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# Microbial Community Dynamics Associated with Rhizosphere Carbon Flow

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**Root-deposited photosynthate (rhizodeposition) is an important source of readily available carbon (C) for microbes in the vicinity of growing roots. Plant nutrient availability is controlled, to a large extent, by the cycling of this and other organic materials through the soil microbial community. Currently, our understanding of microbial community dynamics associated with rhizodeposition is limited. We used a $^{13}C$ pulse-chase labeling procedure to examine the incorporation of rhizodeposition into individual phospholipid fatty acids (PLFAs) in the bulk and rhizosphere soils of greenhouse-grown annual ryegrass (*Lolium multiflorum* Lam. var. Gulf). Labeling took place during a growth stage in transition between active root growth and rapid shoot growth on one set of plants (labeling period 1) and 9 days later during the rapid shoot growth stage on another set of plants (labeling period 2). Temporal differences in microbial community composition were more apparent than spatial differences, with a greater relative abundance of PLFAs from gram-positive organisms (i15:0 and a15:0) in the second labeling period. Although more abundant, gram-positive organisms appeared to be less actively utilizing rhizodeposited C in labeling period 2 than in labeling period 1. Gram-negative bacteria associated with the 16:1ω5 PLFA were more active in utilizing $^{13}C$-labeled rhizodeposits in the second labeling period than in the first labeling period. In both labeling periods, however, the fungal PLFA 18:2ω6,9 was the most highly labeled. These results demonstrate the effectiveness of using $^{13}C$ labeling and PLFA analysis to examine the microbial dynamics associated with rhizosphere C cycling by focusing on the members actively involved.**

It is widely recognized that root-deposited photosynthate serves as an important carbon (C) source for microorganisms in the vicinity of growing roots. In turn, plants rely on the microbially mediated decomposition of organic materials for their supply of available nutrients. Several studies have examined the partitioning of photosynthate throughout the plant-soil system (e.g., see reference 23); a few of these studies have monitored the incorporation of this photosynthate (rhizodeposition) into the soil microbial biomass (e.g., see reference 21). Although these studies have revealed a great deal of information on C partitioning and soil organic matter dynamics, the microbial biomass is generally considered a single entity, which reveals nothing of the structure of the microbial community actively involved in nutrient cycling.

Several molecular and biochemical methods have been developed over the past decade that are useful for studying the great diversity of microorganisms in soil, most of which are unknown and unculturable (32). For example, analyses of microbial DNA and phospholipid fatty acids (PLFAs) have proven extremely useful for describing the general structure of soil and aquatic microbial communities. Analysis of PLFAs has been used to monitor changes in microbial community structure in response to several factors, such as agricultural management activities (6, 27, 28, 36) and heavy metal contamination (12). Analysis of PLFAs has also been utilized to examine the structure of rhizosphere microbial communities (14, 17, 18, 29, 30, 31). Although these studies have provided considerable information regarding the dynamics of microbial community structure in rhizospheres, they did not reveal any information regarding the function of microbial communities associated with rhizosphere C cycling.

Only within the last few years has the use of C isotope techniques coupled with PLFA analysis been exploited (4). Because $^{13}C$-PLFA analysis reveals information on the active portion of the microbial community (33), the isotopic labeling of individual PLFAs has the ability to directly link microbial processes with the groups of organisms involved. Boschker et al. (4) first used this approach with $^{13}C$-labeled acetate and methane to examine which members of aquatic microbial communities were involved in two processes: sulfate reduction coupled to acetate oxidation and methane oxidation. Subsequently, this approach has been used to trace $^{13}C$-labeled C substrates into several cultured strains of bacteria and fungi (1), to examine the activity of soil bacterial and fungal biomarkers through incorporation of $^{13}C$-labeled acetate (2), and to link toluene degradation with specific PLFA biomarkers (16).

The primary objective of this experiment was to trace photosynthetically fixed C ($^{13}C$) through the microbial community associated with the rhizosphere and bulk soils of annual ryegrass (*Lolium multiflorum* Lam. var. Gulf) during two different stages of plant growth. We hypothesized that spatial (i.e., rhi-

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zosphere or bulk soil) or temporal (i.e., growth stage) changes in the quality and/or quantity of rhizodeposition would influence which members of the microbial community would most actively utilize rhizodeposition. To test these hypotheses, we used a $^{13}C$ pulse-chase labeling procedure coupled with PLFA analysis. Another paper (8a) reports on the distribution of photosynthetically fixed $^{13}C$ throughout the plant-soil system along with the turnover of $^{13}C$ through the microbial biomass pool. The present paper describes the dynamics of microbial communities associated with rhizosphere C cycling.

## MATERIALS AND METHODS

**Plant production.** Growing conditions are explained in detail in another paper (8a). Briefly, two recently germinated annual ryegrass seedlings were planted in containers with 500 g of a Nekia silty-clay loam (clayey, mixed, mesic Xeric Haplohumult), obtained from conventionally tilled, high-residue-incorporated grass seed research plots in Marion County, Oreg. The soil had a pH of 5.1, was 35% clay and 3.7% organic C, and had a microbial biomass of 335 mg of C $kg^{-1}$. Soil was adjusted to 25% volumetric water content every 2 days. Light intensity averaged 500 μmol $m^{-2}$ $s^{-1}$ with a 16-h photoperiod. Plants were randomly moved at each watering to reduce any potential bias in growing conditions. Maximum daily temperatures ranged from 21 to 35°C; daily minimum temperatures ranged from 8 to 18°C.

**Labeling procedure.** Two pulse-chase experiments were done: the first was at 41 days after germination, when plants were in transition between active root growth and rapid shoot growth (labeling period 1), and the second was 9 days later during a stage dominated by shoot growth (labeling period 2). In each pulse-chase experiment, 20 annual ryegrass plants were exposed to 112 mg of $^{13}CO_2$-C during a 7-h period. About half of the $^{13}C$ was recovered in the plants and soil 1 day after labeling (8a). Of this $^{13}C$, the amount in the microbial biomass declined from 11.3 to 3.3% between days 1 and 8 of labeling period 1 and from 8.7 to 2.9% between days 1 and 8 of label period 2. Unplanted soil was included in the labeling treatment to test for autotrophic activity, which was not detected (data not shown).

