GLOBAL CHANGE BIOLOGY
BIOENERGY

GCB Bioenergy (2017), doi: 10.1111/gcbb.12428

Cover crop root contributions to soil carbon in a no-till corn bioenergy cropping system

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Abstract

Crop residues are potential biofuel feedstocks, but residue removal may reduce soil carbon (C). The inclusion of a cover crop in a corn bioenergy system could provide additional biomass, mitigating the negative effects of residue removal by adding to stable soil C pools. In a no-till continuous corn bioenergy system in the northern US Corn Belt, we used $^{13}CO_2$ pulse labeling to trace plant C from a winter rye (*Secale cereale*) cover crop into different soil C pools for 2 years following rye cover crop termination. Corn stover left as residue (30% of total stover) contributed 66, corn roots 57, rye shoots 61, rye roots 50, and rye rhizodeposits 25 g C m^{-2} to soil. Five months following cover crop termination, belowground cover crop inputs were three times more likely to remain in soil C pools than were aboveground inputs, and much of the root-derived C was in mineral-associated soil fractions. After 2 years, both above- and belowground inputs had declined substantially, indicating that the majority of both root and shoot inputs are eventually mineralized. Our results underscore the importance of cover crop roots vs. shoots and the importance of cover crop rhizodeposition (33% of total belowground cover crop C inputs) as a source of soil C. However, the eventual loss of most cover crop C from these soils indicates that cover crops will likely need to be included every year in rotations to accumulate soil C.

Keywords: $^{13}CO_2$, bioenergy, cover crops, density fractions, isotopes, microbial biomass C, rhizodeposits, roots, *Secale cereale*, soil carbon

Received 26 September 2016; accepted 23 October 2016

Introduction

Bioenergy production could provide more sustainable energy and reduce dependency on fossil fuels, while using existing infrastructure for fuel delivery (Department of Energy, 2011). Crop stover, not used for food, represents an important potential feedstock for biofuel production, but its removal could lead to lower soil carbon (C) stocks (Clapp *et al.*, 2000; Anderson-Teixeira *et al.*, 2009) and thereby offset some of the greenhouse gas benefits of biofuel production (Gelfand *et al.*, 2010). Removing 25% or 50% of stover biomass has been estimated to reduce soil C by 3 and 8 Mg ha^{-1}, respectively (Anderson-Teixeira *et al.*, 2009). Studies have reported that maintaining soil C pools under corn requires 6–12.5 Mg ha^{-1} yr^{-1} stover input (Zanatta *et al.*, 2007; Pikul *et al.*, 2008; Johnson *et al.*, 2014), depending on edaphic properties and management practices (Wilhelm *et al.*, 2007). For example, Johnson *et al.*, 2006 found that conventional tillage required more stover to maintain soil C than no-till (7.6 vs. 5.3 Mg ha^{-1} yr^{-1}, respectively). By increasing plant C inputs to soil, cover cropping is one of the more promising management practices that could reduce the effects of stover removal on soil C stocks.

In annual temperate agroecosystems, cover crops are often grown during seasonal windows when there are no cash crops (e.g., fall-spring). Cover crops provide many benefits to agricultural systems including weed suppression and soil aggregation and are also known to promote soil C formation (McDaniel *et al.*, 2014a; Kallenbach *et al.*, 2015; Tiemann *et al.*, 2015). Thus, including them in bioenergy cropping systems may counteract the removal of aboveground crop residues by increasing biomass inputs. Belowground cover crop inputs are known to contribute disproportionately to soil carbon (Puget & Drinkwater, 2001; Rasse *et al.*, 2005; Kong & Six, 2010; Mendez-Millan *et al.*, 2010) and, as a result, cover crops may serve as a useful tool for maintaining soil C even if cover crop shoot biomass is harvested as a biofuel feedstock (Moser *et al.*, 2009). While the role of aboveground cover crop biomass in building soil

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organic matter (SOM) has been widely discussed in the literature (e.g., Barber, 1979; Hooker *et al.*, 1982; Campbell *et al.*, 1991; Drinkwater *et al.*, 1998; Marriott & Wander, 2006; Calegari *et al.*, 2008; Steele *et al.*, 2012), little attention has been paid to the role of belowground cover crop inputs (but see Puget & Drinkwater, 2001; Kong & Six, 2010), and whether they might be sufficient to offset stover removal.

Several studies have shown that belowground root-derived inputs contribute disproportionately to soil C compared to aboveground shoot inputs (Balesdent & Balabane, 1996; Clapp *et al.*, 2000; Rasse *et al.*, 2005; Kong & Six, 2010; Mendez-Millan *et al.*, 2010; Clemmensen *et al.*, 2013; Mazzilli *et al.*, 2015). Studies using biomarkers specific to root and shoot tissue (Mendez-Millan *et al.*, 2010; Ji *et al.*, 2015) and natural abundance isotopes (Balesdent & Balabane, 1996; Mazzilli *et al.*, 2015) show more root than shoot C in SOM, as do studies of cover crops using isotope labels (Puget & Drinkwater, 2001; Kong & Six, 2010, 2012).

In addition to root biomass, rhizodeposits are an additional source of belowground C. Annual grain crops allocate 30–50% of photosynthate belowground, and 30–50% of belowground C are attributed to rhizodeposits although values as high as 40% of total plant inputs have been reported (Barber & Martin, 1976; Meharg & Killham, 1991; Puget & Drinkwater, 2001; Kuzyakov *et al.*, 2003; Butler *et al.*, 2004; Jones *et al.*, 2009). Up to 75% of soil C inputs to SOM come from belowground sources including root biomass and rhizodeposits, whereas C from plant shoots is mostly lost via respiration (Gale *et al.*, 2000). As for shoots, the majority of annual crop root biomass turns over in a single pulse at the time of plant death. However, during the growing season rhizodeposits are continuously being added to SOM from root turnover, sloughed or border cells, mycorrhizal hyphae, actively released secretions, and passively released exudates (Jones *et al.*, 2009; Bradford *et al.*, 2012). Rhizodeposits thus represent a significant input to soil C that may differ from biomass inputs because they are continuous, differ in chemical composition, and enter SOM in close physical proximity to soil minerals and microbial communities. Continuous rhizodeposition can stimulate microbial biomass production and activity, a key precursor for SOM formation (Grandy & Neff, 2008; Schmidt *et al.*, 2011; Wieder *et al.*, 2014, 2015; Kallenbach *et al.*, 2015), and rhizodeposits may be preferentially protected in aggregates on mineral surfaces (Rasse *et al.*, 2005; Dungait *et al.*, 2012; Mazzilli *et al.*, 2015).

