



Limits to soil carbon stability; Deep, ancient soil carbon decomposition stimulated by new labile organic inputs



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ARTICLE INFO

Article history:

Received 21 October 2015

Received in revised form

4 April 2016

Accepted 5 April 2016

Keywords:

Alanine

Glucose

Deep carbon

Priming

Global change

ABSTRACT

Carbon (C) buried deep in soil (below 1 m) is often hundreds to thousands of years old, though the stability and sensitivity of this deep C to environmental change are not well understood. We examined the C dynamics in three soil horizons and their responses to changes in substrate availability in a coarse-textured sandy spodosol (0.0–0.1, 1.0–1.3, and 2.7–3.0 m deep). Substrate additions were intended to mimic an increase in root exudates and available inorganic nitrogen (N) that would follow an increase of belowground biomass at depth, as previously found in a long-term CO₂ enrichment experiment at this site. We incubated these soils for 60 days with glucose, alanine, and leaf litter, crossed with an inorganic N amendment equivalent to three times ambient concentrations. The organic substrates were isotopically labeled (¹³C), allowing us to determine the source of mineralized C and assess the priming effect. Enzyme activity increased as much as 13 times in the two deeper horizons (1.0–1.3, and 2.7–3.0 m) after the addition of the organic substrates, even though the deepest horizon had microbial biomass and microbial phospholipid fatty acids below the level of detection before the experiment. The deepest horizon (2.7–3.0 m) yielded the largest priming response under alanine, indicating that microorganisms in these soil horizons can become active in response to input of organic substrates. Inorganic N amendments significantly decreased the priming effect, suggesting that decomposition may not be N limited. However, alanine (organic N) yielded the highest priming effect at every soil depth, indicating the importance of differentiating effect of organic and inorganic N on decomposition. Distinct priming effects with depth suggest that portions of the soil profile can respond differently to organic inputs. Our findings indicate that the deep soil C pools might be more vulnerable to environmental or anthropogenic change than previously thought, potentially influencing net CO₂ exchange estimates between the land and the atmosphere.

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1. Introduction

Deep soil carbon (C) pools (below 1 m) contain more C than the biotic and atmospheric pools combined (Schlesinger, 1997; Lal, 2008). Deep soil carbon is considered to be highly stable due to a

variety of physical and chemical processes that inhibit microorganisms from accessing or efficiently mineralizing organic compounds (Sollins et al., 1996; De Nobili et al., 2001; Davidson and Janssens, 2006; Kuzyakov, 2010; Schmidt et al., 2011), and can remain stored for millennia, provided the ecosystem is not disturbed (Wolf and Wagner, 2005; Ewing et al., 2006). Deep soils are typically dominated by old, slowly cycling organic C (Schimel et al., 1994; Baisden et al., 2002; Schmidt et al., 2011) that is often recalcitrant to microbial mineralization (Ewing et al., 2006; Baisden and Parfitt, 2007). The dynamics of deep C pools have been understudied leaving their response to environmental change poorly understood (Jobbágy and Jackson, 2000; Baisden and Parfitt,

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2007). Soil carbon cycling research has generally focused on surface soils (top 1 m of soil), extrapolating in some cases to deeper depths based on modeling assumptions (Richter et al., 1999; Jobbágy and Jackson, 2000; Six et al., 2002; Baisden and Parfitt, 2007; Lal, 2008; Richter and Yaalon, 2012). However, deep soils have unique C and nutrient cycles (Richter and Markewitz, 1995; Fontaine et al., 2007; De Graaff et al., 2014) that are likely to respond differently to global change than surface soils (Richter et al., 1999; Hamer and Marschner, 2005a; Guenet et al., 2010; Harper and Tibbett, 2013; Mobley et al., 2015).

The mechanisms through which soil organic compounds persist in soil are not fully understood, but include a variety of biological, physical and chemical phenomena (Fog, 1988; Sollins et al., 1996; Six et al., 2002; Ewing et al., 2006; Baisden and Parfitt, 2007; Rasmussen et al., 2007; Schmidt et al., 2011). In deep soils, C may accumulate because of a combination of factors, including: (1) a scarcity of microbial substrates such as labile organic compounds, available nutrients, or molecular oxygen that limit microbial activity or enzyme production (De Nobili et al., 2001; Kuzyakov, 2010) and determine the physiological state or abundance of microorganisms (Thurston, 1994; Blagodatskaya and Kuzyakov, 2008; Dijkstra et al., 2015); (2) environmental conditions such as low temperature or low pH (Cheng and Johnson, 1998; Davidson and Janssens, 2006; Hagerty et al., 2014; Mobley et al., 2015); (3) inaccessibility and physical protection in the soil matrix, whether occluded in aggregates or bound to clays (Garten and Ashwood, 2002; Six et al., 2002; Ewing et al., 2006; Fontaine et al., 2007; Schmidt et al., 2011); or (4) chemically low degradability of the accumulating organic compounds conferred by lignin, phenolics, tanins, or low N content (Sollins et al., 1996; Hamer and Marschner, 2002; Hernes et al., 2007).

The stability of deep soil C pools can change in response to a wide variety of perturbations; among these is an increase in root exudates caused by increased biomass production or deepening of the root profile (Hamer and Marschner, 2002; Fontaine et al., 2003; Waldrop and Firestone, 2004; Ewing et al., 2006; Schmidt et al., 2011). Roots can break aggregates and decrease bulk density (Lal and Shukla, 2004), increasing soil oxygen diffusion rates (Mueller et al., 2015) and the supply of fresh, relatively labile organic compounds deep into the soil profile through root turnover or exudation of sugars, aminoacids, organic acids, and inorganic nutrients, all which can stimulate soil microbial community activity (De Nobili et al., 2001; Hamer and Marschner, 2002; Fontaine et al., 2007; Stumpe and Marschner, 2009; Fischer et al., 2010; De Graaff et al., 2014). Microbial growth requires nutrients such as nitrogen (N), which microbes can actively mineralize from soil organic matter through exoenzyme production when growth is N-limited (nitrogen mining theory; Kuzyakov, 2002; Fontaine et al., 2004). Increased microbial mineralization of soil organic matter in response to an increase in the supply of labile organic inputs is a phenomenon known as “priming” (Blagodatskaya et al., 2009; Cheng, 2009; Fischer et al., 2010; Kuzyakov, 2010). Priming is common in plant-soil systems subject to changes in an organic input or substrate (Kuzyakov et al., 2000; Blagodatskaya and Kuzyakov, 2008; Kuzyakov, 2010).

