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REGULAR ARTICLE

^{13}C labeling of plant assimilates using a simple canopy-scale open air system

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Abstract A simple system based on web-FACE technology was designed and implemented as an approach to label plant-assimilated carbon (C) with ^{13}C. The labeling system avoids the use of a chamber or other enclosure, instead distributing CO_2 heavily enriched in ^{13}C at near atmospheric concentrations to the tree foliage through the use of porous tubing. The system was applied to three plantation grown juvenile larch (*Larix* spp.) trees during the daylight hours over the course of five days in the middle of the growing season. Relative to control trees, fumigation with enriched CO_2 resulted in significantly ^{13}C-enriched foliar respiration and nighttime soil respiration. Enrichment was also created in the foliar tissue, but differences between labeled and control trees were not statistically significant. Temporal and spatial variation in the strength of the isotopic label did occur, and modifications to the system are suggested to limit the variation. The approach should enable the implementation of pulse-chase experiments designed to understand plant source-sink relationships or experiments designed to understand the flux of C from plant roots into the soil food web.

Keywords ^{13}C labeling · Carbon assimilation · Keeling plots · *Larix* spp. (larch) · Open air system

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Introduction

Numerous studies have isotopically labeled plant carbon (C) in order to determine the fate of assimilated C within the plant and the movement of C from plants into the soil. In the past, a majority of these studies have utilized the radio-isotope ^{14}C. However, due to safety and experimental issues associated with this technique, increasing emphasis has been put on use of the stable isotope ^{13}C in experimental labeling schemes (Deléens et al. 1994; Simard et al. 1997). An added benefit is that the rate of discrimination against ^{13}C is lower than that of ^{14}C in metabolic processes (Ehleringer 1991), allowing for more accurate measurements of plant C allocation (Simard et al. 1997), especially under circumstances where the amount of fractionation is unknown.

While a number of studies utilize ^{13}C as a label for plant C, a majority of these studies have been

conducted on agricultural crops or grasses (Simard et al. 1997). Although several investigators have applied an isotopic label to plant C in forested systems, this technique is generally limited to seedlings and branches (Simard et al. 1997; Lacointe et al. 2004; Phillips and Fahey 2005; Wiegner et al. 2005; Nogués et al. 2006) or trees under going treatment with elevated CO_2 derived from fossil fuels highly depleted in ^{13}C (Haile-Mariam et al. 2000; Leavitt et al. 2001; Loya et al. 2003; Steinmann et al. 2004; Johnsen et al. 2005; Pregitzer et al. 2006; Keel et al. 2006). Beyond those studies of elevated CO_2 utilizing free-air carbon dioxide enrichment (FACE), we are aware of no other labeling studies in which the process of labeling photosynthetically derived C does not involve a chamber, bag or other similar enclosure device (Avice et al. 1996; Simard et al. 1997; Stewart and Metherell 1999; Ostle et al. 2000; Lacointe et al. 2004; Wiegner et al. 2005; Phillips and Fahey 2005; Nogués et al. 2006). Chamber conditions can differ significantly from ambient conditions and have been shown to alter conditions that affect plant gas exchange traits (Owensby et al. 1993). Chamber designs are also impractical and/or expensive for labeling mature trees in the field.

Inspired by the web-FACE system in Switzerland that delivers elevated concentrations of CO_2 to the canopy of mature trees (Pepin and Körner 2002), we set out to devise a simple system that would allow investigators to label recent photosynthates. Our goal was to create a practical and low-cost system that could be used by researchers interested in tracing C assimilated in vivo under typical field conditions and near ambient concentrations of CO_2. Here, we present the results of our first web-based system designed to deliver $^{13}CO_2$ to juvenile larch trees. We discuss the successes and limitations of our system, and make suggestions for future improvements.

Materials and methods

Plot preparation

We conducted our experiment in an 8-year old larch (*Larix spp.*) plantation with 2–2.5 m tree spacing on a dry upland site in Houghton County, MI, USA. Soils at the site were well-drained sandy loams. Soil samples collected prior to the experiment from the top 20 cm of soil averaged 0.09% N, 1.6% C, and a $\delta^{13}C$ of −28.3‰.