Recent work suggests occlusion in soil aggregates or mineral association may be more important mechanisms for long-term SOM stability than reduced decomposition rates via chemical recalcitrance (Grandy & Neff, 2008; Dungait *et al.*, 2012; Wieder *et al.*, 2014; Kallenbach *et al.*, 2015, 2016). Thus, organic matter occlusion in soil aggregates or association with minerals influences its accessibility to microbes, and thus potential to persist in soil (Six *et al.*, 2002; Grandy *et al.*, 2009). In order to capture functionally different SOM pools, fractionation methods are used to separate SOM into pools with distinct protection mechanisms (Zimmermann *et al.*, 2007). The simplest density fractionation defines two pools: a light fraction containing minimally processed inputs known as particulate organic matter (POM) and the remaining heavy fraction (Gregorich *et al.*, 2006). Occluded POM can be isolated by breaking up aggregates prior to density fractionation, and sonication can disrupt a variety of associations between organic matter and mineral surfaces. Complex fractionation schemes may seive multiple sizes of soil aggregates or use multiple sonication steps to isolate SOM with increasingly strong mineral associations and, presumably, turnover times (von Lützow *et al.*, 2007). Cover crop residues in free POM may represent a short-term SOM pool compared to cover crop inputs in aggregates or in the heavy, mineral-associated fraction.

While past studies highlight the potential utility of cover crops for restoring soil C lost to stover removal, their potential to do so under different agricultural management scenarios has not been well studied. For instance, tillage intensity is likely to impact the relative contribution of root vs. shoot C to SOM (Allmaras *et al.*, 2004). Presumably incorporation of aboveground cover crop biomass via tillage would lead to increased shoot C storage; however, studies of root and shoot contributions to SOM have usually taken place in tilled systems (Puget & Drinkwater, 2001; Kong & Six, 2010). Thus, it remains uncertain whether cover crop root and/or shoot inputs could help counteract the negative effects of residue removal on soil C in no-till bioenergy cropping systems.

Here, we examine the relative contributions of cover crop root and shoot to soil C in order to determine whether belowground cover crop C could help offset the deleterious effects of residue removal in a no-till continuous corn bioenergy cropping system. We labeled cereal rye (*Secale cereale)*, a common winter cover crop, *in situ* with $^{13}CO_2$, tracked inputs from rhizodeposits during the growing season, and tracked root and shoot inputs into different soil C pools over the following 2 years. We address four specific questions: (1) What are the relative contributions of different cover crop inputs to soil C? (C_{shoot}, C_{root}, and C_{rhizo}; Table 1); (2a) How much C_{rhizo} is incorporated into MBC during cover crop growth; and (2b) is C_{shoot} or C_{bg} preferentially incorporated into MBC during cover crop decomposition? (3) How are C_{shoot} and C_{bg} distributed among three soil density fractions?; and (4) How long do C_{shoot} and C_{bg} persist in soils?

 doi: 10.1111/gcbb.12428

Table 1 Abbreviated terms are defined for four cover crop C inputs, two experiments, and three soil density fractions

Abbreviation	Definition
Cover crop carbon inputs: Pulse labeling of cover crops *in situ* provides realistic estimates of above and belowground C inputs without disturbing root-soil interactions. At the time of cover crop termination we measured root biomass and soil C from cumulative rhizodeposits during cover crop growth. However, after termination it is not possible to distinguish between rhizodeposit ^{13}C, which occurred during the growing season, and ^{13}C from decomposing root biomass following cover crop death. We therefore define a fourth pool of belowground C inputs resulting in four specific plant C inputs, defined below.	
C_{shoot}	Carbon input from cover crop shoot biomass
C_{root}	Carbon input from cover crop root biomass, measured at the time of cover crop harvest
C_{rhizo}	Carbon inputs from rhizodeposits during cover crop growth (e.g., exudates, sloughed cells, root hair turnover)
C_{bg}	Carbon from all belowground cover crop inputs: root biomass and rhizodeposits. (After cover crop termination, C_{root} and C_{rhizo} combine into C_{bg}.)
Experiments: We performed an initial labeling experiment in 2013 to assess the fate of cover crop C inputs following cover crop termination. Early results indicated that growing season rhizodeposits represented a significant source of cover crop C inputs. We therefore performed a second labeling experiment in 2014 to measure belowground cover crop inputs during plant growth.	
EXP1	Experiment one: Labeling took place in five pulse labeling events during April and May 2013. Samples were measured at 0, 5, 12, and 17 months following cover crop termination.
EXP2	Experiment two: Cover crops were labeled in five pulse labeling events during April and May 2014. Samples were measured during cover crop growth 24 h following the first and third labeling events, and 0, 5, and 12 months following cover crop termination.
Soil density fractions: We define three soil density fractions based on separation with 1.6 g L^{-1} sodium polytungstate and calculated as a proportion of sand-free soil.	
FLF	Free light fraction: particulate organic matter <1.6 g L^{-1} density
OLF	Occluded light fraction: particulate organic matter <1.6 g L^{-1} density released by shaking to disrupt soil aggregates
MHF	Mineral heavy fraction: >1.6 g L^{-1} density

Materials and methods

Experimental design and labeling

The Great Lakes Bioenergy Research Center (GLBRC) Biofuel Cropping System Experiment (BCSE, http://glbrc.org/) was established in 2008 at the Kellogg Biological Station LTER site (42° 24′ N 85° 24′ W, 288 m asl) in Southwest Michigan, USA. Temperature at the site ranged from −26.5 °C to 34.4 °C during the period of experimentation (2013–2014), and mean annual air temperature was 8.8 °C in 2013 and 7.6 °C in 2014. Precipitation was 1177 mm in 2013 and 933 mm in 2014. The dominant soil series is Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs; Muñoz & Kravchenko, 2011; Tiemann & Grandy, 2015). Cover crops were added to the no-till continuous corn treatment in 2012. The treatment was replicated in five 30 × 40 m replicate plots with a subplot (4.6 × 13.1 m) in which cover crops were terminated via glycophosphate application. The winter cover crop *Secale cereale* (winter rye) was planted November 10, 2012 and October 29 and 30 of 2013. The cover crop in the subplot was terminated with herbicide just prior to planting of corn (*Zea mays*, Pioneer P8906AM Corn Hybrid) on June 5, 2013 and May 30, 2014.

To assess the fate of cover crop root and shoot C in bulk soils, soil density fractions, and microbial biomass over a 2 year period, we established a reciprocal litter transfer experiment with $^{13}CO_2$ labeled winter rye in the spring of 2013 (EXP1, Table 1). Early results indicated that rhizodeposition could be an important component of belowground C inputs, and we therefore established a second labeling experiment following the same methods in adjacent plots in 2014 (EXP2, Table 1) to estimate belowground inputs to bulk soil and microbial biomass during the growing season. At the start of each experiment (EXP1 and EXP2), we established three 1 m^2 plots in each block of the BSCE. One plot in each block was randomly designated for $^{13}CO_2$ pulse labeling as described below. We chose to establish the plots within the herbicide treatment to minimize transfer of cover crop residues between subplots by farm equipment. However, cover crops in our plots were clipped prior to glycophosphate application (further described below) and thus not terminated by herbicide.