Because of the potential ability of belowground biomass to stimulate soil C mineralization and of inorganic N availability, we designed this study to test the effect of increased root exudates and inorganic N on deep (>2.7 m) soil C pools that have been traditionally considered unreactive. With substrate amendments we intended to simulate an increase in organic inputs to deep soils that may accompany global change at a scrub oak (*Quercus* sp.) forest sandy spodosol (Fig. 1; Hungate et al., 2013a) that will likely experience higher photosynthetic rates and deeper root profiles as atmospheric CO₂ rises (as described in Day et al., 2006; Stover et al.,



Fig. 1. Soil profile with horizons included in this study and depth, modified from Hungate et al. (2013a).

2007; Carney et al., 2007; Day et al., 2013). This forecast is based on a 10-year elevated CO₂ experiment adjacent to our study site (2x ambient; Hungate et al., 2013a) in which increased atmospheric CO₂ increased belowground biomass production over 2 fold (Day et al., 2013) after 11 years, and induced other changes in soil C and N cycling in the upper 1.3 m of the soil profile (Fig. 1). In particular, elevated CO₂ stimulated microbial organic matter mineralization in surface soils (0–0.1 m; Langley et al., 2009; McKinley et al., 2009; Hungate et al., 2013a), and microbial communities in the top 0.1 m of the elevated CO₂ plots were more prone to decompose soil organic matter when presented with fresh plant litter (Carney et al., 2007). Soil enzyme activity and stable isotope probing data suggested that the priming response measured in the surface soil by Carney et al. (2007) was due to a CO₂-induced increase in N demand by fungi. Here, we explore the potential for soil organic matter priming deeper in the profile and assess the vulnerability of deep soil C to changes in labile C and N inputs. The response of these deep, subsurface soil C dynamics to environmental conditions predicted by global change models and their feedback to atmospheric CO₂ concentration are among the largest uncertainties in current climate change projections (Jobbágy and Jackson, 2000; Fontaine et al., 2007; Guenet et al., 2010; Carvalhais et al., 2014; Friend et al., 2014).

We hypothesized that: (1) microbial community responses to substrate addition will be faster at the soil surface than at depth, where microbial population sizes and activity are expected to be relatively low; (2) substrate addition will induce a larger priming effect (i.e., more μg soil-derived C per gram of soil) in deep soils than surface soils because microbial activity is likely to be more strongly substrate limited at depth (Bardgett et al., 2008; Langley et al., 2009); and (3) the addition of substrates containing N (organic or inorganic) will cause less soil organic matter (SOM) priming than substrates that lack N because microbes can acquire N through the direct uptake of the added substrate, alleviating their N demand (Kuznyakov, 2002; Fontaine et al., 2004). Hypothesis 3 assumes that N availability limits microbial activity throughout the soil profile.

2. Materials and methods

2.1. Site description

This research was conducted at the Merritt Island National Wildlife Refuge, on the east coast of subtropical central Florida, USA (28°38'N, 80°42'W). Temperatures range between 33 °C in July and a 9.6 °C in January; precipitation averages 131 cm y^{-1} , falling mostly (about 65%) in the months of June to October. Three perennial evergreen oaks, *Quercus myrtifolia* Willd., *Quercus geminata* Small., and *Quercus chapmanii* Sarg., constitute up to 90% of the above-ground biomass at the site (Schmalzer and Hinkle, 1992). This is a short-statured forest which experiences a fire return interval of 8–12 years (McKinley et al., 2009). Soils are classified as Orsino series (Huckle et al., 1974), i.e. very deep and moderately well drained sandy deposits with low water holding capacity (hyperthermic, uncoated Spodic Quartzipsamments) and very low to nonexistent silt and clay mineral contents (as addressed in Carney et al., 2007; Langley et al., 2009; and McKinley et al., 2009). The horizons identified below the surface 0–1.0 m have been described in McKinley et al. (2009) and Hungate et al. (2013a) as a buried A horizon at ~1 m, an albic horizon at about 1.9 m, and a spodic horizon at 2.7 m deep (Fig. 1). The spodic horizon occurs near the average depth of the water table (2.35–2.45 m; Hungate et al., 2013a).

2.2. Sampling protocol

As in McKinley et al. (2009) and Hungate et al. (2013a), we collected 10 cores to a depth of 3 m, and divided them into 10 cm increments from 0 to 100 cm, and in 30 cm increments from 100 to 300 cm. The soil cores were collected in two groups of five, with about 25 m separating the groups to physically average the natural variability in the area (Allmaras and Kempthorne, 2002). We composited the depth increments separately for the five cores in each group, yielding two sets of composite soil profile samples. The study was designed to test variation in microbial processes as a function of soil depth. The collection of five cores per site ensured enough composite soil sample at each depth to run the laboratory experiment (i.e., seven treatments triplicated per composite soil sample, as described below), and for soil characterization (Table 1 and Fig. 1, from Hungate et al., 2013a). We selected three depths for this experiment that represented the surface (0–0.1 m), buried A horizon (1–1.3 m), and spodic horizon (2.7–3.0 m), as defined in McKinley et al. (2009) and Hungate et al. (2013a). Roots were removed and the soil was sieved through a 2-mm mesh and mixed thoroughly to obtain a homogeneous composite sample for analyses and experimentation.

2.3. Soil characterization

Homogenized soil was analyzed for inorganic N (N-NH_4^+ and N-NO_3^-), gravimetric water content, cation exchange capacity, and total C and N (Table 1), all performed at the Oklahoma Soil Testing Laboratory (Stillwater, OK). Mean radiocarbon (^{14}C) conventional age (years BP; Stuiver and Polach, 1977) and $^{13}\text{C}/^{12}\text{C}$ ratio (‰) were determined for each soil horizon at Beta Analytic Radiocarbon Dating Laboratory (Miami, FL), who followed Talma and Vogel (1993) for conversion to years (Table 1). A subset of soil samples was used for initial (time = 0) microbial assays, including microbial C and N biomass, microbial phospholipid fatty acids (PLFA), and phenol oxidase and peroxidase activity.