**Harvesting procedure.** For the work reported here, four labeled plants were harvested 1 and 8 days following labeling. One unlabeled plant was harvested on each sample day to serve as a control for background $\delta^{13}C$ values. PLFA profiles and associated $\delta^{13}C$ values were determined on rhizosphere and bulk soils. Extra plants were grown, two of which were used for plant biomass assessment prior to labeling and two were used to determine if plants had mycorrhizae.

On each sampling day, randomly selected plants were removed from their containers and weighed. The root-soil systems were sliced down the middle and shaken up in plastic bags until approximately four-fifths of the initial weight was in the bag—this portion was considered bulk soil. The remaining one-fifth that was still 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 were stored in plastic bags at −20°C until analyzed.

**Mycorrhiza staining.** A standard staining procedure was used to assess the presence or absence of mycorrhizae (8). Briefly, roots were rinsed in tap water, placed in Tissue-Tek plastic capsules (Fisher Scientific Co., Pittsburgh, Pa.), and steamed for 45 min at 100°C in a 10% KOH solution. The KOH solution was decanted, and the roots were thoroughly rinsed in tap water. Cleared roots were stained by steaming them in a staining solution of 0.5% trypan blue in lactoglycerol. After staining, roots were rinsed in tap water and examined with a dissecting microscope for the presence of diagnostic features of arbuscular mycorrhizae.

**PLFA analysis.** PLFAs were analyzed on frozen (−20°C) rhizosphere and bulk soils sampled 1 and 8 days following $^{13}CO_2$ labeling. Lipids were extracted according to the procedure of Bligh and Dyer (3). Briefly, sufficient potassium phosphate buffer (100 mM, pH 7.1) and water were added to 15 g of wet soil to achieve a final concentration of 50 mM phosphate and mixed with chloroform and methanol at a ratio of 0.8:1:2. Lipids were extracted the following day by centrifuging (210 × *g* for 5 min) and filtering the supernatant through Whatman no. 1 filter papers. This step was repeated (5) once after the remaining soil had been resuspended with additional chloroform and methanol (same 1:2 ratio). Following filtration, 15 ml of 2 M NaCl was added to the chloroform-methanol-buffer solution in order to separate the phases (9). The bottom (chloroform) phase was removed and immediately dried under a stream of ultrahigh purity (UHP) $N_2$ (99.998%). Dried lipid extracts were redissolved in chloroform and separated into neutral lipids, glycolipids, and phospholipids on solid-phase extraction columns containing 500 mg of silica (Supelco, Inc., Bellefonte, Pa.) with chloroform, acetone, and methanol, respectively (36). The methanol, containing phospholipids, was immediately dried under UHP $N_2$.

Dried phospholipids were then converted to fatty acid methyl esters (FAMEs) through mild alkaline methanolysis by dissolving in 1 ml of 1:1 methanol-toluene and 1 ml of 0.2 M KOH and heating for 15 min in a water bath at 38 to 42°C (34). FAMEs were then extracted by adding 2 ml of deionized water, 0.3 ml of 1 M acetic acid, and 0.5 ml of hexane (5). The hexane phase was transferred to a fresh test tube and dried under UHP $N_2$. Dried samples were redissolved in hexane and transferred to glass inserts (200 μl) placed inside 2-ml amber vials (Agilent Inc., Palo Alto, Calif.) and analyzed by capillary gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Data are expressed as mole percentages, calculated as the area of each PLFA peak relative to the summed area of all PLFA peaks, after first adjusting for the number of C atoms per mole of PLFA.

The $\delta^{13}C$ values of individual PLFAs were determined with an Agilent 6890 gas chromatograph (Agilent Inc.) equipped with a 30-m HP Innowax column (internal diameter, 0.25 mm; film thickness, 0.25 μm) connected to a Europa ORCHID on-line combustion interface in line with a Europa 20-20 mass spectrometer (PDZ Europa Ltd., Northwich, Cheshire, England). The carrier gas was He, and the oven temperature was ramped from 120 to 260°C at a rate of 5°C per min with a 5-min hold at 260°C. Carbon dioxide of known isotopic composition was injected at the beginning and end of each run. Individual PLFA peaks were determined by comparison with retention times of authentic standards. PLFA peaks were confirmed by comparing peaks run on a 25-m HP Ultra-2 column (internal diameter, 0.2 mm; film thickness, 0.33 μm) with the MIDI system (Microbial ID, Inc., Newark, Del.) and by GC-MS. In addition, PLFA extracts of similar soils were analyzed on the HP Innowax column and cross-referenced with several standards: a mixture of 37 FAMEs (FAME 37 47885-4; Supelco, Inc.), a mixture of 24 bacterial FAMEs (P-BAME 24 47080-U; Supelco, Inc.), 18:2ω6,9c, 10Me16:0, and 10Me18:0 (Matreya, Pleasant Gap, Pa.), and MIDI standards (Microbial ID, Inc.). Standard curves were produced with tridecanoic FAME (Supelco, Inc.). In the standard curves, isotopic discrimination was observed in peaks smaller than 52 pmol; these peaks were therefore excluded from the data set.

During the methylation step, when fatty acids are cleaved from phospholipids to form FAMEs, an additional C atom is added to the fatty acid molecule. This additional C atom, of known $\delta^{13}C$ value (−44‰), was accounted for in the $\delta^{13}C$ values of the original PLFAs with the following equation:

$$\delta^{13}C_{PLFA} = [(C_{PLFA} + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}]/C_{PLFA}$$

where $C_{PLFA}$ and $\delta^{13}C_{PLFA}$ refer to the number of C atoms and the $\delta^{13}C$ value, respectively, of the PLFA, $\delta^{13}C_{FAME}$ refers to the $\delta^{13}C$ value after derivatization, and $\delta^{13}C_{MeOH}$ refers to the $\delta^{13}C$ value of the methanol used for methylation.