Pulse labeling was carried out five times between snow melt in early April and cover crop termination in late May. At the start of each labeling event, the designated plot was enclosed under a 1 m^2 adjustable height chamber constructed of PVC and clear vinyl sheeting. To seal the chamber to the soil, we placed sandbags along the vinyl where it met the ground. We monitored the concentration of CO_2 in the chamber continuously using a portable infrared gas analyzer (Qubit CO_2 Analyzer, Model S-151; Qubit Systems, Kingston, ON, Canada) and deployed a small fan inside the chamber to maintain an even distribution of CO_2. We recorded initial CO_2 concentration in the chamber and added 99 atom percent enriched $^{13}CO_2$ at the rate of 1 L min^{-1} for 2–3 min to a maximum level of roughly double ambient CO_2

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concentration (actual mean 853 ppm). The chamber was left in place until the CO_2 concentration returned to ambient levels, the duration of the period between peak CO_2 concentration and removal of the chamber ranged from 18 to 96 min (mean 41 min). Because photosynthetic rate varies throughout the day, all labeling occurred between the hours of 10 : 00 and 15 : 00 and the blocks were visited in random order each time. Labeled plots were re-covered with the chambers at night to capture $^{13}CO_2$ lost from nighttime respiration for re-assimilation the following morning, thereby increasing our labeling efficiency.

Treatment establishment and plant sampling

Following pulse labeling and prior to corn planting, we terminated rye cover crop by clipping aboveground biomass to ground level on May 24, 2013 in EXP1 and May 24, 2014 in EXP2. We collected rye and weeds separately, air-dried, weighed, and cut the shoots into 2.5 cm pieces before returning the material to the soil surface. Aboveground biomass was transferred among three plots within each block to create a root plot containing labeled roots and unlabeled shoots, a shoot plot containing unlabeled roots and labeled shoots, and a control plot containing unlabeled roots and shoots.

We estimated root biomass at the time of rye termination in EXP2 by isolating roots from bulk soil cores; four soil cores (5 cm diameter, 15 cm deep) were collected per plot, and roots larger than 2 mm diameter were isolated by sieving fresh soil collected at the rye termination date. To ensure we were accurately estimating root biomass, two air-dried soil samples from control plots were later wet sieved to 250 μm and fine roots and all discernible root material were collected under a dissecting microscope. Because wet sieving resulted in negligible increases in root biomass estimates, we consider soil sieved to 2 mm root free. Root biomass in EXP1 plots were estimated using the aboveground biomass measures in EXP1 and the ratio of total root biomass to shoot biomass in EXP2 plots. Additional rhizodeposits were estimated using the $\delta^{13}C$ values of bulk soil collected from the root plots at the time of cover crop termination and sieved to 2 mm. Belowground C inputs based on bulk soil $\delta^{13}C$ ‰ values at the time of cover crop termination were calculated as described below. The final isotopic composition in the EXP1 rye was 757 (±105) $\delta^{13}C$ ‰ for the shoots and 701 (±83) $\delta^{13}C$ ‰ in the roots; and 787 (±149) $\delta^{13}C$ ‰ in shoots and 719 (±60) $\delta^{13}C$ ‰ in roots for EXP2 rye.

Soil sampling

To assess rhizodeposits, we collected soil 24 h after the first and third labeling events during the spring cover crop labeling period in EXP2. To calculate the relative contribution of rhizodeposit C at the time of rye termination, we collected soils at the time of rye termination and treatment establishment in both EXP1 and EXP2, and to evaluate changes in cover crop C from aboveground or belowground sources over time, we sampled after 5 months of cover crop residue decomposition in both EXP1 and EXP2, and after 12 and 17 months of residue decomposition in EXP1. We calculated bulk density using four 5 cm diameter, 10 cm long cores taken from each plot.

Density fractionation

To assess the contribution of root and shoot C to different soil fractions, we performed sodium polytungstate (NaPT) fractionation on EXP1 soils collected 5 and 17 months following rye termination. We followed a standard protocol for density fractionation (Sohi *et al.*, 2001) with modifications described below. Air-dried soils were first rewetted using capillary action (Haney & Haney, 2010). About 50 g of air-dried soil from each sample was added to a beaker with holes drilled in the bottom, which was placed in a glass jar (473 mL) with 10 mL deionized water on a glass microfiber filter (Whatman, GF/D 1823 - 043, GE Healthcare Life Sciences, Buckinghamshire, UK). Soils were monitored and in the case that the soil surfaces were dry after 1 h, water was added in 1 or 2 mL increments up to 5 mL (15 mL added total) until moisture had permeated the soil sample.

Following an 8 h incubation, a soil subsample was dried at 70 °C to assess gravimetric water content, and 10 g of moist soil were added to each of three 50 mL centrifuge tubes along with 30 mL of NaPT at 1.7 g mL^{-1} density (final density after addition of wet soil was 1.68 g mL^{-1}). Tubes were then rolled along the counter one full rotation to promote mixing of the soil with the NaPT and then allowed to settle overnight. The floating light fraction (hereafter free light fraction (FLF, Table 1)) was then vacuumed from the surface and collected on preweighed, ash-free 8 μm pore size filter paper (Whatman, 1540- 055, GE Healthcare Life Sciences, Buckinghamshire, UK). The centrifuge tubes with soil were then placed on a shaker at 250 rpm for 3 h to break apart aggregates, and tubes were subsequently removed from the shaker and placed in a rack overnight. The floating particulate organic matter (hereafter occluded light fraction (OLF, Table 1)) was vacuumed from the surface and processed as for the FLF. Approximately 5 g of the remaining soil was rinsed of residual NaPT by adding 25 mL deionized water, shaking for 1 h at 200 rpm and centrifuging at 966 *g* for 2 min; the supernatant was removed, and the rinse was repeated once more. Following centrifugation, the sample contained the mineral heavy fraction (MHF, Table 1) at the surface, increasing concentrations of sand toward the bottom. A small portion of sand-free MHF collected from the surface was dried and analyzed for ^{13}C and total C and N contents. Sand content was determined on approximately 10 g of air-dried bulk soil after dispersing soil in 2.5 mL 5% hexametaphosphate and collecting the sand on a 53 μm sieve.

Microbial biomass

We measured microbial biomass C (MBC) and ^{13}C content in bulk soil on three sample dates in EXP1, 5, 12, and 17 months following termination, and four sample dates in EXP2, 24 h following the first and third labeling events, at time of termination, and 5 months after termination. Soils were subsampled after sieving to 2 mm, and subsamples were transported on ice to the laboratory where they were refrigerated at 4 °C and analyzed for MBC within 5 days of sampling. MBC was extracted from five grams of field moist soil using a modified chloroform fumigation and extraction with 40 mL of 0.5 M K_2SO_4 (McDaniel *et al.*, 2014b).