Inorganic N was extracted with 2 M KCl and quantified colorimetrically using the indophenol blue method for N-NH_4^+ and cadmium reduction followed by diazotization with sulfanilamide for $\text{N-NO}_2^-/\text{N-NO}_3^-$ (Mulvaney, 1996). Total soil C and N was determined by dry combustion using a LECO Elemental CN Analyzer. Cation exchange capacity (CEC) was determined by displacement with ammonium acetate (Summer and Miller, 1996). Gravimetric moisture content was determined on soil subsamples that were oven dried at 105 °C until constant weight. Microbial phospholipid fatty acids (PLFAs) were analyzed following the modified White and Ringleberg (1998) method. The 21 fatty acid methyl esters (FAMES) identified from the phospholipid fraction were grouped into saturated, gram positive and gram negative bacteria, and fungi (according to McKinley et al., 2005). Microbial biomass C and N was determined with the chloroform-fumigation extraction method (Horwath and Paul, 1994), in which soil samples are fumigated with ethanol-free chloroform and extracted with 0.5 M K_2SO_4 . The activity of phenol oxidase and peroxidase enzymes was measured following Sinsabaugh et al. (2002) and Carney et al. (2007), using 50 mM pyrogallol as substrate and measuring absorbance at 460 nm. Soil characterization was intended to represent the initial conditions ($t = 0$) of the priming experiment.

2.4. Priming experiment

Soil samples were pre-conditioned for 10 days at 7% gravimetric moisture (about 20% water holding capacity) content (i.e., 70 mg water g^{-1} soil) and 25 °C, to allow for degradation of the pulse of labile carbon liberated by disturbing the soil samples in establishing the incubation, and to allow microbial communities to stabilize (Marstorp, 1996; Carney et al., 2007; Stumpe and Marschner, 2009). After 10 days, triplicates of each soil sample from the three soil depths were assigned to 6 different substrate amendments plus a control without amendments. The mass of subsamples used in the incubations increased with depth to account for decreasing soil carbon content. These subsamples consisted of 15 g of surface soil (0–0.1 m), 20 g of buried A horizon soil (1–1.3 m), and 30 g of spodic horizon soil (2.7–3.0 m), dry weight equivalent, and were placed in 120-mL airtight glass serum bottles fitted with a PTFE-lined septum for headspace sampling. Soil water content was then raised to 10% gravimetric moisture content (equivalent to 30% water holding capacity, maintained throughout the incubation period), and the substrates were added. The substrates (described in detail below) were glucose, alanine, plant litter, glucose + N, alanine + N, and litter + N.

Bottles were capped and headspace CO_2 concentration was measured regularly throughout the 60-day incubation period by drawing a 1 mL headspace sample with a syringe through the septum and analyzing it immediately on a modified LI-COR LI-7000 (LiCor, Lincoln, NE). During the incubation period, aerobic conditions were maintained in the bottles by periodically opening them (3–5 times during the 60-day incubation, depending on CO_2

Table 1

Soil characterization and initial soil conditions of the three soil depths studied (surface, buried A, and deep spodic horizon) before the 60-day incubation.

	Surface	Buried A	Spodic
Depth (m)	0–0.1	1–1.3	2.7–3
Age			
Conventional Radiocarbon age (yr BP)	–	430 ± 40	14,600 ± 90
¹³ C/ ¹² C Ratio (‰)	–28.26	–25.70	–25.88
Chemistry			
CEC (meq 100 g ⁻¹)	5.7	1.9	4.9
Total C (g C kg ⁻¹)	33.85	21.75	8.48
Total N (g N kg ⁻¹)	1.07	0.59	0.19
NH ₄ ⁺ -N + NO ₃ ⁻ -N (ppm)	1.54	1.81	1.06
C : N	31.36	36.75	43.09
Biology			
Microbial biomass C (μg C g ⁻¹)	82.5 ± 8.8	0.9 ± 2.9	nd
Microbial biomass N (μg N g ⁻¹)	10.0 ± 1.4	0.1 ± 0.3	nd
Total PLFA (ηmol g ⁻¹)	39.96 ± 9.52	7.35 ± 1.55	0
Bacteria : fungi	0.73	3.05	–

CEC, cation exchange capacity; PLFA, phospholipid fatty acid; yr BP, years before 1950 AD; nd, not detected.

production) after taking an additional sample for $\delta^{13}\text{C}$ –CO₂ analysis. The opened bottles were allowed to equilibrate with atmospheric CO₂ and O₂; after 2 h they were sealed again, and the CO₂ of the headspace was measured immediately. For $\delta^{13}\text{C}$ –CO₂, 10 mL of headspace gas was taken, stored in evacuated Exetainers, and measured on a Delta Plus Advantage gas isotope-ratio mass spectrometer at the Colorado Plateau Stable Isotope Laboratory in Northern Arizona University.

Organic substrates additions were chosen to represent a range of C:N ratios, and inorganic N amendment were intended to help separate the relative importance of C versus N substrates that would be naturally available under increased belowground biomass on soil organic carbon (SOC) priming. For the glucose and alanine amendments, we added 1 mg substrate (0.4 mg substrate-C) per dry g of soil (as in Shen and Bartha, 1996), representing 12 μg substrate-C mg⁻¹ SOC in our surface soil (similar to the 13 μg substrate-C mg⁻¹ SOC added in Hamer and Marschner, 2005b). The litter amendment consisted of 100 mg of dried and ground plant biomass (following the protocols of the earlier surface priming experiment at this site by Carney et al., 2007). These litter amendments correspond to an increase of 7, 5, and 3 mg substrate per g of dry soil in the surface, buried A horizon, and spodic horizon, respectively, reflecting the declining total organic matter content with depth (Table 1). Inorganic nitrogen treatments received (NH₄)₂SO₄ at 2 μg N per dry g of soil, an addition approximately equivalent to three times the ambient KCl-extractable N for each of the three horizons. The change in inorganic N availability due to NH₄ addition was similar across the three horizons because of approximately similar ambient inorganic N concentrations across horizons (Table 1). The glucose and alanine additions were also consistent across horizons, so that comparisons would be matched in terms of stoichiometry.

All substrates had unique $\delta^{13}\text{C}$ that differed from the native soils. Uniformly-labeled glucose (Icon Isotopes) and alanine (Isotec Sigma) had $\delta^{13}\text{C}$ values of 307 and 142‰, respectively. Plant litter was collected from an adjacent experiment in which plants that had been grown at elevated CO₂ for 11 years, resulting in a $\delta^{13}\text{C}$ of –38‰ due to the fossil-fuel derived CO₂ used to elevate the CO₂ (Carney et al., 2007). The soil samples had distinctly different $\delta^{13}\text{C}$ than these substrates (–28‰ in the surface soil, and –26‰ in the buried A and spodic horizon samples; Table 1), which allowed for the differentiation of the source of the C being mineralized during the incubation period. The partitioning of soil-derived CO₂ and substrate-derived CO₂ was calculated using a two-member mixing model (Schimel, 1993; Carney et al., 2007; Cheng, 2009; among others):

$$F_S = (C_T - C_C) / (C_S - C_C) \quad (1)$$

where, F_S is the proportion of substrate-derived CO₂, C_T is the $\delta^{13}\text{C}$ of the total CO₂ produced in the treatment incubation, C_C is the $\delta^{13}\text{C}$ of the CO₂ produced in the control incubation, and C_S is the $\delta^{13}\text{C}$ of the substrate added to the treatment incubation.