Standard nomenclature is used to describe PLFAs. The number before the colon refers to the total number of C atoms; the number(s) following the colon refers to the number of double bonds and their location (after the 'ω') in the fatty acid molecule. The prefixes "Me," "cy," "i," and "a" refer to the methyl group, cyclopropane groups, and iso- and anteiso-branched fatty acids, respectively.

We used an HP Innowax column to achieve complete separation of the fungal marker, 18:2ω6,9, which allowed us to directly compare C flow to fungi and bacteria; however, some common PLFAs were not separated by this column. These include: 16:1ω9, 16:1ω7, and 10Me16:0, which merged into one peak (designated 16:1ω7+), and 18:1ω9, 18:1ω7, and 10Me18:0, which also merged into one peak (designated 18:1ω7+). Consequently, we used 12 peaks, consisting of 16 PLFAs and comprising approximately 70% of the total PLFA peak area, for data analysis.

**Light fraction.** Because several of the common microbial PLFAs overlap with those found in plant material, it was necessary to determine if the PLFA profiles obtained and their $\delta^{13}C$ values were representative of the soil microbial community and not plant material. To do this, we separated floatable, plant-derived debris (light fraction) by suspending 15-g soil samples in 20 ml of 50 mM phosphate buffer, centrifuging for 5 min at 8,400 × *g*, decanting the supernatant, and vacuum filtering it through Whatman no. 1 filter papers to collect the light fraction. The centrifugation and filtering procedure was repeated twice after the soil was resuspended prior to centrifugation. Phospholipids were extracted as described above. The light fraction was extracted for PLFAs or dried in an oven for 24 h at 65°C, weighed, ground with a mortar and pestle, and analyzed for $^{13}C$ abundance. The $\delta^{13}C$ values of the light fraction were incorporated into a simple mixing model to determine the maximum amount of root material in the light fraction:

TABLE 1. Average mole percentages of individual PLFAs from days 1 and 8 of each labeling period

| PLFA | Mol % (mean ± SE, $n = 5$) of PLFA in[a]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Labeling period 1 | | | | Labeling period 2 | | | |
| | Day 1 | | Day 8 | | Day 1 | | Day 8 | |
| | Rhizosphere | Bulk | Rhizosphere | Bulk | Rhizosphere | Bulk | Rhizosphere | Bulk |
| 16:0 | 12.5 ± 0.2 | 12.8 ± 0.1 | 12.4 ± 0.5 | 12.6 ± 0.2 | 12.8 ± 0.2 | 12.2 ± 0.6 | 12.3 ± 0.1 | 12.0 ± 0.4 |
| 18:0 | 4.4 ± 0.2 | 4.2 ± 0.1 | 4.6 ± 0.5 | 4.4 ± 0.2 | 4.2 ± 0.4 | 4.4 ± 0.6 | 5.0 ± 0.3 | 5.1 ± 0.4 |
| 16:1ω5 | 4.5 ± 0.1 | 4.7 ± 0.2 | 4.9 ± 0.2 | 4.8 ± 0.2 | 4.7 ± 0.2 | 4.5 ± 0.1 | 4.3 ± 0.2 | 4.5 ± 0.2 |
| cy17:0 | 7.1 ± 0.4 | 7.1 ± 0.3 | 7.5 ± 0.1 | 7.4 ± 0.2 | 7.2 ± 0.3 | 8.3 ± 0.4 | 7.9 ± 0.1 | 7.8 ± 0.4 |
| cy19:0 | 10.1 ± 0.3* | 8.7 ± 0.1 | 9.2 ± 0.2 | 9.2 ± 0.4 | 8.8 ± 0.2 | 8.6 ± 0.7 | 9.8 ± 0.4* | 8.3 ± 0.1 |
| i15:0 | 9.0 ± 0.2 b | 9.9 ± 0.4 | 9.4 ± 0.5 ab | 10.5 ± 0.5 | 11.3 ± 0.3 a | 10.8 ± 1.1 | 11.1 ± 0.6 a | 11.6 ± 1.0 |
| a15:0 | 7.2 ± 0.1 | 7.9 ± 0.2 | 7.7 ± 0.3 | 8.1 ± 0.3 | 8.5 ± 0.3 | 8.1 ± 0.5 | 8.1 ± 0.3 | 8.4 ± 0.4 |
| i16:0 | 5.7 ± 0.1 | 5.9 ± 0.6 | 5.7 ± 0.4 | 6.3 ± 0.3 | 6.4 ± 0.4 | 6.3 ± 0.2 | 6.7 ± 0.1 | 6.1 ± 0.3 |
| i17:0 | 4.0 ± 0.2 | 4.3 ± 0.1 | 4.5 ± 0.5 | 4.0 ± 0.2 | 4.0 ± 0.1 | 4.4 ± 0.3 | 4.5 ± 0.2 | 4.1 ± 0.2 |
| 18:2ω6,9 | 5.9 ± 0.3 | 5.4 ± 0.3 | 5.3 ± 0.5 | 5.3 ± 0.2 | 5.4 ± 0.3 | 6.3 ± 1.1 | 5.7 ± 0.4 | 5.7 ± 1.0 |
| 16:1ω7+ | 9.6 ± 0.1 | 10.1 ± 0.1 | 9.8 ± 0.3 | 9.7 ± 0.2 | 9.6 ± 0.1 | 9.4 ± 0.4 | 8.8 ± 0.5 | 9.4 ± 0.2 |
| 18:1ω7+ | 20.0 ± 0.5 a | 18.9 ± 0.2 | 19.0 ± 0.4 ab | 17.8 ± 1.1 | 17.1 ± 0.5 bc | 16.5 ± 0.2 | 15.7 ± 0.7 c | 17.0 ± 0.8 |

[a] Dissimilar letters within a rhizosphere or bulk sample indicate significant time differences ($P = 0.01$). *, significant difference between rhizosphere and bulk soil samples on that day ($P = 0.01$).

$$\% \text{ of } {}^{13}C \text{ derived from roots} = 100 \times$$

$$[(\delta^{13}C_{\text{labeled LF}} - \delta^{13}C_{\text{unlabeled LF}})/(\delta^{13}C_{\text{labeled RT}} - \delta^{13}C_{\text{unlabeled LF}})]$$

where LF and RT refer to light fraction and roots, respectively.