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^{13}C measurements

Plant and soil ^{13}C values were analyzed on a Finnigan Delta Plus XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a peripheral Costech 4010 elemental analyzer (Costech Analytical Technologies, Valencia, CA, USA) at the University of New Hampshire Stable Isotopes Laboratory at the Institute for study of Earth, Oceans and Space. Samples were ground to a fine powder in a ball mill grinder (SPEX SamplePrep 8000D Mixer/Mill, Metuchen, NJ, USA), and ground, homogenized samples were weighed into Costech aluminum tins (9–11 mg soil or MHF and 2–2.5 mg plant material, FLF, or OLF). Microbial biomass extracts were analyzed for C content and ^{13}C content at the Stable Isotope Facility at the University of California, Davis using an O.I. Analytical model 1030 TOC Analyzer (OI Analytical, College Station, TX, USA) interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) with a GD-100 Gas Trap Interface (Graden Instruments, Oakville, ON, Canada).

Calculations

The fraction of a given C pool (bulk soil C, FLF C, OLF C, MHF C, and MBC) coming from a cover crop source material (F_{cover}) was calculated using the following mixing model:

$$f_{cover} = \frac{\delta_{sample} - \delta_{control}}{\delta_{source} - \delta_{control}}, \quad (1)$$

where δ_{sample} refers to the $\delta^{13}C$ value of the soil sample in question, $\delta_{control}$ refers to the relevant control (or unlabeled plot), and δ_{source} refers to the labeled plant source material (root or shoot). We used the $\delta^{13}C$ value of root material as the δ_{source} for f_{cover} of rhizodeposits (Puget & Drinkwater, 2001). The f_{cover} value was multiplied by the C concentration of each sample material to calculate the new C incorporated from each source material (C_{new}). The $\delta^{13}C$ value of MBC (δMBC) was calculated as:

$$\delta\text{MBC} = \frac{\left(\left(\delta^{13}C_{FUM} * C_{FUM}\right) - \left(\delta^{13}C_{UF} * C_{UF}\right)\right)}{\left(C_{FUM} - C_{UF}\right)}, \quad (2)$$

where $\delta^{13}C_{FUM}$ and $\delta^{13}C_{UF}$ are the $\delta^{13}C$ value of the fumigated and unfumigated samples, respectively, and C_{FUM} and C_{UF} are the carbon content of the fumigated sample and unfumigated samples, respectively.

We calculated a standardized measure of root vs. shoot contribution to soil C (i.e., the relative contribution factor) as

$$\frac{C_{bg}\text{ in soil}/C_{bg}\text{ inputs}}{C_{shoot}\text{ in soil}/C_{shoot}\text{ inputs}} \quad (3)$$

(Rasse *et al.*, 2005). Thus, a relative contribution factor greater than one indicates preferential storage of C_{bg} to C_{shoot} in soil C.

Statistical analysis

All data manipulation and statistical analyses were performed in R (R Core Team 2014). The following response variables were log-transformed to meet the assumptions of normality: F_{cover} of bulk soil C, F_{cover} of MBC, new FLF C, new OLF C, and $\delta^{13}C$ in EXP2 bulk soil. To test differences between F_{cover} in bulk soil or MBC in root and shoot plots at each sampling date after termination (5, 12, and 17 months in EXP1; 2 weeks and 5 months in EXP2), we performed separate one-way ANOVAs at each date. To compare whether F_{cover} in MBC plots was different from zero, we performed separate one-way *t*-tests for root and shoot treatments at each date. To compare C inputs from cover crop material in soil density fractions, we performed separate one-way ANOVAs on FLF, OLF, and MHF fractions in EXP1 at 5 and 7 months. To test whether $\delta^{13}C$ values increased in bulk soil in root treatment plots during the growing season in EXP2 plots, we compared root and control plots in a two-way ANOVA with date and treatment as discrete independent variables and performed one-way *t*-tests on the difference in $\delta^{13}C$ values ($\delta^{13}C$ root plot – $\delta^{13}C$ control plot). We report mean values ± standard error and consider $\alpha \leq 0.05$ a statistically significant effect.

Results

In EXP1, 151 (±7.3) g m^{-2} and in EXP2, 123 (±5.9) g m^{-2} aboveground rye biomass was added to each subplot (oven dry weight), corresponding to 66.9 ± 3.2 and 54.5 ± 2.6 gC m^{-2} (C_{shoot}, Table 1). In EXP2, 151 (±37.2) g m^{-2} of belowground biomass (44.8 ± 11.0 gC m^{-2}; C_{root}, Table 1) was measured in each subplot and belowground biomass inputs in EXP1 were estimated to be 186 g m^{-2} (55.0 ± 2.7 gC m^{-2}). When rye was cut in EXP1, plots contained 26.6 (±8.7) g m^{-2} of rhizodeposit C (in addition to measured root biomass, C_{bg}, Table 1) and in EXP2, this value was 23.4 (±3.3) gC m^{-2} (Table 3).

After 5 months, the contribution of C_{bg} to bulk soil C in EXP1 was approximately four times greater than that of C_{shoot} (14.5 ± 2.5 gC m^{-2} vs. 3.5 ± 0.8 gC m^{-2}, Fig. 1a; $F_{(1,8)} = 26.3$, $P < 0.001$). One year following cover crop termination, seven times more C_{bg} remained in bulk soil (14.8 ± 1.3 gC m^{-2} or 0.7% of total soil C) than C_{shoot} (2.33 ± 2.2 g m^{-2} or 0.1% of total soil C; Fig. 1a; $F_{(1,8)} = 17.6$, $P = 0.003$). However, after 17 months, there was no difference in bulk soil C derived from C_{bg} and C_{shoot} in EXP1 (Fig. 1a, $F_{(1,8)} = 0.3$, $P = 0.63$). During the period of cover crop growth in EXP2 plots, C_{bg} accumulated in bulk soil in the root plots (Fig. 1a, Treatment $F_{(2,66)} = 33.2$, $P < 0.0001$; Date $F_{(1,66)} = 5.59$, $P = 0.02$). As soon as 24 h following the first labeling event, 12.7 ± 1.9 gC m^{-2} was attributable to belowground cover crop inputs. This number peaked at 23.4 ± 3.3 gC m^{-2} at the time of rye termination. C_{bg} remained more abundant than C_{shoot} up to 5 months following rye termination, similar to observations in EXP1 (10.9 ± 3.4 g m^{-2} C_{bg} and 3.29 ± 2.0 g m^{-2} C_{shoot}; Fig. 1a, $F_{(1,9)} = 6.7$, $P = 0.03$).