From F_S , we calculated the amount of C derived from substrate decomposition multiplying it by the total amount of CO₂ produced during the incubation (both expressed in μg C–CO₂ g⁻¹ soil). Similarly, the amount of C derived from SOC decomposition was calculated by multiplying the total amount of CO₂ produced during the incubation by $(1 - F_S)$. The priming effect (PE) was calculated as the difference between the soil-derived C–CO₂ in the control incubations and in the amended incubations (μg C–CO₂ g⁻¹ soil; Kuzyakov, 2010). We also calculated the percentage of initial soil C that was mineralized during the incubation under each substrate amendment.

Before and after the 60-day incubation period, we measured enzyme activity (phenol oxidase and peroxidase, following the method described above) and calculated the response ratio (activity after incubation over activity before incubation) to determine if the changes in soil C mineralization were associated with an increase of enzyme activity in the soil. We also measured extractable N (following the method described above) to determine the net N mineralization rate (μg N g⁻¹ soil day⁻¹) by subtracting the extractable N before the incubation from the extractable N by the end of the incubation (McKinley et al., 2009).

2.5. Data analysis

Samples from each site of each treatment (control, glucose, alanine, litter, glucose + N, alanine + N, and litter + N) per depth were incubated independently in triplicate. After the incubation period, we compared the SOC-derived CO₂ produced with 2-way ANOVA with site and depth as blocks to determine if soils from both sites were producing the same response to the treatments. Site had no effect on SOC-derived CO₂ ($p > 0.5$), and its interaction with the treatment was not significant ($p > 0.9$). We also compared SOC-derived CO₂ of each depth between sites with Students *t*-test and found the same variability and no significant differences between sites ($p > 0.3$). Based on this evidence we pooled the SOC-derived CO₂ replicates from the two sites to yield $n = 6$ per depth per treatment. There was a site by depth interaction ($p < 0.05$) that was driven entirely by differences in the surface-most horizon, with no significant differences in the two deep horizons. Thus, the

possibility of non-independence between depths and site is isolated to surface horizon.

We compared the SOC-derived CO₂ of each treatment with its control for each depth with a Student *t*-test to determine if there was a significant priming effect with respect to the controls (Hamer and Marschner, 2005a). We used ANOVA with Tukey HSD (at $\alpha < 0.1$) to determine differences in the priming effect of the organic substrate amendments (glucose, alanine, and litter) at each depth, to determine if the change in enzyme activity (natural log of the response ratios) was significantly different between soil horizons, and to assess if the addition of inorganic N to the organic substrates influenced the priming effect. We used ANOVA with Tukey HSD (at $\alpha < 0.1$) to determine if there were effects of depth or treatments on net N mineralization and the proportion of soil C mineralized. Differences among the three depths (surface, buried A, and spodic horizon) and the effects of substrate additions are summarized in Table 2. All statistical analyses were performed with SPSS version 22.0 for Macintosh (SPSS Inc.).

3. Results

3.1. Soil property differences with depth

The mean C-14 label of carbon in the buried A horizon had a conventional radiocarbon age of 430 ± 40 years BP (before 1950 AD) with $\Delta^{14}\text{C} -54.5 \pm 4.7\%$, while the mean radiocarbon age of spodic horizon was $14,600 \pm 90$ years BP with $\Delta^{14}\text{C} -837.6 \pm 1.8\%$ (Table 1). Total soil C and N content decreased with depth from 33.85 to 8.48 g C kg⁻¹ and from 1.07 to 0.19 g N kg⁻¹ (Table 1). Soil CEC was low throughout the entire sandy soil profile. Microbial biomass as estimated both by chloroform fumigation and PLFA was highest in the surface horizon and declined sharply with depth; neither assay detected any microbial biomass in the deepest soil horizon (see Table 1). Fungal biomass was a relatively larger component of total microbial biomass in the surface horizon ($46 \pm 3\%$ bacterial and $53 \pm 3\%$ fungal), compared to the buried A horizon ($78 \pm 2\%$ bacterial and $28 \pm 1\%$ fungal; Table 1).

3.2. Responses of soil carbon dynamics to substrate availability with depth

Microbial respiration (CO₂ production) declined sharply with soil depth in the control treatments (Fig. 2). In the amended treatments, the total CO₂ evolved during the incubation period was a combination of soil-derived and substrate-derived C as calculated from the isotopic mass balance. Substrate amendments consistently increased total CO₂ production compared to the non-amended controls. Conversely, crossing the organic amendments with inorganic N did not increase total CO₂ production (e.g., glucose and glucose + N amendments yielded similar soil respiration). While the effect of glucose addition on respiration was immediate, especially in the surface and buried A horizons, alanine required up to three days to reach maximum total respiration in every soil. In the buried A horizon, the glucose and alanine amendments produced similar total amounts of CO₂ over the incubation period

(Fig. 2). However, in this horizon the glucose amendment produced most of its CO₂ from the respiration of the substrate, while under alanine most of the CO₂ was soil derived (Fig. 3). Alanine consistently resulted in the highest soil-derived C mineralization at every soil depth ($p < 0.01$; Fig. 3), especially in the deeper two horizons where alanine caused five times more soil-derived mineralization than any other substrate in the buried A horizon, and six times more than any other substrate in the spodic horizon. Overall, the surface horizon had the greatest proportion of soil C mineralized ($p < 0.005$; Fig. 3). Treatment and depth had a significant effect on the proportion (%) of SOC mineralized, but they did not interact (Fig. 3; Table 2).

The response ratio in the oxidative enzyme activity (i.e., activities of phenol oxidase and peroxidase after incubation over the activities before the incubation), or relative enzymatic activity, revealed that substrate amendments increased enzyme activity during the 60-day incubation. This increase in activity was greatest in the deeper horizons, as indicated by the consistently positive values at depth in Fig. 4 and the significant effect of depth in Table 2. Before the incubation most of the enzyme activity was localized in the surface horizon, but by the end of the incubation period all horizons had similar enzyme activity, thereby producing the largest response ratio in the deep spodic horizon for phenol oxidase ($p < 0.05$; Fig. 4a) and in the buried A and spodic horizon for peroxidase (marginally significant, $p < 0.15$; Fig. 4b).