Within the plantation, eight larch trees were selected for study, all ~2 m in height. We randomly selected half of the trees for labeling with ^{13}C-enriched CO_2, with the remaining trees used as controls. Thirty days prior to the start of the ^{13}C fumigation, we dug a circular trench 0.6 m deep and 2 m across around the base of each tree. Construction grade plastic sheeting was laid in the trench to prevent root infiltration and respiration from surrounding plants, and the trench was backfilled. After trenching, all competing vegetation within the trenched area was removed; weeding was repeated during the experiment as needed. To help trees cope with possible trenching-induced water stress, natural precipitation was supplemented by adding 5 l of water to each tree two nights per week. Prior to the fumigation, one tree appeared to display signs of water stress and was removed from the experiment. Thereafter, we reduced the sample size to three for both the control and $^{13}CO_2$-enriched "fumigated" treatments.

After trenching the trees, we took eight equally spaced soil cores (depth: 20 cm, diameter: 10 cm) 50 cm from the base of each tree. The cores were sieved through a 4-mm sieve, removing coarse roots and other large organic material. Half of the cores were fertilized by mixing into the sieved soil N–P–K fertilizer with the concentration of added N equivalent to 200 kg N ha^{-1} (42 kg P ha^{-1} and 83 kg K ha^{-1}). The sieved soil (with or without fertilizer) was placed back into the original core cavity.

Labeling system

The labeling system used to deliver the ^{13}C-enriched CO_2 to the trees consisted of a pressurized source of 99% $^{13}CO_2$, battery-operated pumps to mix the $^{13}CO_2$ with atmospheric air, and microporous tubing to deliver the ^{13}C-enriched mixture in close proximity to the foliage on the trees (Fig. 1). The pressurized gas was combined with air in two stages, using high flow air pumps (Boxer Model 3114/12, Clark Solutions, Cambridge, MA, USA) to ensure uniform and rapid mixing of the ^{13}C around the tree needles. We ran the labeling system during the daylight hours for 5 days,

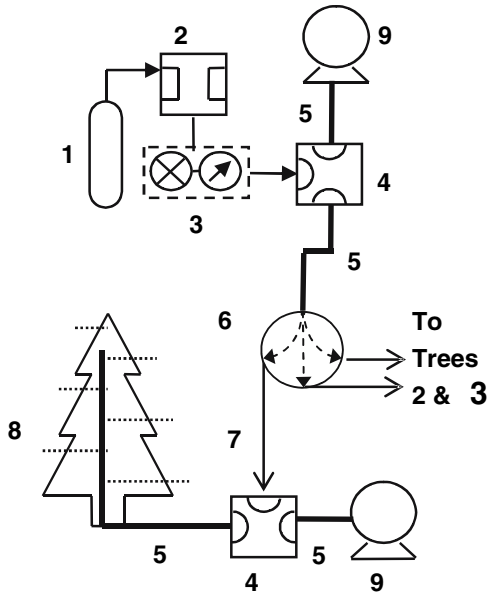

Fig. 1 Schematic diagram of the gas distribution system used to create isotopic enrichment in plant assimilated C in juvenile larch trees. Labeling for the diagram is as follows: 1—Pressurized $^{13}CO_2$; 2—Adapter, regulator, and tubing connector; 3—Flow meter; 4—Tee junction; 5—3/8″ Transfer tubing; 6—Manifold; 7—¼″ Transfer tubing; 8—Microporous tubing; 9—Air pump and 12 V battery

beginning on Julian day 174 and ending when the $^{13}CO_2$ supply was exhausted during day 178 (23 Jun–27 Jun 2005).

To create the labeling system, gas from the 50 l pressurized ^{13}C source cylinder was first depressurized to ~30 kPa and the flow rate of the gas was adjusted to ~75 ml min^{-1} using a volume flowmeter equipped with a needle valve (Bel-Art Scienceware Flowmeter). The gas was then sent to a manifold (12-outlet HDPE drip head, The Drip Store, San Marcos, CA, USA), with one manifold inlet connected to a pump that was pumping air at 30 l min^{-1}. From the manifold, three outlets were connected to each of the three fumigated trees and the remaining outlets were blocked using rubber caps (TOP caps, The Drip Store). At each of the three fumigated trees, a second pump (also pumping air at 30 l min^{-1}) was used to further dilute the air-$^{13}CO_2$ mixture. This was achieved by combining the line running from the manifold to the enrichment plots with a line from the outlet of the second pump through a Tee-joint (3/8″-1/4′-3/8″ Tee, Swagelok Corp., Solon, OH, USA).