Total MBC in soil ranged from 56.1 ± 19.3 mg MBC kg^{-1} soil to 240.1 ± 20.9 g MBC kg^{-1} for the different sampling events across EXP1 and EXP2 (Table 2). Microbial biomass in EXP1 contained over three times

 doi: 10.1111/gcbb.12428

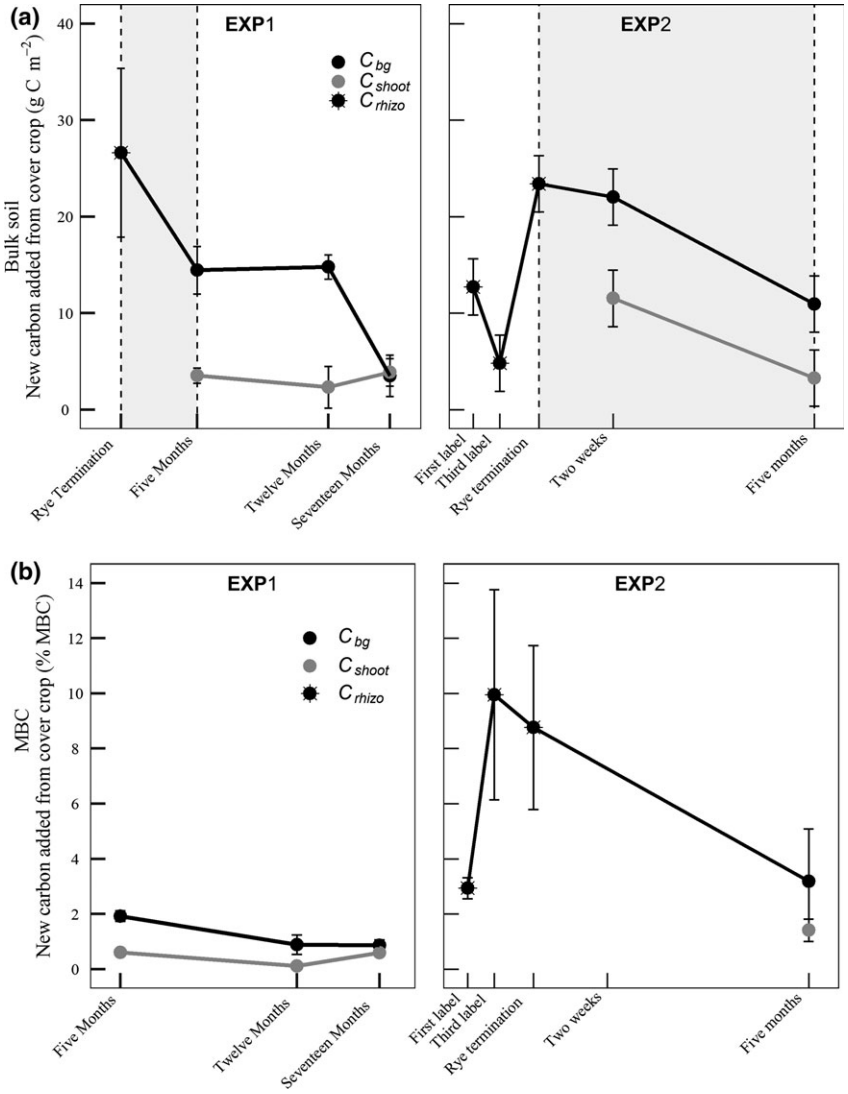

Fig. 1 (a) New cover crop carbon from C_{bg} (black) and C_{shoot} (gray) sources in EXP1 (left) and EXP2 (right) plots. About 26.6 ± 8.7 g m^{-2} of C_{rhizo} was present in bulk soil at the time of cover crop termination in EXP1, and significantly more C_{bg} than C_{shoot} was present after up to 1 year of decomposition. However, after a second growing season (17 months after termination), there was no detectable difference between root and shoot carbon present in the soils (left). Similarly, 23.41 ± 3.3 g m^{-2} of C_{rhizo} was present in bulk soils in EXP2 at the time of termination and the label in the bulk soil decreased during the first 5 months of cover crop decomposition (right). Gray boxes represent comparable periods after cover crop termination (0–5 months of decomposition). Error bars display standard error. (b) Percent of total MBC comprised of C_{bg} (black) and C_{shoot} (gray) in EXP1 (left) and EXP2 (right) plots. Belowground C accumulated in EXP2 root label plots during cover crop growth (right) and C_{bg} was present in the MBC up to 17 months following rye termination in EXP1 (left). MBC contained more C_{bg} than C_{shoot} up to 1 year following cover crop termination, but after a second growing season, there was no difference between C_{bg} and C_{shoot} in MBC in EXP1 (left).

more C_{bg} (1.9 ± 0.2% of MBC) as compared to that of C_{shoot} (0.6 ± 0.1% of MBC) after 5 months (Fig. 1b, $F_{(1,8)} = 58.2$, $P < 0.001$) and seven times more C_{bg} (0.8 ± 0.4% of MBC) than C_{shoot} (0.1 ± 0.1% of MBC) 12 months following rye termination (Fig. 1b, $F_{(1,8)} = 6.4$, $P = 0.04$). Following 17 months of cover crop decomposition, there was no difference in the fraction of MBC derived from C_{bg} or C_{shoot} in EXP1 ($F_{(1,8)} = 0.78$, $P = 0.41$) although both C_{bg} (0.8 ± 0.2%, $P = 0.01$) and C_{shoot} (0.6 ± 0.06%, $P = 0.002$) were present in MBC. We measured significant C_{bg} inputs to MBC in EXP2 at each sampling date during cover crop growth ($P < 0.01$ at each date, Fig. 1b), comprising 2.9 ± 0.4% of MBC 24 h following the first labeling event. The fraction of MBC traced to C_{bg} peaked after the third label at 10.0 ± 3.8% of total MBC. At rye termination in EXP2, 8.8 ± 3.0% of MBC was derived from C_{bg}. There was no significant difference in the proportion of MBC from C_{bg} (3.2 ± 31.9%)

 doi: 10.1111/gcbb.12428

Table 2 Soil properties in the continuous corn plus cover crops rotation at the Great Lakes Bioenergy Research Center biofuel cropping systems experiment. Soil fractions (FLF, OLF, and MHF) are calculated as a proportion of sand- free soil and values are averaged for 5 and 17 months sample dates in EXP1 (n = 30)

	Mean (SE)
Total soil C (%)	1.1 (0.02)
pH	6.7 (0.2)
Sand content (%)	51 (12)
Bulk density (g cm^{-3})	1.47 (0.03)
MBC (mg kg^{-1} soil)	115.8 (12.4)
FLF (mg g^{-1} soil)	7.28 (2.3)
OLF (mg g^{-1} soil)	6.03 (0.6)
MHF (mg g^{-1} soil)	476.57 (21.6)

and the proportion from C_{shoot} (1.3 ± 0.5%) after 5 months of cover crop decomposition in EXP2 ($F_{(1,8)}$ = 0.27, P = 0.62) although both C_{bg} (P = 0.004) and C_{shoot} (P = 0.002) were present in MBC (%MBC > 0).