Light fraction samples were analyzed for $^{13}C$ abundance with a PDZ Europa 20-20 isotope ratio mass spectrometer interfaced with a Europa Roboprep elemental analyzer. By convention, $^{13}C$ abundances were expressed relative to the Pee Dee Belemnite standard as either $\delta^{13}C$ or atom fraction $^{13}C$ excess (7).

**Statistics.** Multivariate analysis of variance (MANOVA) was used to determine the overall effects of time on mole percentages and $\delta^{13}C$ values of PLFAs using the SAS statistical software package (SAS/STAT user's guide, version 6.12; SAS Institute, Cary, N.C.). Labeling periods were analyzed together for mole percentage data and separately for $\delta^{13}C$ value data. 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 for individual PLFAs were determined using Tukey's honestly significant difference method. Significance for all data are reported at the $P = 0.01$ level. Data are reported as means of four replicates unless otherwise noted.

Nonmetric multidimensional scaling (NMS) based on Sørensen's distance was used to provide a graphical representation of PLFA profile relationships (19, 24; Multivariate Analysis of Ecological Data, version 4.0; MjM Software, Gleneden Beach, Oreg.). NMS approximates community relatedness among samples based on the distance measure with synthetic axes. It is a nonparametric ordination method that performs an iterative search for a placement of $n$ entities on $k$ dimensions (axes) that minimizes the stress of the $k$-dimensional configuration (Multivariate Analysis of Ecological Data, version 4.0). PLFA mole percentage data underwent the "general relativization" option in PC-ORD to standardize individual PLFA across all samples. NMS was constrained to two axes and the "slow-and-thorough" autopilot mode of NMS in PC-ORD used the best of 40 runs with a random starting configuration using the real data and 50 runs using randomized data for a Monte Carlo test of significance. Final stability was evaluated for each run by examining plots of stress (a measure of the dissimilarity between ordinations in the original $p$-dimensional space and in the reduced dimensional space) versus number of iterations.

## RESULTS

**PLFAs.** A list of the PLFAs used in the data analysis, along with their mole percentages on days 1 and 8 of each labeling period, is shown in Table 1. Figure 1 shows the NMS plot of PLFA community profile data for rhizosphere and bulk soils sampled on days 1 and 8 of each labeling period. Because the control samples grouped with their labeled counterparts, we assumed that the actual labeling treatment (i.e., sitting in a chamber for 7 h) did not affect overall PLFA profiles and included the control samples in further analysis of the mole percentage data. In the NMS plot, axis 1 and axis 2 explained 55 and 38% of the variation, respectively (Fig. 1). With the exception of 16:1ω5, each PLFA was significantly correlated with at least one NMS axis, but only a few, such as the gram-positive-bacterial markers a15:0 and i15:0, showed correlations greater than 0.75 (Fig. 1). The majority of the samples from the first labeling period had positive axis 2 values whereas the majority of samples from labeling period 2 had negative axis 2 values. Within each labeling period, rhizosphere and bulk soil samples grouped together.

There were very few significant differences in mole percentages of individual PLFAs between the rhizosphere and bulk soils (Table 1), which confirmed the lack of a noticeable effect of soil origin in the NMS ordination (Fig. 1). Of all the PLFAs, only cy19:0 showed a consistent trend between rhizosphere and bulk soils, being equal or higher in the rhizosphere, significantly so on two days.

Based on MANOVA, there were no significant temporal effects on the overall PLFA profiles in the bulk soil (Wilks' lambda = 0.195), but marginally significant changes were seen in rhizosphere soil (Wilks' lambda = 0.100). In the rhizosphere samples, time and plant age each had significant effects on two PLFAs (Table 1). Generally, the gram-positive PLFA markers i15:0, a15:0, and i16:0 increased with time, but significantly only for i15:0. The combined PLFA 18:1ω7+, which includes 10Me18:0, a PLFA found in actinomycetes (22), decreased significantly with time.

**$^{13}C$ incorporation into microbial PLFAs.** The $\delta^{13}C$ values of PLFAs in the unlabeled, planted control soils varied from −35.2 to −28.6‰, slightly depleted relative to that of soil organic C (−27.2‰), and there were no consistent trends in the $\delta^{13}C$ values for each of the PLFAs in rhizosphere soil compared to bulk soil. Furthermore, 8 of the 12 peaks were not significantly different between the rhizosphere and bulk soil of unlabeled controls; thus, all 10 unlabeled control samples (rhizosphere and bulk soils from the four sampling days) were