The final mixture of air at 422 μl l^{-1} of CO_2 and with a calculated estimate of $\delta^{13}C$ of +15,500‰ was delivered to the fumigated trees using impervious rubber tubing (3/8″) that led from the pump along the main stem of each tree for its entire height. Tees were inserted into this tubing at ~15 cm intervals, and a section of Irrigro® lightweight drip-irrigation tubing (standard width, International Irrigation Systems, Niagara Falls, NY, USA) made of micro-porous Tyvek® was attached to each tee. The tubing was then wrapped around the branches of each tree and secured in place using cable ties. No part of the foliage was more than 6 cm away from the nearest section of irrigation tubing.

Respiration sampling

We used $\delta^{13}C$ of foliar-respired and soil-respired CO_2 determined using the Keeling plot method (Pataki et al. 2003; Xu et al. 2004) as measures of the amount of enrichment resulting from fumigation. Keeling plots estimate source $\delta^{13}C$ by creating a regression between the values for $\delta^{13}C$ (y-axis) and the inverse of the concentration of CO_2 ($[CO_2]$, x-axis) for a temporal sequence of gas samples. The source $\delta^{13}C$ is equal to the point where the regression line crosses the y-axis. Soil respiration was sampled beneath the fumigated trees on three nights before the label was applied. On one of these pre-fumigation nights, soil respiration was also collected beneath the unfumigated trees. Foliar respiration samples were collected from all trees on three nights prior to fumigation, and all trees were sampled for foliar and soil respiration during four nights after fumigation had begun. Foliar and soil respiration were sampled on concurrent nights, except for the final two sampling periods when we delayed soil respiration sampling by 24 h to account for the lag between assimilation by the leaves and respiration belowground.

To sample foliar respiration (Xu et al. 2004), we placed a gas tight bag fitted with a rubber septum (Chemware Tedlar Gas Sampling Bags) around a single branch. An airtight seal was created by applying modeling clay and electrical tape to both the opening of the bag and the tree branch. Bags were placed on three separate branches ~1 m in height located around each tree. Once sealed around a branch, 7 ml gas samples were extracted from each bag at 20, 40, 60, and 80 min. At each time interval,

samples from all three branches were bulked inside of the syringe for distribution into two separate helium-flushed vials for determination of $\delta^{13}C$ and [CO_2]. Samples taken for CO_2 concentration were stored in 3 ml Wheaton Serum vials with Wheaton 7×13 mm^2 butyl rubber stoppers sealed with Wheaton aluminum crimp seals. Samples used for determination of $\delta^{13}C$ were stored in 12 ml Exetainer vials (Labco 738W) and Exetainer caps with a pierce-able rubber septum (Labco Limited, Buckinghamshire, UK).

To sample soil respiration (Pregitzer et al. 2006), static chambers were created from opaque one liter wide-mouth polypropylene bottles with the bottoms removed. These bottles were then placed on top of each of the cores and in four additional undisturbed areas located between the experimental cores. The bottles were placed over the cores 2 days before the first sampling and remained in place during sampling to minimize the influence of disturbance on soil CO_2 efflux. The bottle lids were fitted with rubber septa for gas sampling and remained off the bottles until sampling. The time sequence of soil CO_2 sampling varied over the course of the study in an attempt to increase the strength of our $\delta^{13}C$ estimates. Initially (Julian day 170), gas samples were extracted with a syringe at 80, 100, 120, and 140 min after capping the bottles. Later (days 171 and 172), samples were extracted at 60, 80, 100, and 120 min or at (all subsequent sampling dates) 30, 45, 60, 80, and 100 min after capping. At each time interval, samples taken over similar cores (fertilized, unfertilized, and uncored soil) were combined resulting in three different soil respiration samples for each tree and distributed into two separate helium-flushed vials for analysis as noted above.