There was more new C incorporated from C_{bg}, compared to C_{shoot}, in OLF and MHF 5 months following rye termination in EXP1 (Fig. 2). In the OLF, four times more C was derived from C_{bg} (6.84 ± 1.7 gC m^{-2}) than from C_{shoot} (1.54 ± 0.5 gC m^{-2}) representing 1.64 and 0.39% of total OLF, respectively ($F_{(1,8)}$ = 10.4, P = 0.01; Fig. 2). In the MHF, six times more C_{bg} was present (11.0 ± 2.3 g C m^{-2}) than C_{shoot} (1.68 ± 1. g C m^{-2}) representing 0.77 and 0.18% of total MHF (F = 15.0, P = 0.005; Fig. 2). A year later, 17 months following cover crop termination in EXP1, there were no differences in C_{bg} and C_{shoot} contributions to FLF, OLF, and MHF C (FLF: $F_{(1,8)}$ = 0.004, P = 0.95; OLF: $F_{(1,8)}$ = 0.10, P = 0.76; MHF: $F_{(1,8)}$ = 0.07, P = 0.79; Fig. 2).

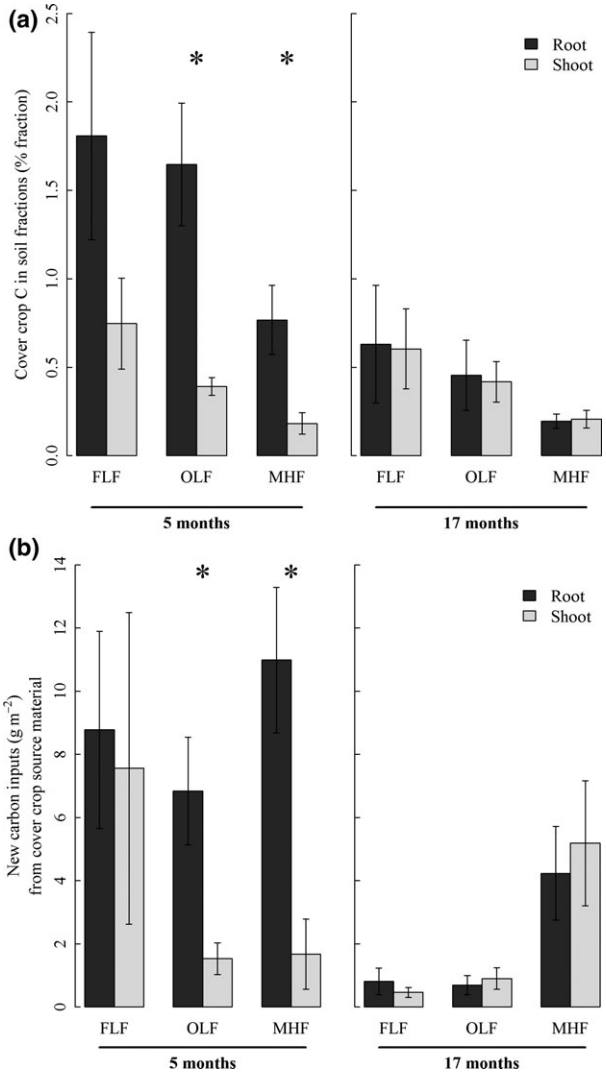

Fig. 2 (a) Percent of soil carbon coming from C_{bg} (black) or C_{shoot} in three density fractions 5 months following rye termination in EXP1 and 17 months following rye termination in EXP1. A greater proportion of the occluded light fraction (OLF) and mineral heavy fraction (MHF) were comprised of C_{bg} than of C_{shoot} after 5 months (left); however, after a second growing season (17 months), there was no significant difference in the fraction of soil carbon coming from C_{bg} or C_{shoot} sources in any density fraction (right). (b) New carbon inputs from belowground cover crop inputs (C_{bg}, black) and shoots C_{shoot} in three density fractions of bulk soil carbon 5 and 17 months following rye termination in EXP1. Total belowground C inputs to the OLF and MHF were greater than those from shoot sources after 5 months (left); however, there was no difference in the C remaining from C_{bg} vs. C_{shoot} after 17 months and a second growing season (right).

Discussion

Our $^{13}CO_2$ pulse labeling results showed belowground cover crop C inputs (C_{bg}) present in higher concentrations than cover crop shoot biomass C inputs (C_{shoot}) in bulk soil, MBC, OLF, and MHF for at least 12 months following cover crop termination. The relative contribution factor suggests that C_{bg} is three times more likely to be maintained in potentially stable soil pools than C_{shoot} 12 months following cover crop termination, and C_{bg} was most abundant in the mineral-associated fraction.

What are the relative contributions of cover crop shoots, rhizodeposits, and total belowground C inputs (C_{shoot}, C_{rhizo}, and C_{bg}) to soil C?

Total C_{shoot} and C_{bg} inputs (cover crop shoot C inputs and belowground C inputs, Table 1) were similar, although a high proportion of C_{shoot} may be lost via respiration before entering the soil; one study estimated that approximately 75% of C entering the soil was from C_{bg} (Gale & Cambardella, 2000). A substantial portion of C_{bg} inputs were attributed to C_{rhizo} (Table 3). We

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estimate that rhizodeposition during cover crop growth accounted for 33% and 34% of total C_{bg} inputs in EXP1 and EXP2, respectively (Table 3). These values are similar to those reported in the literature, which range from 30% to 50% of total C_{bg}, although values as high as 40% of total plant inputs have been reported (Barber & Martin, 1976; Meharg & Killham, 1991; Puget & Drinkwater, 2001; Kuzyakov *et al.*, 2003; Butler *et al.*, 2004; Jones *et al.*, 2009).

We observed significantly more C_{bg} than C_{shoot} in all measured soil pools, except FLF, for up to 1 year following termination. Our mean relative contribution factor across the two experiments is 3.06 (3.36 in EXP1 and 2.77 in EXP2), indicating that on average 3.06 times more C_{bg} than C_{shoot}, per unit C input, was converted to SOM. This value is comparable to others reported in the literature; for example, Puget & Drinkwater (2001) estimated a relative contribution factor of 3.7 using *in situ* isotopic labeling of a hairy vetch cover crop and Kong & Six (2010) found a relative contribution factor of 3.24 for a hairy vetch cover crop in a maize/tomato rotation. Rasse *et al.* (2005) compared the relative contribution factor of roots to shoots across various *in situ* studies of different plant types and found relative contribution factors ranging from 0.77 to 3.7 and an average value of 2.4.