Net N mineralization, calculated as the difference between controls and treatments, changed significantly within the soil profile ($p < 0.05$; Fig. 5; Table 2). Throughout the soil profile alanine and alanine + N had the greatest increase in net N mineralization (an order of magnitude more than the other amendments; $p < 0.001$), and this increase was larger with depth. All substrate amendments increased net N mineralization of the surface soil compared to the controls, with the smallest increase observed for the litter amendment. In the buried A and spodic horizons all amendments except for alanine and alanine + N reduced net N mineralization compared to the controls. Furthermore, net N mineralization in the spodic horizon did not respond to addition of any substrate except alanine and alanine + N.

3.3. Priming effect with depth

Soil organic carbon mineralization was significantly higher under glucose, alanine, and litter amendment at every depth compared to the controls ($p < 0.05$ in the surface, $p < 0.001$ in the buried A and in the spodic horizon), and the overall priming effect of all the substrates (difference in SOC mineralization between treatments and controls) was similar between horizons due to the wide range of mineralization induced by the substrates ($p > 0.3$; Fig. 6). Priming effects at every depth and under every treatment were positive (i.e., they increased SOC mineralization) except for the glucose amendment to the spodic horizon, which induced a negative priming effect (i.e., it decreased SOC mineralization). In the buried A and spodic horizons, all amendments with the exception of glucose + N had a significant effect on SOC mineralization ($p < 0.1$; Fig. 6). Overall, the treatments yielded a significant

Table 2

Summary of *p*-values of 2-way ANOVA with Tukey post-hoc test, using treatment and depth as independent variables and proportion of soil C mineralized, priming effect, phenol oxidase activity, peroxidase activity, and N mineralization as dependent variables.

	Proportion soil C mineralized	Priming effect	Phenol oxidase activity	Peroxidase activity	Net N mineralization
Treatment	≤ 0.1	< 0.005	ns	ns	< 0.001
Depth	< 0.001	ns	< 0.05	< 0.15	< 0.05
Interaction	ns	ns	ns	ns	< 0.05

ns, not significant.

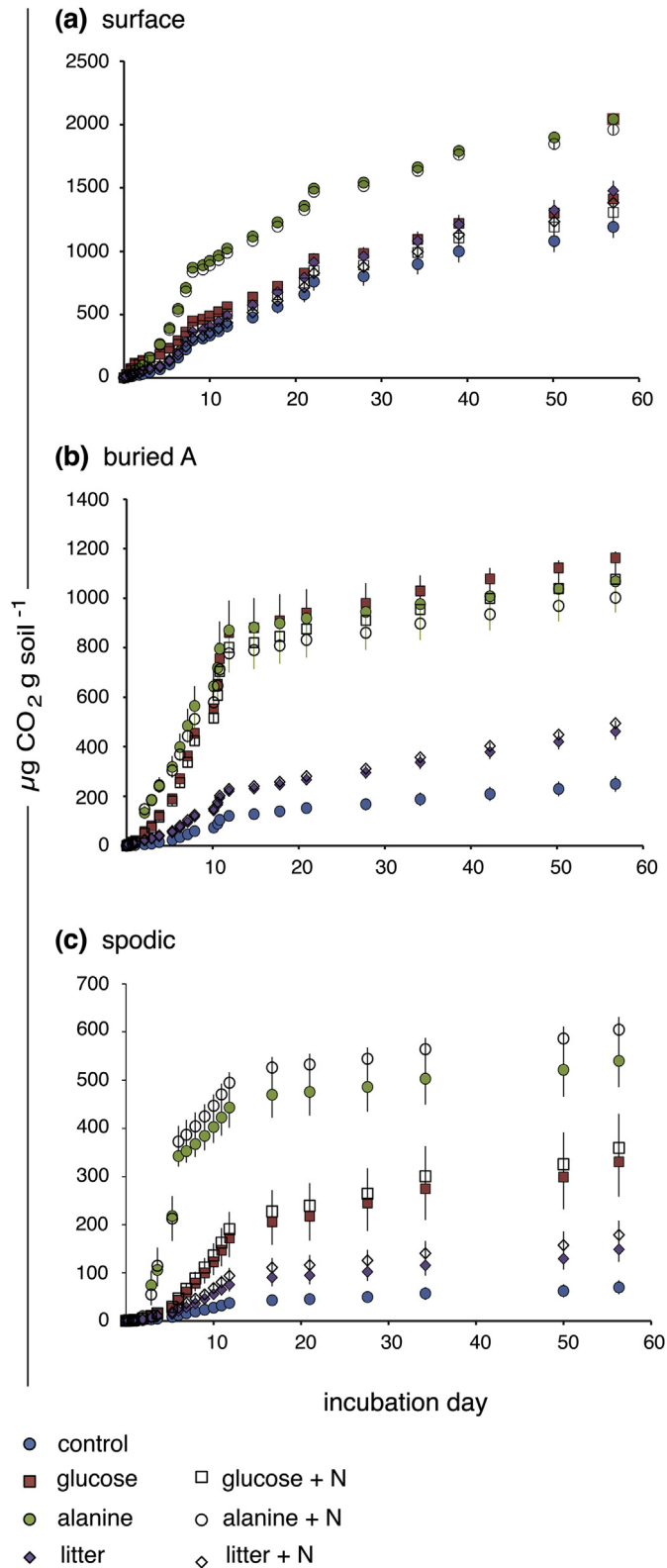


Fig. 2. Cumulative respiration ($\mu\text{g CO}_2 \text{ g soil}^{-1}$) measured during the 60-day incubation in (a) surface, (b) buried A, and (c) spodic horizon samples after substrate amendment (glucose, alanine, litter, glucose + N, alanine + N, litter + N) and under un-amended control conditions. Values are means ($n = 6$) and error bars represent standard error of the mean. Note the different scales of Y-axes.