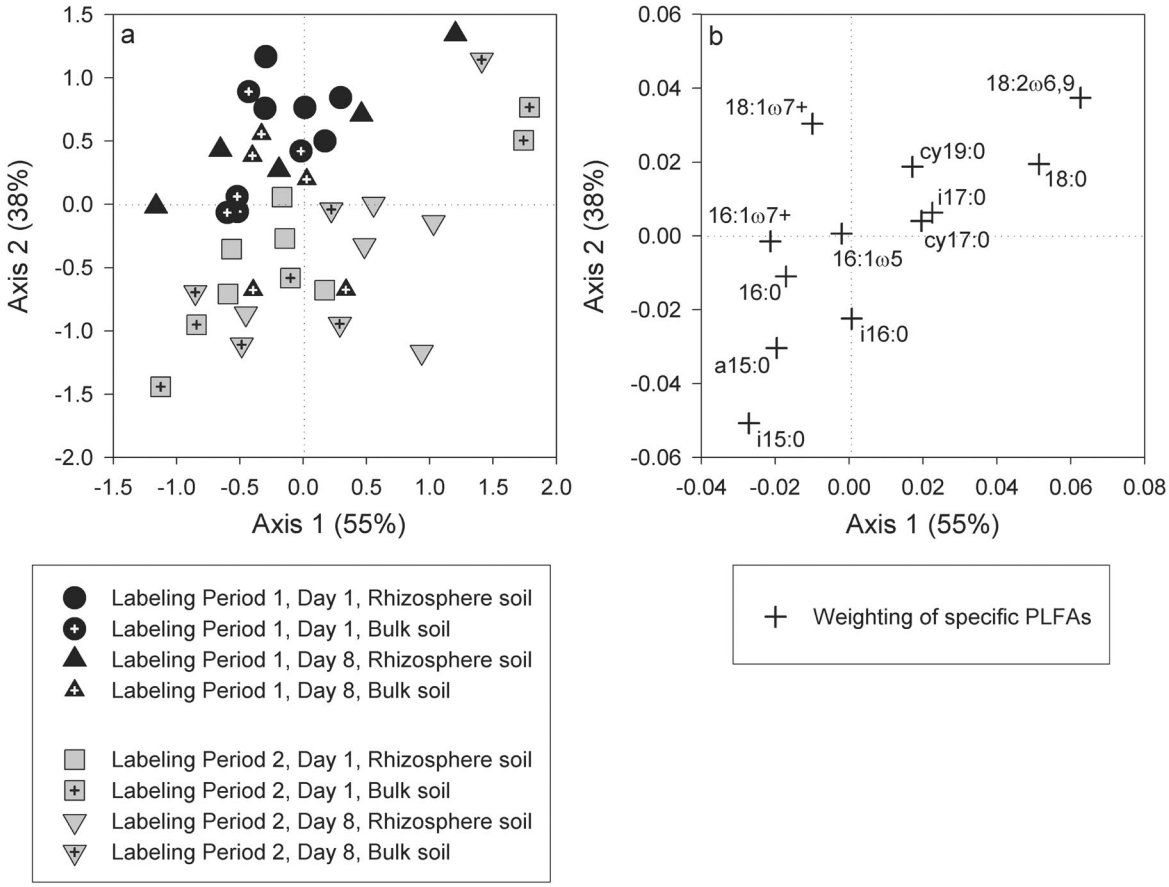


FIG. 1. Nonmetric multidimensional scaling plots of: mole percentages of PLFAs of rhizosphere and bulk soils sampled days 1 and 8 of each labeling period (a) and weighting factors of individual PLFAs (b). The proportion of variance explained by each axis is indicated in parentheses.

combined to determine the statistical significance of the labeled samples (Fig. 2).

Significant differences in the $\delta^{13}C$ values of PLFAs were found for the bulk (Wilks' lambda = 0.001) and rhizosphere (Wilks' lambda = 0.005) soils during labeling period 1. In the first labeling period, most of the PLFAs were significantly enriched in $^{13}C$ to some extent on at least one of the two sampling days (Fig. 2a), with the exception of cy17:0 and 18:0 and cy19:0 in the bulk soil. In both the rhizosphere and bulk soils in the first labeling period, the most label was incorporated into 18:2ω6,9 (rhizosphere, 274 ± 35‰; bulk, 48 ± 16‰) and 16:0 (rhizosphere, 77 ± 12‰; bulk, −0.8 ± 1.3‰). There were also significant differences in the labeling of PLFAs of the rhizosphere relative to the bulk soil in the first labeling period. One day following labeling, 16:0, cy19:0, 18:2ω6,9, 16:1ω7+, and 18:1ω7+ were significantly more enriched in $^{13}C$ in the rhizosphere than in the bulk soil. By the eighth day of the chase period, the $\delta^{13}C$ values in the rhizosphere had declined significantly in 16:0, i15:0, a15:0, i16:0, 18:2ω6,9, 16:1ω7+, and 18:1ω7+, whereas in the bulk soil, only the 16:0 showed a significant decline relative to day 1 (Fig. 2b). Furthermore, only 16:0, 18:2ω6,9, and 18:1ω7+ were still significantly more enriched in $^{13}C$ in the rhizosphere soil relative to the bulk soil on day 8.

Much like what was observed in the first labeling period, the PLFAs that incorporated the most $^{13}C$ in the rhizosphere and bulk soils in the second labeling period were 18:2ω6,9 (rhizosphere, 127 ± 21‰; bulk, 18 ± 11‰) and 16:0 (rhizosphere, 28 ± 8‰; bulk, −10 ± 2‰); however, 16:1ω5 was also highly enriched and had a higher $\delta^{13}C$ value than 16:0 (rhizosphere, 43 ± 6‰; bulk, 22 ± 6‰) (Fig. 2c). In contrast to labeling period 1 (Fig. 2a), in the second labeling period, many of the PLFAs did not incorporate significantly more $^{13}C$ on day 1 relative to unlabeled controls, including: 18:0, cy17:0, cy19:0, i15:0, i16:0, i17:0, and 16:1ω7+ in the rhizosphere and bulk soil and a15:0 in the bulk soil only (Fig. 2c). By day 8 of the second labeling period, however, most of the PLFAs were significantly enriched in $^{13}C$, except for 18:0, cy17:0, and cy19:0, and i16:0 in the bulk soil only (Fig. 2d). Just two PLFAs were more enriched in $^{13}C$ in the rhizosphere than in the bulk soil, 16:0 and 18:2ω6,9 (Fig. 2c). Compared to the first labeling period, where several of the PLFA $\delta^{13}C$ values declined substantially between days 1 and 8 in the rhizosphere, in the second labeling period only 16:1ω5 declined significantly. Conversely, the bulk soil showed a slight but significant increase in the $\delta^{13}C$ values of a15:0, 16:1ω7+, and 18:1ω7+ and a decrease of 16:1ω5.