The $\delta^{13}C$ of CO_2 was analyzed on a GasBench II (Finnigan MAT, Breman, Germany) connected to a ThermoFinnigan Delta^{plus} Continuous Flow Isotope Ratio Mass Spectrometer (IRMS) located at the Michigan Technological University Forest Ecology Analytical Laboratory. Samples were measured against a CO_2 reference gas calibrated with IAEA reference materials. The standard deviation of repeated measurements of a laboratory CO_2 standard analyzed every 12 samples was 0.1‰ for $\delta^{13}C$. The IAEA reference materials had a reported precision of 0.2–0.25‰. Other studies have made linearity corrections on $\delta^{13}C$ measurements based on sample CO_2 concentrations (Van Vuuren et al. 2000), however other work with Keeling plots in our laboratory (Pregitzer et al. 2006) obtained good estimates of $\delta^{13}C$ without using this correction. CO_2 concentrations were analyzed on an Agilent 6890N gas chromatograph with an HP-Plot Q capillary column from J&W Scientific and a TCD detector (at 220°C). The accuracy of this instrument is ±5% of the reported value. Analysis of gas samples was completed within 5 days of sampling, adequate to avoid meaningful storage effects on sample $\delta^{13}C$ (Tu et al. 2001).

Data were analyzed using SAS version 9.1.3 (SAS Institute, Cary, NC, USA) with a significance level of 0.05. Enrichment of respiration samples (foliar and soil) from fumigation and differences between the three types of soil respiration samples were tested using split-plot (with core type as the split plot) repeated measures in PROC GLM with additional GLM analyses to test individual sampling dates. Soil respiration data from the final sampling period (day 179) were not included in the statistical analysis because a laboratory error caused the loss of all of the samples from one of the fumigated trees.

Tissue sampling

For each tree, four major branches at approximately each cardinal direction were removed at three heights (0.3, 0.7, and 1.20 m from the tree base) following the last foliar respiration sampling. The sampled branches were cut into 15 cm segments. Side branches extending from the main branch were trimmed at 10 cm in order to make estimates of labeling radially in the tree crown. Samples representing each of the four cardinal directions were combined by height and distance from the bole. The needles were stripped from the branches, oven-dried, and ground for isotope (IRMS) analysis. These data were analyzed in separate two-way (height and fumigation; radial distance and fumigation) ANOVA using SAS PROC GLM.

Results and discussion

For foliar dark respiration, the fumigation system created significant enrichment on three of the four sampling periods and a significant overall enrichment (P = 0.004, Fig. 2a). However, the level of

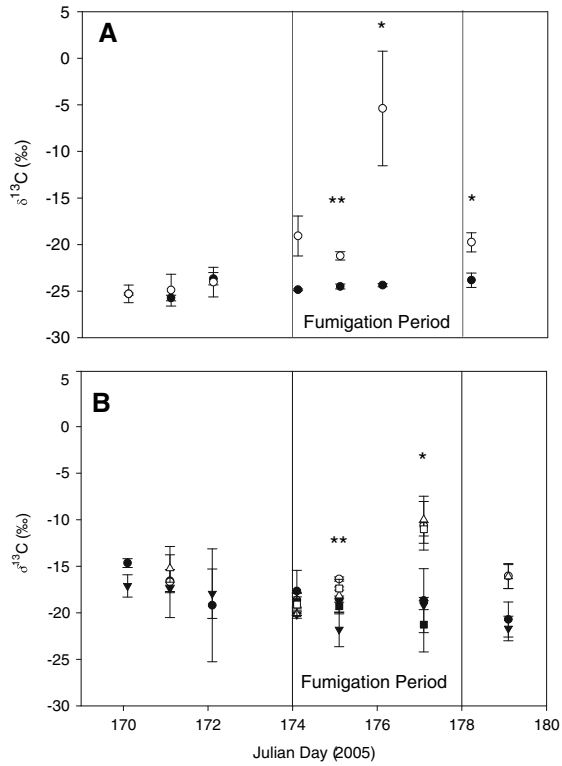

Fig. 2 Average (±1 SE) $\delta^{13}C$ of foliar respiration (**a**) and soil respiration (**b**) measured using the Keeling plot method for juvenile larch trees undergoing fumigation with ^{13}C-enriched CO_2 and unfumigated controls ($n = 3$). For both figures *filled symbols* represent respiration samples from unfumigated trees and *open symbols* represent respiration samples from fumigated trees. For the soil respiration figure, *circles* represent respiration over cores with added fertilizer, *triangles* represent cores without fertilizer, and *squares* represent samples taken over intact soil that was not cored and sieved. Overall fumigation enrichment was significant for both foliar ($P = 0.004$) and soil respiration ($P = 0.046$). Levels of significance for fumigation differences on individual days are represented by the number of *asterisks*: $*P < 0.05$, $**P < 0.01$. Differences among the soil types (fertilized, unfertilized, and intact) were not significant