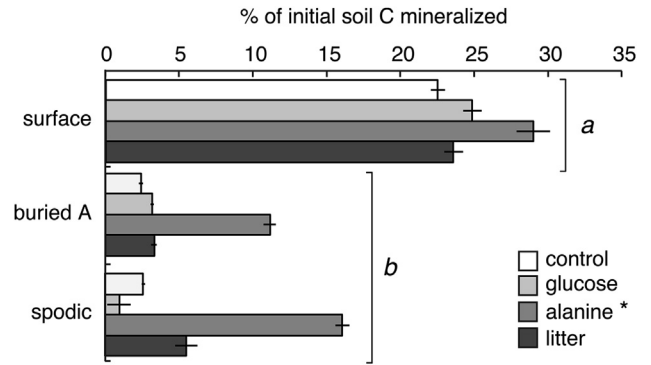


Fig. 3. Percentage of the initial C contained in the soil that had been mineralized after 60-day incubation under substrate amendment (glucose, alanine, and litter) and under un-amended soil controls, for surface, buried A, and spodic horizon samples. Bars represent averages ($n = 6$) with standard errors; letters represent significant differences between horizons ($p < 0.005$), and the asterisk in the legend significant difference between treatments ($p < 0.01$).

priming effect that did not interact with depth (Table 2). Looking at treatment effects individually, alanine had a significantly larger priming effect than the glucose and litter ($p < 0.01$). In fact, the lowest priming effect caused by alanine (in the deep soil) was significantly larger than the largest priming effect induced by any of the other substrate amendments ($p < 0.005$). The addition of inorganic N reduced SOC priming ($p \leq 0.05$) for all substrates at all depths, with the exception of glucose + N in the spodic horizon, where adding N increased priming caused by glucose.

4. Discussion

4.1. Deep soil carbon

Our results showed that deep (~3 m) soil C in this scrub oak can be mineralized in response to fresh substrate additions. Previous studies have shown that deep C is typically old and slow cycling due to diverse stabilization processes and scarce disturbance (Fog, 1988; Schimel et al., 1994; Sollins et al., 1996; Richter et al., 1999; Baisden et al., 2002; Six et al., 2002; Ewing et al., 2006; Baisden and Parfitt, 2007; Rasmussen et al., 2007; Schmidt et al., 2011; Richter and Yaalon, 2012). The radiocarbon age of our soil horizons indicates that the C was also old (centuries below 1 m deep, millennia at 3 m; Table 1) and slow cycling. Interpretation of radiocarbon ages in mixed pools requires caution because the age is the mean of all sources and may include dissolved C translocated from other soil horizons (Raymond and Bauer, 2001; Baisden et al., 2002; Sanderman et al., 2009). Although our site has a fluctuating water table that could transport old dissolved C throughout the soil profile (Hungate et al., 2002), we suspect that this processes does not explain the age of deep soil C pool; these soils are not expected to accumulate dissolved and mobile C because they are sandy and well-drained, and have low CEC and low water holding capacity (Huckle et al., 1974; Langley et al., 2009; McKinley et al., 2009; Hungate et al., 2013a).

The presence of relatively old, deep soil carbon in this scrub oak forest may be attributed to a combination of factors, one of which is C and N limitation of microbial activity. Evidence of this interpretation is the low to undetectable microbial activity and biomass in the buried A and deep horizons prior to our experiment (Table 1), low mineralization rates after a 60-day incubation under control conditions (i.e. without substrate addition; Fig. 2), and increased mineralization rates with C substrate addition. It is possible that a portion of the SOM pool is stable because of chemical recalcitrance

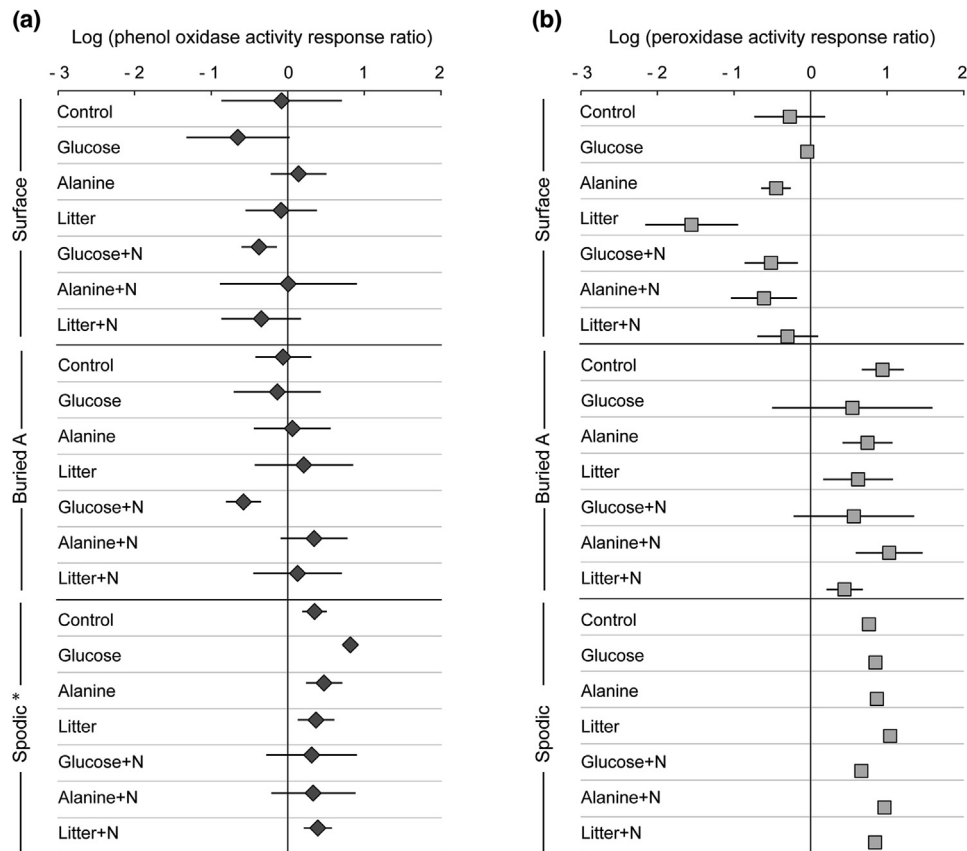


Fig. 4. Logarithm of the response ratio of oxidative enzyme activity of (a) phenol oxidase and (b) peroxidase produced after 60 days of incubation respect to the initial soil conditions, in surface, buried A, and spodic horizon samples under glucose, alanine, litter, glucose + N, alanine + N, and litter + N. The asterisk in the spodic horizon indicates that response ratio of this horizon was significantly different ($p < 0.05$) to the ratio in the buried A and surface horizons.

and would have persisted despite substrate addition had the incubations continued for >60 days.

