Soil respiration is not limited by reductions in microbial biomass during long-term soil incubations.

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ABSTRACT

Declining rates of soil respiration are reliably observed during long-term laboratory incubations. However, the cause of this decline is uncertain. We explored different controls on soil respiration to elucidate the drivers of respiration rate declines during long-term soil incubations. Following a long-term (707 day) incubation (30°C) of soils from two sites (a cultivated and a forested plot at Kellogg Biological Station, Hickory Corners, MI USA), soils were significantly depleted of both soil carbon and microbial biomass. To test the ability of these carbon- and biomass-depleted (“incubation-depleted”) soils to respire labile organic matter, we exposed soils to a second, 42 day incubation (30°C) with and without an addition of plant residues. We controlled for soil carbon and microbial biomass depletion by incubating field fresh (“fresh”) soils with and without an amendment of wheat and corn residues. Although respiration was consistently higher in the fresh versus incubation-depleted soil (2 and 1.2 times higher in the fresh cultivated and fresh forested soil, respectively), the ability to respire substrate did not differ between the fresh and incubation-depleted soils. Further, at the completion of the 42 day incubation, levels of microbial biomass in the incubation-depleted soils remained unchanged, while levels of microbial biomass in the field-fresh soil declined to levels similar to that of the incubation-depleted soils. Extra-cellular enzyme pools in the incubation-depleted soils were sometimes slightly reduced and did not respond to addition of labile substrate and did not limit soil respiration. Our results support the idea that available soil organic matter, rather than a lack microbial biomass and extracellular enzymes, limits soil respiration over the course of long-term incubations. That decomposition of both wheat and corn straw residues did not change after major changes

in the soil biomass during extended incubation supports the omission of biomass values
from biogeochemical models.

INTRODUCTION

Following cessation of labile carbon inputs, soil respiration rates decline (Kelly et al., 1997; Conant et al., 2011; Schmidt et al., 2011), whether in the field (as bare fallow experiments; Cambardella and Elliott, 1992; Six et al., 2002) or in laboratory incubations (Conen et al., 2008; Creamer et al., 2011; Haile-Mariam et al., 2008; Liu et al., 2006; Plante et al., 2010). If this decline in respiration is controlled by a reduction of the available soil organic matter (SOM) pool as labile inputs diminish or by a decreased microbial biomass remains uncertain.

The chief objective of this work is to disentangle various drivers that might limit the decomposition of SOM to CO₂ in a laboratory incubation setting. Specifically, this work seeks to understand whether (1) the size of the available SOM pool or (2) microbially mediated depolymerization and respiration of available SOM limits respiration during a long-term soil incubation. Here we define available SOM as organic compounds susceptible to enzymatic depolymerization that are not bound in aggregates or on mineral surfaces.

In their 2008 paper, Kemmitt et al. reported that despite culling up to 90% of the native microbial biomass, there was no significant decrease in soil respiration when soils were incubated under controlled conditions. This was unexpected since the process of SOM respiration to CO₂ is known to be conducted by living microorganisms. The authors of the study suggested that abiotic mechanisms of physical stabilization in microaggregates and chemical associations with clay and silt particles (Six and Jastrow, 2002) release a slow “trickle” of SOM to the available pool. Once SOM enters the available pool, it is depolymerized, assimilated, and respired by an excess supply of

microbial extracellular enzymes and biomass. The authors concluded that the rate of soil respiration during incubations is independent of the size of the microbial biomass pool and is instead limited by physico-chemical drivers.

This finding marks a departure from a body of literature that seeks to understand how changes in microbial activity are reflected in the processes they mediate (e.g. depolymerization and respiration of SOM), and how this in turn drives overall changes in soil respiration rates observed during laboratory incubations. For example, Kuzyakov (2000) described that some increases in soil respiration in response to substrate addition are unexplained by the amount of substrate addition alone. Rather, this additional respiration is the result of a “priming effect”, whereby a labile substrate amendment stimulates a spike in microbial activity, yielding an immediate increase in older, less labile SOM decomposition (Blagodatskaya et al., 2011; Kuzyakov, 2006; Kuzyakov et al., 2009; Kuzyakov, 2010; Neff et al., 2002). Under the priming scenario, the interactions among microbiota, substrate and native carbon determines soil respiration rates.