**PLFAs and light fraction.** Although considerable effort went into removing all visible root material from soil during harvest, it was possible that the highly labeled PLFAs (i.e., 18:2ω6,9 and 16:0, which are abundant in plant cells) were coming from

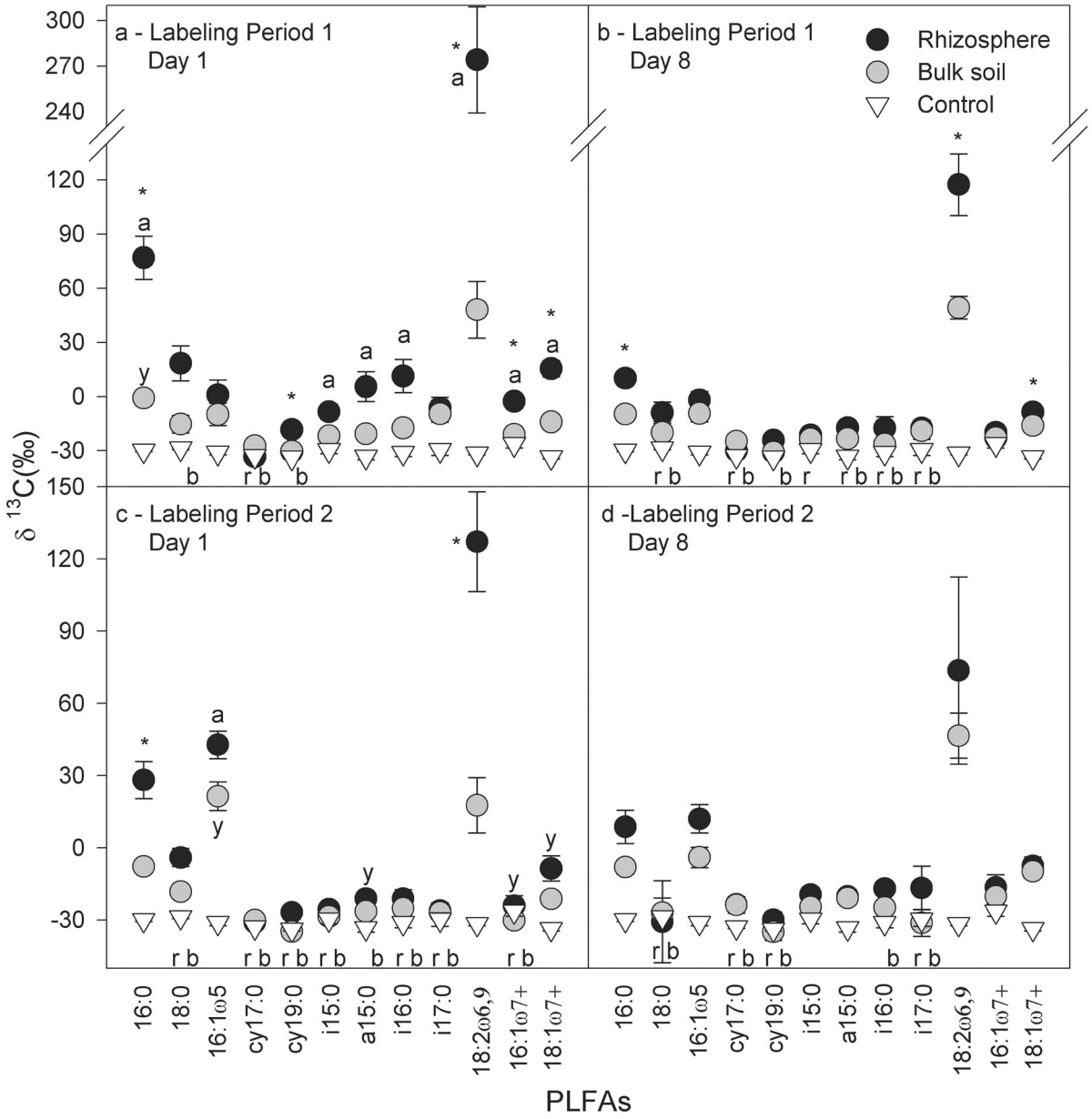


FIG. 2. Mean $\delta^{13}C$ values (with standard errors, $n = 4$) of individual PLFAs extracted from rhizosphere and bulk soils. "r" (rhizosphere) and "b" (bulk) indicate that there is no significant difference from the unlabeled control soils ($n = 10$). *, significant difference between rhizosphere and bulk $\delta^{13}C$ values on that day. Within each labeling period, significant time differences (day 1 versus day 8) are designated by "a" for the rhizosphere data and "y" for the bulk soil data.

root material remaining in the soil. We addressed this concern by extracting PLFAs from the light fraction and from soil with the light fraction removed to see how the $\delta^{13}C$ values of PLFAs would be affected. Light fraction removed from labeled soil was also analyzed for $\delta^{13}C$.

We used 16:0, a dominant PLFA in plants and microorganisms, to determine if plant-derived PLFAs were present and thus contributing to the $\delta^{13}C$ values observed in soil. The $\delta^{13}C$ values of 16:0 in rhizosphere soils increased upon removal of the light fraction material whereas those of bulk soils changed little (±2‰), providing evidence that the labeling of PLFAs in soil was not coming from plant-derived PLFAs (data not shown). It is not clear why the $\delta^{13}C$ values of 16:0 increased in the rhizosphere upon removal of the light fraction, although it is possible that there were more unlabeled organisms associated with the light fraction in the rhizosphere and they were lost during the fractionation process.