4.2. Substrate additions

Belowground biomass has been found to stimulate soil organic matter mineralization due to an increase in the supply of fresh organic substrates via root exudation and root biomass turnover, a response that increases the availability of inorganic N in some cases (Fontaine et al., 2004; Blagodatskaya and Kuzyakov, 2008; Kuzyakov, 2010). Our study was intended to assess the effect of an increase in organic substrates and inorganic N on deep (<3 m) soil C pools, a scenario that was demonstrated by a long-term elevated CO_2 experiment at our site (Stover et al., 2007; Day et al., 2013; Hungate et al., 2013b). This long-term experiment showed how elevated atmospheric CO_2 can alter the distribution of C and N inputs and cycling through changes in ecosystem structure and function (Hungate et al., 1997; Carney et al., 2007; Langley et al., 2009; McKinley et al., 2009; Hungate et al., 2013a).

The addition of substrates to the three soil horizons induced a priming effect. A common explanation for priming is the alleviation of a resource limitation that constrained microbial growth, thereby allowing microbial communities to consume portions of the native soil organic matter that they could not consume efficiently in the absence of the substrate addition (Kuzyakov, 2002; Fontaine et al., 2004; Hamer and Marschner, 2005a; Waldrop et al., 2004; Carney et al., 2007; Kuzyakov, 2010). The magnitude of the priming effect measured in our experiment varied with substrate and soil horizon, with alanine causing the largest priming response. Alanine has a C

content similar to glucose, but it also contains organic N and was the most N-rich organic substrate. This suggests that microbial activity was partly N-limited, and that the priming mechanism was sensitive to organic N availability. Evidence of N limitation includes the wide C:N ratios of SOM increasing with depth (Table 1) and net N microbial immobilization (Fig. 5) in the buried A and spodic horizons after the addition of the substrates. Only alanine increased N mineralization compared to the controls in the deep horizons, perhaps because the alanine-N itself was being mineralized during the incubation rather than the soil organic N.

Priming responses to inorganic N were smaller and more variable than to organic substrates, suggesting either that priming is less sensitive to inorganic N than organic N, or that the amount of inorganic N added was too low to cause a priming effect. Although we increased the ambient ammonium pool by three times, it is possible that the relative small amount added ($2 \mu\text{g N per g dry soil}$) was fixed into clays and thus remained unavailable to soil microbes. We suspect that this was not the case because clay content is very low in these soils (Table 1; Carney et al., 2007; Langley et al., 2009; McKinley et al., 2009), and inorganic N addition did have a significant negative effect on mineralization in some cases. We do not know how soil microorganisms would have responded to larger ammonium additions more comparable to our alanine addition ($157 \mu\text{g N per g dry soil}$), but both additions fell in the range of concentrations reported for unfertilized rhizosphere soil (Shen and Bartha, 1996; Hamer and Marschner, 2005b; McKinley et al., 2009), the scenario that we intended to simulate.

Our results are consistent with previous studies showing that alanine addition often stimulates SOC mineralization whereas

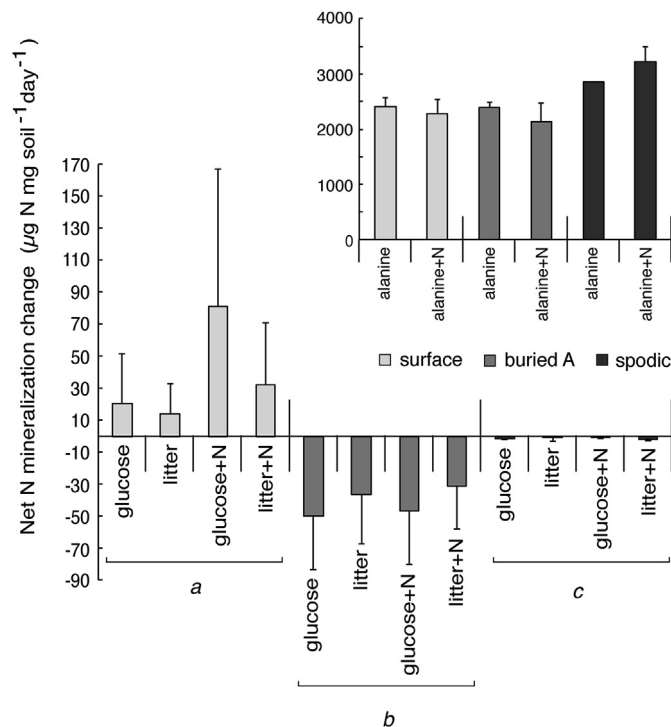


Fig. 5. Change in net N mineralization ($\mu\text{g N mg soil}^{-1} \text{day}^{-1}$) caused by substrate amendment (glucose, alanine, litter, glucose + N, alanine + N, litter + N) compared to the controls (i.e., net N mineralization in amendments minus controls) in surface, buried A, and spodic horizon, in the 60-day incubation. Error bars represent standard errors of the mean; letters represent significant differences between horizons ($p < 0.05$); alanine and alanine + N treatments (upper graph) results were significantly different ($p < 0.001$) to the other treatments (lower graph).

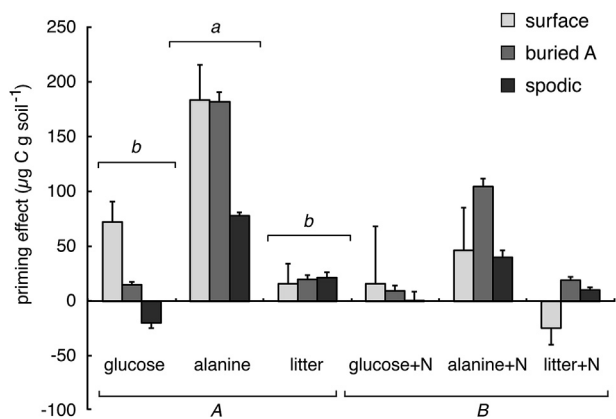


Fig. 6. Priming effect ($\mu\text{g C-CO}_2 \text{ g soil}^{-1}$) calculated for each substrate amendment (glucose, alanine, litter, glucose + N, alanine + N, litter + N) in surface, buried A, and spodic horizon. Bars represent averages ($n = 6$) with standard errors; lower case letters represent significant differences between treatments ($p < 0.01$); upper case letters represent significant differences between organic substrates and organic substrates with inorganic N ($p \leq 0.05$).