enrichment varied by day ($P = 0.03$), ranging from 4 to 19‰. The variation in enrichment of foliar respiration was probably the result of differences in wind velocity, with the most enriched day (176) having a low average wind speed and the lowest maximum wind speed of any day during the fumigation period (National Climatic Data Center, Houghton County Airport Observation Post, 10 km away). Despite only 5 days of fumigation, the enrichment was strong enough to create differences in bulk needle tissue $\delta^{13}C$ (data not shown), although the fumigation effects were not statistically significant. The enrichment of needle tissues in the fumigated trees was observed at all horizontal and vertical levels within the canopy with a mean (±SD) of 1.7 ± 0.9‰.

Enrichment of soil respired CO_2 was also significant over the duration of the fumigation period ($P = 0.046$, Fig. 2b). Soil respired CO_2 mirrored those trends found in the foliar respiration (day × fumigation interaction: $P < 0.001$), but with an apparent 1-day lag. Initial enrichment did not occur until the second day of fumigation and the peak in enrichment of soil respired CO_2 followed the peak in foliar respiration enrichment by 1 day.

The enrichment from fumigation was so strong that the effect was significant despite difficulty in achieving reliable Keeling plot estimates of soil respiration $\delta^{13}C$. Our Keeling intervals were larger than in other applications of the technique (Tu et al. 2001; Tu and Dawson 2005; Göttlicher et al. 2006; Pregitzer et al. 2006) and were problematic. Five-minute intervals were used in some preliminary tests prior to fumigation, but were subsequently abandoned. Low soil respiration rates produced low-sample CO_2 concentrations during the 5-min intervals. Analysis of these samples created weak IRMS CO_2 peak amplitudes and decreased the precision of the IRMS. Longer intervals were adopted to maintain IRMS precision and to try to create a wide range in $[CO_2]$ that would produce more accurate Keeling estimates (Pataki et al. 2003). However, the high-sample concentrations that occurred during the longer sampling intervals may have created equilibrium conditions that favored exchange of respired CO_2 with soil CO_2 and atmospheric CO_2.

Susfalk et al. (2002) observed the phenomenon of atmospheric mixing with soil respiration in their attempts to measure the $\delta^{13}C$ of soil CO_2 in a dry sandy clay loam soil after observing $\delta^{13}C$-values that were more enriched (−20‰) than plant and soil $\delta^{13}C$-values (−26 to −27‰). We observed a similar result experimenting in dry sandy loam, with soil respired CO_2 from the unfumigated plots (∼−20‰) more enriched than either pre-fumigation bulk soil C (−28.3‰) or unfumigated foliar respired CO_2 (∼−25‰). We did not encounter these issues on a previous test of our enrichment system on a potted larch tree growing in a finer textured soil with higher respiration rates and smaller Keeling sampling inter-

vals. Ultimately, the use of longer sampling intervals probably reduced R^2 correlations between 1/[CO_2] and δ^{13}C-values. These correlations ranged from 0.973 to 0.002, with many too low to achieve the precise estimates of δ^{13}C normally afforded by Keeling plots (Fig. 3). However, the δ^{13}C label was strong enough to be distinct despite our methodological difficulties. Susfalk et al. (2002) also observed horizontal diffusion of CO_2 within the soil. This pattern of diffusion likely obscured any differences in δ^{13}C between the three sets of soil respiration samples (fertilized, unfertilized, intact) in our study, which did differ significantly from one another. This suggests that future work with nutrient baiting should include barriers to horizontal diffusion.