An average of 15.8 mg of light fraction was separated from each of the 15-g soil samples during centrifugation (Table 2). When the light fraction was extracted for PLFAs, all peaks were below the limit of reliable $^{13}C$ measurement; therefore, no PLFA data were obtained. The maximum percentage of plant material that could potentially be residing in the light fraction, and therefore influencing its $\delta^{13}C$ value, was <1.8%

TABLE 2. Abundance of $^{13}C$ and amount of light fraction separated from 15-g soil samples (i.e., the amount used in PLFA extractions)[a]

| Sample | LF amt (mg/15 g of soil) | $\delta^{13}C$ abundance (‰) in: | | % Roots in LF[b] |
|---|---|---|---|---|
| | | Roots | LF | |
| Control rhizosphere | 16.9 | −26.7 | −28.5 | |
| Day 1 rhizosphere | 16.4 | 173 | −24.9 | 1.8 |
| Day 8 rhizosphere | 15.9 | 267 | −24.8 | 1.3 |
| Control bulk | 17.4 | −26.7 | −27.9 | |
| Day 1 bulk | 14.7 | 173 | −26.6 | 0.7 |
| Day 8 bulk | 13.6 | 267 | −28.8 | NL |

[a] All soils were from labeling period 1. Rhizosphere and bulk soils were analyzed from the same plant.
[b] Calculated from the equation in the text. NL, not labeled.

in the rhizosphere soil and <0.7% in the bulk soil (Table 2). This provides further evidence that the soil PLFA $\delta^{13}C$ values were not coming from roots. Furthermore, this calculation provides a liberal estimate of root material that could be in the light fraction because the $\delta^{13}C$ values of the light fraction could include microbial biomass or other labeled organic matter and not only root material. Nevertheless, we think it is important to remove any root material from soil prior to PLFA extraction, as the presence of highly labeled plant-derived PLFAs has the potential to severely alter data interpretation.

**Mycorrhizal infection.** Neither of the plants harvested at the start of each labeling period had the diagnostic morphological features of active mycorrhizae (arbuscules and vesicles) in their root systems, although fungal hyphae in close contact with the root system were evident.

## DISCUSSION

Our results demonstrated that coupling of $^{13}C$ pulse-chase labeling and PLFA analysis was a useful tool for obtaining information on the structure of the microbial communities actively involved in cycling of rhizodeposition. Although recently fixed $^{13}C$ was distributed similarly between plants and soil in each of the two labeling periods (8a), there were several fundamental differences associated with the movement of $^{13}C$-labeled rhizodeposits through PLFAs of the microbial community, which suggested that some ecological or physiological changes had occurred within the plant/soil system between the first and second labeling periods.

**Microbial community dynamics.** Because many PLFAs are present to some degree in most, if not all, organisms (e.g., 16:0), relating PLFAs to specific groups of organisms must be done with caution. Nevertheless, several PLFA biomarkers have been recognized in the literature (35). Additionally, because we were unable to separate some PLFAs (16:1ω9, 16:1ω7, and 10Me16:0; 18:1ω9, 18:1ω7, and 10Me18:0) in our GC-C-IRMS, we are limited in our ability to interpret the ecological significance of these PLFAs in this experiment.

We were surprised to find few differences between the PLFA profiles of rhizosphere and bulk soils. This may have been because our operational separation of rhizosphere soil left too much bulk soil adhering to the roots or because high root density caused all soil to be affected by roots. Nevertheless, a study that used compartmentalized pots to unambiguously separate roots of the perennial grass *Agrostis stolonifera* from bulk soil also failed to find a strong difference between rhizosphere and bulk soil microbial communities during active shoot growth (31).

Temporal trends in PLFA profiles of rhizosphere soils were apparent, the most significant of which were the increasing proportion of i15:0, which is generally synthesized by gram-positive bacteria (25), and a decreasing proportion of 18:1ω7+, which contains monounsaturated PLFAs associated with gram-negative bacteria and 10Me18:0 associated with actinomycetes (25). In contrast, Steer and Harris (31) observed a decrease of PLFAs associated with gram-positive bacteria and an increase in the proportion of several monounsaturated fatty acids with increasing plant age (70 to 160 days). These conflicting results may reflect differences in plant species, experimental duration, and root growth activity (8a, 31).

**$^{13}C$ PLFAs.** Phospholipids are rapidly degraded upon cell death and are not found in storage lipids (36); therefore, they serve as useful indicators of living microorganisms. The incorporation of rhizodeposits into PLFAs was slower in the second labeling period (e.g., several of the PLFAs were more enriched in $^{13}C$ on day 8 than on day 1) than in the first labeling period, which suggests that the microorganisms incorporating the label were less active or growing more slowly. This observation is consistent with the smaller amount of the soil $^{13}C$ incorporated into microbial biomass on the first day of the second labeling period relative to the first period (8a) and suggests that rhizodeposition occurred at a lower rate during labeling period 2 and/or was comprised of less readily available substrates than in labeling period 1.

PLFAs were differentially labeled with $^{13}C$ from rhizodeposition, a phenomenon observed even with simple, widely utilized C substrates, such as glucose (16) or acetate (2). As in those studies, we found 18:2ω6,9 to be the most highly labeled PLFA (Fig. 2). 18:2ω6,9 is often used as a biomarker for fungi (10) because it is present in high abundance in most fungi (although not in arbuscular mycorrhizal fungi [26]). This suggests that nonmycorrhizal fungal populations in the rhizosphere and bulk soils were most actively utilizing rhizodeposition. This is consistent with the presence of fungal hyphae growing in close association with the root systems we examined and increases in 18:2ω6,9 observed when amounts of synthetic root exudates added to soil were increased (14).