ammonium addition often does not (Fog, 1988; Hamer and Marschner, 2005b; Hobbie, 2005; Stumpe and Marschner, 2009). This pattern may reflect a condition where microbial growth is limited by C rather than N (Stumpe and Marschner, 2009). In soils with low N availability, low quality (chemically recalcitrant) C can be an overriding constraint on SOM decomposition rates (Hobbie, 2000, 2005). In such a case, inorganic N addition may only increase the decomposition of relatively recalcitrant forms of SOM

when accompanied by enough labile organic carbon to alleviate C limitation (Hobbie, 2000, 2005). In the soil studied here, the surface and buried A horizons showed a positive priming response to all forms of carbon addition (glucose, alanine and litter), suggesting that some portion of the soil carbon pool was unavailable to soil microbes due to carbon limitation of microbial growth (Fontaine et al., 2007, Table 1). This pattern held in the deepest (spodic) horizon for organic compounds that contained N (alanine and litter), but not for glucose which lacks nitrogen. This suggests that microbial activity in the deepest horizon was co-limited by C and N.

4.3. Soil microbe responses

Lag phases in CO_2 evolution after substrate additions could indicate a substrate limitation. However, a frequent explanation for the immediate peak in total CO_2 evolution under glucose addition and the delayed peak of a few days under alanine addition (Fig. 2) is that the two amendments stimulated different soil microbial communities or changed the existing ones (Griffiths et al., 1999; Hamer and Marschner, 2002, 2005b; Waldrop and Firestone, 2004; Fontaine et al., 2007; Fischer et al., 2010). The relatively rapid increases in SOC mineralization after the addition of glucose may reflect a growth response by fast-growing bacteria (Hamer and Marschner, 2005b), or a rapid change in metabolism by a wide variety of microorganisms (Stotzky and Norman, 1961; Hungate et al., 2015). One reason that glucose usually produces an immediate respiratory response (Fig. 2) is because many types of organisms use it as a substrate (Fog, 1988; Hopkins et al., 1994; Stumpe and Marschner, 2009). By comparison, the alanine response had a lag phase that suggests it influenced a different community of slow-growing bacteria, even though it can also be used by a wide range of microbes (Hamer and Marschner, 2005b; Fischer et al., 2010).

The PLFA analysis revealed that fungi were 53% of microbial biomass in surface soil, but dropped sharply below the surface horizon to 22% (buried A) and 0% (spodic; Table 1). Total microbial biomass also decreased sharply from $82.5 \mu\text{g C g}^{-1}$ in the surface to $0.9 \mu\text{g C g}^{-1}$ in the buried A horizon. By comparison, the decrease in soil C content with depth was smaller and more gradual (from 34 g C kg^{-1} in the surface to 22 g C kg^{-1} in the buried A horizon). Based on soil C content alone, we would have expected the buried A horizon to support more microbial biomass than we measured. Because microbial biomass generally increases with the size of the labile soil C pool (Hamer and Marschner, 2005b; Fischer et al., 2010; Bird et al., 2011), the sharp drop with depth reflects the relatively recalcitrant character of the SOC contained in the buried A and spodic horizons.

The spodic horizon showed no detectable microbial biomass by chloroform fumigation or by PLFA in the spodic horizon before substrate addition (Table 1). However, low rates of CO_2 production ($22.7 \pm 5.0 \mu\text{g C-CO}_2 \text{ g}^{-1} \text{soil}$) and the increase in enzyme activity after incubation at (Fig. 4) indicate that microbes were present and possibly dormant. In addition to the evidence presented here that substrate availability limits microbial biomass in the spodic horizon, it is possible that anoxic conditions may prevail at times in the spodic horizon due to its proximity to the water table creating oxygen limitation.

5. Conclusions

Distinct priming effects with depth suggest that portions of the soil profile can respond differently to organic inputs. We found that SOC mineralization is high in the surface soils with and without substrate additions (Fig. 3), suggesting that microbial communities in surface soils with active rhizospheres are adapted to the levels of

substrate additions used in our experiment. Roots in this site do not presently reach to the buried A and spodic horizons but the root zone is expected to deepen as atmospheric CO₂ increases (Day et al., 2006; Stover et al., 2007; Carney et al., 2007; Day et al., 2013), affecting soil depths below the root zone by acting as a closer source of C. Organic C and N additions in deep soils (1–3 m) stimulated SOC mineralization, a priming effect that demonstrates deep soil C is vulnerable to environmental change. The size and direction of SOC priming varied with substrate (Fig. 6). The addition of labile C (glucose) increased SOC mineralization in the two surface horizons but not the spodic horizon, which was deepest and contained the least labile organic C (Table 1, Fig. 1). When labile C addition was combined with labile organic N (alanine), soil C and N mineralization increased by orders of magnitude at every depth (Fig. 3; Fig. 5; Fig. 6), including in the spodic horizon that had no detectable microbial biomass and activity before the experiment (Table 1). On the contrary and as reported by other studies in surface soils (Fog, 1988; Hobbie, 2000, 2005; Hamer and Marschner, 2005b; Stumpe and Marschner, 2009), the addition of inorganic N did not have the stimulating effect of organic N (Fig. 5; Fig. 6).

Sub-tropical spodosols like those at our site are relatively low in organic matter compared to croplands and grasslands (Fig. 1; Mobley et al., 2015). However, they are typically very deep and contribute to a large pool of deep soil C pool that remains vastly underestimated in global C budgets (Richter and Markewitz, 1995; Richter et al., 1999; Jobbágy and Jackson, 2000; Baisden and Parfitt, 2007; Richter and Yaalon, 2012; Harper and Tibbett, 2013). Deep soils C pools are typically old and recalcitrant, mostly because they remain inaccessible to microbial degradation (De Nobili et al., 2001; Hamer and Marschner, 2005a; Ewing et al., 2006; Baisden and Parfitt, 2007; Fontaine et al., 2007). Here we show that deep soil C pools can become unstable in the presence of increased availability of labile organic substrates that may accompany climate change (Fig. 6). The dynamics of deep soil C are not well understood, but they are key to fully understanding the potential impacts of environmental change on soils C pools and C exchange with the atmosphere.

Acknowledgments

This work was supported by a grant from the National Science Foundation (DEB-0445324). This project was accomplished with assistance of Maria Ceron-Castilla and Dr. Jason Keller. We acknowledge the help of Dr. Sean McMahon in the data analysis, and the support of NASA Kennedy Space Center, Dynamac 457 Corp. and US Fish and Wildlife Service, Merritt Island National Wildlife Refuge.

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