The greater abundance of C_{bg} vs. C_{shoot} in SOM, and its higher efficiency of conversion to SOM, is likely due to differences in the size, chemical composition, location, and timing of the two inputs (Rasse *et al.*, 2005; Loecke & Robertson, 2009a,b; Mendez-Millan *et al.*, 2010; Dungait *et al.*, 2012). Greater physical protection of C_{bg} may result from the small size and close proximity of belowground inputs to soil aggregate formation (Tiemann & Grandy, 2015). Many belowground inputs, especially rhizodeposits, are orders of magnitude smaller in size than shoot inputs that must be shredded, leached, or otherwise broken down prior to incorporation in soil aggregates (Jones *et al.*, 2009). Roots play an important role in structuring soil and contribute to aggregate formation; therefore, rhizodeposits are inherently positioned to be enmeshed in soil aggregates (Puget & Drinkwater, 2001; Denef & Six, 2006; Clemmensen *et al.*, 2013). Increased mineral association of C_{bg} may be facilitated by the close proximity of rhizodeposits to mineral surfaces and the chemical composition of soluble rhizodeposits.

It has also been suggested that belowground inputs decompose slowly due to the chemically complex components of root biomass (e.g., lipids or waxes such as suberin; Rasse *et al.*, 2005; Mendez-Millan *et al.*, 2012). However, given that long-term SOM storage is not driven by chemical recalcitrance of direct plant inputs (Dungait *et al.*, 2012; Cotrufo *et al.*, 2013) as much as it is by the physical protection of plant and especially microbial products by association with minerals (Grandy & Neff, 2008; Heckman *et al.*, 2013). Thus, an alternative mechanism is that through transformation via microbial consumption, root inputs may enhance microbial processes that result in the preservation of root-derived C. For example, the continuous input of low molecular weight substrates in the rhizosphere may

Table 3 Mean biomass and C inputs from cover crop and corn plant fractions in 2013 (EXP1) and 2014 (EXP2), values in parentheses represent standard error

	Biomass (g m^{-2})		Carbon (gC m^{-2})	
	EXP1	EXP2	EXP1	EXP2
Cover crop inputs				
Shoot	151.3 (7.3)	123.2 (5.9)	66.9 (3.2)	54.5 (2.6)
Root	185.8 (9.0)†	151.3 (37.2)	55.0 (2.7)‡	44.8 (11.0)
Rhizodeposits	–	–	26.6 (8.7)	23.4 (3.3)
Belowground total	–	–	81.6	68.2
Total cover crop inputs	337.1	274.5	148.5	122.7
Corn residues				
Harvested stover	367.0 (7.5)	499.3 (39.5)	163.2 (3.3)	222.0 (17.6)‡
Stover residue	148.6 (11.4)	213.3 (15.2)	66.2 (5.2)	94.9 (6.7) ‡
Root	128.1 (14.5)	177.0 (5.7)†	57.3 (6.0)	79.5 (5.7)‡
Stover total	515.6	712.6	229.3	316.9
Total corn inputs	276.8	390.4	123.5	396.4

†Values for belowground productivity were estimated for cover crop in EXP1 and corn in EXP2 based on corresponding allometry (root: shoot) in EXP2 and EXP1, respectively.

‡Values for cover crop root C inputs in EXP1 and corn C inputs in EXP2 were estimated based on corresponding cover crop chemistry in EXP2 and corn in EXP1, respectively.

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promote greater carbon use efficiency (CUE), which may in turn lead to greater rates of C retention in soils (Puget & Drinkwater, 2001; Kallenbach *et al*., 2015; Roller & Schmidt, 2015). Further, recent work suggests that the majority of stabilized C in soils has been previously transformed by microorganisms or else is composed of microbial necromass. (Grandy *et al*., 2007; Kindler *et al*., 2009; Miltner *et al*., 2012). The rapid incorporation of root inputs to microbial biomass may further promote its protection by association with minerals (Grandy & Robertson, 2007; Tiemann & Grandy, 2015).

How much C_{rhizo} is incorporated into MBC during cover crop growth and is C_{shoot} or C_{bg} preferentially incorporated into MBC during cover crop decomposition?

Given the assertion that microbial belowground inputs may be more rapidly incorporated into microbial biomass and that microbial biomass serves as an important pathway for the C stabilization, we examined the incorporation of $C_{rhizo,}$ $C_{shoot,}$ and C_{bg} into MBC. Twenty-four hours following the first labeling event in EXP2, 0.83 (±0.1) g m^{-2} C_{rhizo} was present in MBC, constituting 2.9% of total MBC. At the time of rye termination, 2.31(±1.1) g m^{-2} C_{rhizo} was in MBC, which constituted 8.8% of total MBC. Pulse chase labeling studies have found photosynthate in MBC as soon as 1 h following fixation, with peak concentrations occurring roughly 3 h after fixation (Minchin *et al*., 1994; Rattray *et al*., 1995; Dilkes *et al*., 2004). Rhizodeposit C comprised 8.8% of total MBC at time of rye termination, which is lower than the 25–30% of MBC reported by Williams *et al*. (2006) in a system of ryegrass and clover with belowground biomass of 200–210 g m^{-2} (compared to 151–186 g in our study, Table 3). Fungi and bacteria in the rhizosphere produce polysaccharides and other binding agents, and transformation by microbial decomposers can be an important precursor to SOM protection on mineral surfaces (Six *et al*., 2006; Grandy & Neff, 2008; Miltner *et al*., 2012; Mardhiah *et al*., 2014; Kallenbach *et al*., 2015). The greater abundance of C_{bg} than C_{shoot} in MBC 5 months to 1 year following termination indicates more belowground C is entering the microbial biomass, which may help explain the higher concentrations of C_{bg} in mineral-associated fractions.

How are C_{shoot} and C_{bg} distributed among three soil density fractions?

In an effort to understand the turnover and stabilization dynamics of C_{bg} and C_{shoot}, we measured the incorporation of cover crop C into three different soil fractions. Cover crop C derived from either C_{bg} or C_{shoot} accumulated most in MHF C, followed by FLF C, then OLF C (Fig. 2b) and cover crop C comprised the greatest proportion of FLF C, followed by OLF C, and MHF C (Fig. 2a). Slowed decomposition of C_{bg} due to chemical recalcitrance should result in a buildup of particulate organic matter (POM); thus, if greater retention of C_{bg} than C_{shoot} is simply due to greater chemical recalcitrance of roots (which are not likely to result in long-term SOM accumulation), we would expect more C_{bg} than C_{shoot} in the FLF. If physical protection within aggregates is primarily driving greater abundance of C_{bg} than C_{shoot} in soil, we would expect to find a greater abundance of C_{bg} than C_{shoot} in the OLF. More C_{bg} than C_{shoot} in the MHF would suggest the possibility that direct mineral association is playing a role in slowing the turnover of C_{bg}.

We did not observe differences between C_{bg} and C_{shoot} in FLF C, where POM is not physically protected and decomposition is primarily driven by chemical recalcitrance. If decomposition of C_{root} was slower due to chemical recalcitrance, we would expect to find a greater proportion of C_{bg} in the FLF C pool. We did observe greater quantities of C_{bg} compared to C_{shoot} in FLF C, and the lack of statistical significance between these two sources may be due to greater variation in the quantity of cover crop C in FLF C compared to the OLF and MHF fractions. However, recent evidence suggests that chemical recalcitrance and unprotected POM such as that found in FLF contribute little to SOM stability (Carrington *et al*., 2012; Dungait *et al*., 2012). Physical protection may result in more C_{bg} POM in the OLF C pool. Root exudates and secretions may play a role in promoting aggregation, and the small size and close proximity of C_{bg} POM to aggregate formation may result in greater occlusion of C_{bg} POM compared to C_{shoot} POM.