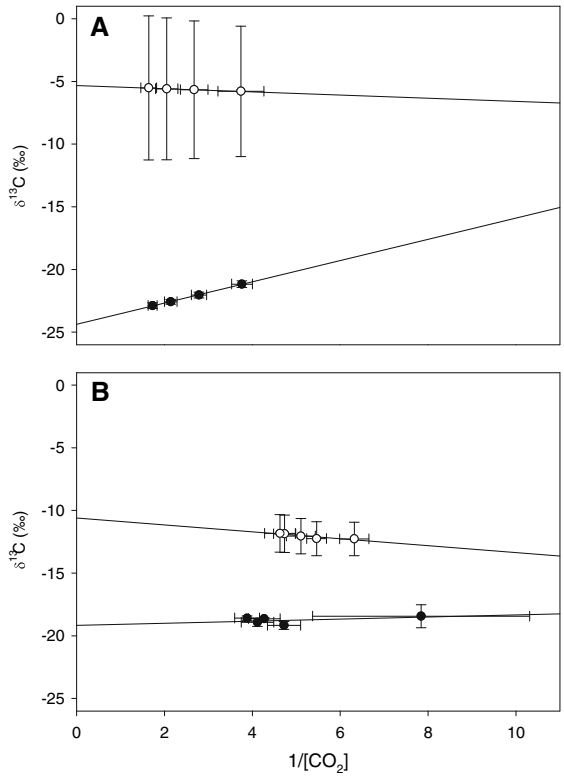

Fig. 3 Average Keeling plots (±SE) for foliar (**a**) and soil (**b**) respiration on Julian days 176 (foliar) and 177 (soil) for fumigated (*open symbols*) and unfumigated (*filled symbols*) trees. Concentrations of CO_2 in liters CO_2 per liter. Data points represent average values at each sampling interval for the three trees in each fumigation treatment. Soil respiration values were averaged across core types (fertilized, unfertilized, and intact) at the tree level. R^2-values for foliar respiration (both fumigated and unfumigated) were 0.99. R^2-values for soil respiration were 0.82 and 0.22 for fumigated and unfumigated trees, respectively

Potential improvements and applications

Our system for ^{13}C labeling of assimilated plant C has the advantages of being cost effective and relatively simple. Few substantial costs were incurred beyond isotopic analysis and the supply of $^{13}CO_2$. Unlike a chamber approach, application of this technique on the canopy or perhaps the stand scale is realistic and has little risk of disturbing plant gas exchange. We feel this approach is particularly applicable to trees with a simple branching structure, as in many conifers. It is important to note that although this system was effective, it could be improved to reduce variability in label strength. Temporal variability could be reduced through the use of pumps that allow for flow rates to be increased on days with high-wind speeds. We observed some variation in label strength within the canopy. The fumigation enrichment was roughly 1‰ stronger in the interior than exterior needles and was 0.7‰ stronger at the bottom of the canopy than the top. We feel that these differences could be reduced by using less porous canopy tubing (Laser Drilled Soaker Hose, 6′ spacing, The Drip Store). A preliminary labeling experiment used this tubing, and we feel that it more evenly distributed the $^{13}CO_2$. Also, running the impervious tubing first to near the top of each tree and then branching off irrigation lines to the foliage could reduce this label strength variation. Both changes would help maintain a more constant pressure among the fumigation lines and promote a more even distribution of $^{13}CO_2$. Overall, we feel that a consistent label for assimilated C could be achieved using our simple labeling system and the minor modifications suggested here. Collaboration with others providing basic engineering expertise could further increase system precision.

The motivation for developing our simple open-air ^{13}C labeling system was the desire to be able to trace the movement of C from the plant into the soil. Rigorous mass-balance estimates of C assimilation and whole-plant allocation would require many assumptions, and making this type of estimate was not the motivation for the development of this technique. However, results suggest that studies of the timing of C flux through the plant and into the soil food web, and the allocation of plant C into factorial soil treatments (e.g., nutrient baiting experiments),

should be feasible using the approach we have developed.

Our attempts to estimate the belowground label strength through Keeling plots were problematic, however other experiments approaches could be applied to take advantage of our simple labeling system. For instance, periodic measurements of phloem sap $\delta^{13}C$ (Pate and Arthur 1998; Barbour et al. 2000) during labeling and shortly after labeling could be used to estimate the total belowground flux of $\delta^{13}C$. This belowground flux could then be partitioned into fine roots, rhizosphere soil, bulk soil (Deléens et al. 1994; Simard et al. 1997; Phillips and Fahey 2005; Johnsen et al. 2005), and even partitioned among microbial communities (Boschker and Middelburg 2002) and plant C pools (sugars, lipids, etc., Wanek et al. 2001). Using these processes, the labeling system could be applied to analyze changes in the fate of recently assimilated C under different environmental conditions such as fertilization, temperature change or drought. The merits of this labeling system are that it is very simple, open to the atmosphere, portable, easily implemented in a variety of field situations, and that it requires only modest battery power. Hopefully, this ^{13}C pulse-chase labeling approach can be further refined and used to unravel some of the factors that control the timing and movement of plant assimilates into the soil food web.

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