thus, it is not surprising that we observed slower incorporation of rhizodeposition into the MBC in the bulk soils, relative to the rhizosphere soils.

Five days after pulse-labeling perennial ryegrass with $^{14}$CO$_2$, Kuzyakov et al. (2001, 2002) found between 0.5 and 1.7% of the total $^{14}$C incorporated into the microbial biomass pool, which corresponded to approximately one-third of the total soil $^{14}$C (presumably the entire container was considered rhizosphere soil with nine plants and 2.2 or 3.5 kg soil). Similarly, in our study, between 1 and 3% of the plant/soil system $^{13}$C on day 5 resided in each of the microbial biomass pools (data not shown). Although there were slight variations between the labeling periods, these values generally correspond to approximately one-third of the $^{13}$C in the soil (Table 1); the remaining two-thirds had therefore been incorporated into soil organic matter.

During the 8-day chase periods, the decline of $^{13}$C in the microbial biomass C pool followed an exponential relationship with time (Fig. 5). Average turnover rates and turnover times for the recently assimilated C in the microbial biomass pools in each of the two labeling periods were estimated by fitting the data to a negative exponential model

$$F^* = F_{0e}^{-kt}$$  \hspace{1cm} (3)

where $F_0$ is the atom fraction excess $^{13}$C at time $t = 0$, $F^*$ is the atom fraction excess $^{13}$C at time $t$, and $k$ is the rate constant. Average atom fraction $^{13}$C of the soluble C pool was used to determine the atom fraction excess $^{13}$C of MBC because we assumed that the majority of rhizodeposited $^{13}$C entering the MBC pool had originated from readily available compounds in the soluble C pool as suggested by de Neergaard and Magid (2001). Furthermore, this calculation of average turnover time is based on the assumption of steady-state conditions (i.e., constant microbial biomass pool size). Although not strictly true (MBC varied significantly at a few time points; Fig. 5), given the high variability and random up/down fluctuations, this would seem a reasonable approximation.

Average rate constants ($k$) and associated turnover times (the inverse of the rate constant, $k$) for the rhizosphere soils were, $0.32 \pm 0.07$ day$^{-1}$, with a turnover of 3.2 days for Labeling Period 1, and $0.24 \pm 0.05$ day$^{-1}$ for Labeling Period 2, with a turnover time of 4.2 days. Bulk values were: $0.18 \pm 0.02$ day$^{-1}$ with a turnover time of 5.5 days in Labeling Period 1, and $0.14 \pm 0.07$ day$^{-1}$ and a turnover time of 7.1 days in Labeling Period 2. Turnover times were nearly twice as fast in the rhizosphere compared to the bulk soil in each labeling period. This suggests that the microorganisms in the rhizosphere were more active than in the bulk soil, which would be expected.

Faster turnover rates are generally observed in the rhizospheres of younger root systems (Kuzyakov et al., 2001), thus it is likely that we would have seen turnover rates faster than 3 days had we labeled in earlier stages of root growth. Furthermore, the slower turnover times observed in both the rhizosphere and bulk soils of Labeling Period 2 suggest that the microbial biomass was more stable at the later stage of plant growth. Norton et al. (1990) estimated 9 days for the turnover of $^{14}$C labeled microbial biomass utilizing rhizodeposition under ponderosa pine seedlings and the data of Ostle et al. (2003) suggest a 7-day turnover for soil RNA in a $^{13}$CO$_2$ pulse-chase experiment with grass soil.

5. Conclusion

Our results confirm that recently assimilated C moves through the plant/soil system at a very rapid pace. Within 24 h after labeling, approximately 12% of the total $^{13}$C retained in the plant/soil system resided in the soil, much of which had already been incorporated into the soil microbial biomass. Although the overall C distribution patterns were similar in the two labeling periods, our results show fundamental differences in the way in which rhizodeposition was cycled through the total microbial biomass during a transition stage of plant development and in a stage more dominated by shoot growth. The use of $^{13}$C pulse-chase labeling was an effective approach for examining the microbial dynamics associated with rhizosphere cycling. Application of this methodology to a range of plant species, developmental growth stages, and environmental conditions has the potential to greatly enhance our knowledge of the dynamics of rhizosphere processes.

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