In fact, we did observe a greater content of C_{bg} than C_{shoot} in the OLF fraction, which indicates greater abundance of root material than shoot residues as POM in soil aggregates. The belowground POM inputs such as root hairs, mycorrhizal hyphae, and to a lesser extent fine roots are small enough to be incorporated into microaggregates, which are usually defined as <250 μm but may be especially stable at the 2–20 μm scale (Krull *et al*., 2003), which corresponds to the scale of the most active components of mycorrhizal hyphae and root hairs (Rasse *et al*., 2005). Conversely, shoot material must be fragmented from the cm to μm scale before incorporation into stable soil aggregates and some C may be lost due to leaching or respiration during that process. Thus, the relatively smaller size of some C_{bg} components may promote enhanced physical protection of belowground POM in soil aggregates. The decomposition of POM in aggregates may be slowed by physical

isolation from decomposers and low oxygen concentrations (Six *et al*., 2002; Grandy & Robertson, 2007; Dungait *et al*., 2012).

Mineral association represents another mechanism by which C_{bg} may be preferentially stabilized in soil (Dungait *et al*., 2012; Cotrufo *et al*., 2013; Wieder *et al*., 2014; Tiemann *et al*., 2015), and we did observe a greater proportion of MHF derived from C_{bg} than from C_{shoot} after 5 months (Fig. 2a). Soluble rhizodeposits include organic acids produced by plants such as lactate, acetate, oxalate, malate, and citrate, which adsorb to clay mineral surfaces via polyvalent cation bonding (Kraffczyk *et al*., 1984). The highest contribution of rhizodeposits happens at the root tip, especially via mucilages, sloughed cells, and secretions (Dakora & Phillips, 2002; Farrar *et al*., 2003; Carvalhais *et al*., 2011), and as the root tip grows between soil pores, organic acids and mucilages are wiped along the surface of soil minerals. Thus, C_{rhizo}, contributing one-third of total C_{bg} in this system, may have greater likelihood of coming in contact with mineral surfaces. Additionally, root inputs are in close proximity to soil microbial communities, which can facilitate sorption on mineral surfaces. Greater mineral association of C_{bg} than C_{shoot} could result in greater long-term storage of C_{bg}.

How long do C_{bg} and C_{shoot} persist in soil C?

Given six times greater abundance of C_{bg} than C_{shoot} in soils 12 months following termination, representing three times greater relative contribution (relative to inputs, see *eq* 3) of C_{bg} than C_{shoot}, we would expect this to result in the accumulation of SOM in the long term as has been suggested in previous studies (Puget & Drinkwater, 2001; Kong *et al*., 2005; Rasse *et al*., 2005; Mendez-Millan *et al*., 2010). There are many cases in which the presence of cover crops have led to increases in soil C (Mullen *et al*., 1998; Mazzoncini *et al*., 2011; Wang *et al*., 2012; Higashi *et al*., 2014; McDaniel *et al*., 2014a; Tiemann *et al*., 2015), but others have shown no effect (Kaspar *et al*., 2006; Steele *et al*., 2012). We did not detect a difference between C_{bg} and C_{shoot} in soils 17 months following termination in EXP1. The majority of both C_{bg} and C_{shoot} were mineralized after 17 months. This may indicate short-term persistence of cover crop C in this system despite the lack of physical disturbance from tillage, but further study could reveal long-term stabilization of C_{bg} in greater proportion than C_{shoot} as observed in previous studies (Gale & Cambardella, 2000; Puget & Drinkwater, 2001; Rasse *et al*., 2005; Kong & Six, 2010; Mendez-Millan *et al*., 2010). A stronger isotopic label may be required to detect the long-term persistence of a single season's cover crop inputs in stable soil C pools, or repeated annual input of labeled materials may reveal the accumulation of cover crop carbon over time. The benefits of cover crop to building soil may thus depend on continuous use of cover crops in annual rotation with main crops.

Cover cropping could support partial harvest of corn stover for biofuel production

Biofuel crop residues and cover crop biomass may provide substantial feedstock for bioenergy production (Perlack *et al*., 2005; Graham *et al*., 2007), but this removal of potential soil C inputs could lead to reduced SOM (Anderson-Teixeira *et al*., 2009; Blanco-Canqui & Lal, 2009). However, several studies have found that decreased aboveground inputs do not necessarily correlate with decreased SOM (Tonitto *et al*., 2006; Steele *et al*., 2012; Adler *et al*., 2015). One potential mechanism for this discrepancy is a disproportionate contribution to SOM from belowground inputs (Balesdent & Balabane, 1996; Rasse *et al*., 2005; Kong & Six, 2010; Mendez-Millan *et al*., 2010). A meta-analysis of residue inputs required to maintain soil carbon in corn systems estimated that a mean of 638 ± 219 g m^{-2} corn stover is required to maintain soil carbon (Johnson *et al*., 2014), a value roughly equivalent to total annual stover input at the GLBRC BCSE site (Table 3).

We estimate that the use of cereal rye as a winter cover crop in a no-till continuous corn rotation at this site could replace about 80% stover removal based on productivity and carbon content of crops in 2013 and 2014, although conversion rates of stover to soil carbon will vary with litter quality, soil type, management practices, climate, and other factors. Total cover crop C inputs are roughly equal to 80% of harvested stover C, whereas belowground cover crop carbon inputs are roughly equal to 42% of harvested stover C. As C_{bg} is three times more likely to be stored in soil C than C_{shoot}, the incorporation of winter cover crop roots could substantially remediate the effects of stover removal in this system in the short term. Including winter cover crops in annual rotation could increase biofuel feedstocks directly and indirectly; aboveground cover crop residues could contribute to biofuel feedstocks and belowground cover crop inputs could offset the C removal associated with the use of main crop stover as a biofuel feedstock.

Acknowledgements

We would like to thank the Grandy Soil Biogeochemistry Lab at The University of New Hampshire and two anonymous reviewers for helpful comments and manuscript revisions. This study was supported by the Department of Energy's Great

 doi: 10.1111/gcbb.12428

Lakes Bioenergy Research Center (DOE Office of Science, DE FC02-07ER64494 and Office of Energy Efficiency and Renewal, DE-ACO5-76RL01830), the National Science Foundation LTER program and the University of New Hampshire Agricultural Experiment Station. We are also grateful to the W.K. Kellogg Biological Station, the Great Lakes Bioenergy Research Center, and in particular to Rick Corder and Stacey VanderWulp for assistance in the field.

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