We also found significant $^{13}C$ in 16:0 and 18:1ω7+ peaks (Fig. 2). Because 16:0 is ubiquitous in cell membranes of all organisms and 18:1ω7+ was comprised of several PLFAs, these are not particularly useful in identifying active members of the microbial community. We note, however, the PLFAs in these two peaks were also highly enriched when $^{13}C$-labeled glucose or acetate was added to soils (2, 16).

A fundamental difference between the two labeling periods was the incorporation of $^{13}C$ into the 16:1ω5 PLFA (Fig. 2), which is found in gram-negative bacteria (11) and arbuscular mycorrhizal fungi (26). Arbuscular mycorrhizal fungi do not appear to be a major contributor to the 16:1ω5 peak, because our plants showed no signs of mycorrhizal infection. Thus, it appears that gram-negative bacteria were the major contributors to the 16:1ω5 peak in our study.

Gram-negative bacteria associated with 16:1ω5 incorporated some $^{13}C$-labeled rhizodeposits in each of the labeling periods;

TABLE 3. Average percentages of $^{13}C$ incorporated into each PLFA peak relative to the amount of $^{13}C$ incorporated in the 16:0 of rhizosphere and bulk soils on day 1 of each labeling period

| PLFA | % of $^{13}C$ incorporated[a] | | | |
|---|---|---|---|---|
| | Labeling period 1 | | Labeling period 2 | |
| | Rhizosphere | Bulk | Rhizosphere | Bulk |
| i15:0 | 20 | 25 | 7 | 6 |
| a15:0 | 31 | 26 | 13 | 10 |
| i16:0 | 38 | 40 | 15 | 31 |
| 16:0 | | | | |
| 16:1ω7+ | 30 | 43 | 18 | 20 |
| 16:1ω5 | 29 | 63 | 119 | 203 |
| i17:0 | 26 | 76 | 14 | 30 |
| cy17:0 | NL | 14 | 2 | 7 |
| 18:0 | 46 | 51 | 45 | 53 |
| 18:1ω7+ | 43 | 56 | 38 | 43 |
| 18:2ω6,9 | 274 | 235 | 253 | 180 |
| cy19:0 | 14 | 12 | 12 | NL |

[a] Percentages were determined by dividing the difference between the $\delta^{13}C$ value of the PLFA in the labeled and unlabeled samples by the difference in $\delta^{13}C$ value of the labeled and unlabeled 16:0. Numbers greater than 100 indicate that those peaks were more highly labeled than 16:0. NL, not labeled.

however, there was a dramatic difference in their activity between labeling periods. In labeling period 1, 16:1ω5 was labeled only 29% relative to 16:0 in the rhizosphere and 63% relative to 16:0 in the bulk soil, whereas in labeling period 2, 16:1ω5 was labeled slightly higher than 16:0 in the rhizosphere and was twice as $^{13}C$ enriched as 16:0 in the bulk soil (Table 3). It is possible that a change in rhizodeposition quality led to a change in the activity of the gram-negative bacterial community, resulting in the gram-negative organisms associated with 16:1ω5 actively utilizing more rhizodeposited C in labeling period 2. Interestingly, though, these gram-negative bacteria were likely utilizing the more readily available rhizodeposition during the second labeling period, considering that they incorporated $^{13}C$ into PLFAs within the first 24 h after labeling. This is also shown by the significant decline in the 16:1ω5 $\delta^{13}C$ value in the rhizosphere and bulk soil between days 1 and 8 of labeling period 2 (Fig. 2c and d), suggesting rapid turnover of these organisms. In contrast, the $\delta^{13}C$ values of 16:1ω5 in labeling period 1 were not different between the rhizosphere and bulk soils, nor did they change between days 1 and 8 (Fig. 2a and b), suggesting that there was little turnover of these gram-negative bacteria in the first labeling period.

Clearly, 16:1ω5 represented the biggest difference in the fate of rhizodeposition among the gram-negative bacterial community in the two labeling periods. Because of overlapping peaks, we were unable to differentiate between changes in the activity of gram-negative bacteria associated with the 16:1ω7+ or 18:1ω7+ peaks. The cyclopropyl PLFAs, also associated with gram-negative bacterial PLFAs, are formed from the precursors 16:1ω7 and 18:1ω7 during periods of substrate-limited growth (15). These cyclopropyl PLFAs, cy17:0 and cy19:0, were the least labeled relative to 16:0 (Table 3), and this was similar in each of the two labeling periods. Thus, $^{13}C$-labeled rhizodeposits were used to synthesize cyclopropyl PLFAs; however, this was not a major component of the overall activity of the gram-negative bacterial community.

In the first labeling period, gram-positive bacteria actively utilized $^{13}C$-labeled rhizodeposits, as shown by the labeling of i15:0, a15:0, i16:0, and i17:0 relative to 16:0 in the rhizosphere and bulk soils on day 1 (Table 3). In contrast, in the second labeling period, these gram-positive PLFAs were only slightly enriched, and in many cases they were not significantly different from the unlabeled control soils (Fig. 2c and d). Although there was an increase in gram-positive organisms (mole percentage of i15:0) (Table 2) between the two labeling periods, it appears that gram-positive bacteria in the second labeling period either did not compete well for rhizodeposited C or simply utilized soil organic matter preferentially. Perhaps it is possible that the increase in gram-positive organisms in the second labeling period resulted from some type of priming effect (an accelerated decomposition of soil organic matter), which results from rhizodeposition in the later stages of plant development (20). It has been suggested that the presence of certain groups of microorganisms in the rhizospheres of different plant species is related to the variations in exudates produced (13). Thus, temporal variation in rhizodeposition by a plant species is also likely to influence the activity and presence of certain microorganisms.

**Conclusions.** Results from this study show fundamental differences in the way in which rhizodeposition was cycled through microbial communities during two stages of plant development. The use of PLFA analysis coupled with $^{13}C$ pulse-chase labeling appears to be a promising and effective approach for examining the microbial dynamics associated with rhizosphere C 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 rhizosphere processes by focusing on the members actively involved in the cycling of nutrients within this complex system.

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