It is from these divergent conceptualizations of SOM respiration controls that our research emerges. To distinguish the effects of available SOM limitation from reduced microbial biomass on respiration rates, we used soil from a long-term (707 day, 30°C) incubation sufficient to significantly deplete soils of both labile SOM and microbial biomass. A subsequent, shorter-term (double 21-day, 30°C) incubation was used to test the hypotheses that SOM decomposition rates are limited by substrate availability or microbial biomass. To assess whether substrate availability limits SOM decomposition rates, we alleviated potential substrate limitation by adding wheat and corn residues to

soil with fresh and reduced microbial biomass. To test whether a reduction in microbial biomass limits respiration rates, we alleviated potential microbial constraints by resampling the same sites to compare the respiration from the biomass-depleted soils to that of field fresh soils with their field levels of microbial biomass and extra-cellular enzyme pools. This experimental design represents a novel approach to investigating potential mechanisms of soil respiration limitation during laboratory incubations.

METHODS

Establishing different levels of microbial biomass and substrate availability

In late 2007, four soil samples were collected from a cultivated site and three soil samples from a forested site (20 cm-deep cores; dia.=1.8 cm) at the Kellogg Biological Station Long-Term Ecological Research site (Robertson, 1991) (Table 1) for the initial, 707 day incubation (30°C). The cultivated plot was converted from deciduous hardwood forest in the early 1900s and was cropped in a mixed small grain corn–soybean rotation for many years and under alfalfa just prior to 1993, when it was converted to current management: continuous, conventionally-tilled corn. The forested plot has been under forest since at least the late 1800s and is currently a late-successional deciduous hardwood forest. Each site was selected to be broadly representative of common land use types at the Kellogg Biological Station.

After collection, soil samples were transported to the laboratory and stored at 4°C in sterile, plastic bags for roughly one week. Soils were then passed through a 2mm-mesh sieve and large (>2 mm) surface and belowground plant matter was removed. Samples of sieved, air-dried soil were then analyzed for carbonates (none were detected, using a

standard technique of 1M HCl drops to detect effervescence at ambient temperature) and total organic carbon and nitrogen was determined using a LECO CHN-1000 analyzer (LECO Corp., St. Joseph, MI).

The processed soils were then subjected to incubation under constant 30°C temperature and moisture for 707 days. Soil samples (200 g) were placed in 250 mL uncovered glass beakers, which were placed within sealed, half-gallon sized jars. The soils were maintained at 50% water filled pore space throughout the incubation. Air samples from the headspace of the sealed mason jars were drawn through septa, transferred to evacuated vials, and CO₂ concentrations were measured using a Li- Cor LI-6252 (Li-COR Biosciences, Lincoln, NE) infrared gas analyzer.

Carbon dioxide in the headspace of each jar was measured every two to five days at the outset of the incubation, when respiration rates were at their highest. Between days 14-707, samples were taken every 7 to 28 days. Jars were flushed with CO₂-free air after every measurement and lost moisture and worn septa were replenished as needed, roughly six times throughout the incubation (modified from Haddix et al., 2011 and Follett et al., 1997). Incubating the soils for 707 days depleted them of soil carbon (18-20% soil C lost) (Table 1) and reduced microbial biomass.

After 707 days of incubation, all soil samples were removed from the incubator, and subjected to further experimentation (hereafter referred to as “incubation-depleted”). In 2010, we re-sampled the forested and cultivated sites at the Kellogg Biological Station (hereafter referred to as “fresh” soil), processing the samples in the same manner as those collected in 2007.

Substrate amendment experiments

To assess respiration from soils with and without substrate constraints, we added substrate to both incubation-depleted and fresh soil samples (hereby referred to as “amended”). We assessed microbial biomass constraints on respiration from the amended and unamended incubation-depleted and fresh soil samples by measuring respiration concurrent with microbial biomass and enzyme activity midway (Day 21) and at the end (Day 42) of the short-term incubation.

Substrate amendments were added as 600 $\mu\text{g C g}^{-1}$ dry soil of dried and finely ground aboveground wheat or corn tissue. This amount of substrate was equivalent to the amount of microbial biomass C of the fresh, non-depleted soil. Wheat or corn substrate was added to the forested soils, while wheat only was added to the cultivated soil in an attempt to use $\delta^{13}\text{C}$ to track whether or not substrate amendment elicited a microbial priming response, and to trace the origin of respired CO_2 from the substrate amendment versus native SOM.

All soil samples (amended and unamended, incubation-depleted and fresh) were mixed thoroughly by stirring, and then adjusted to 50% water filled pore space and subjected to two consecutive 21 day incubations (30°C). Soil samples (27 g dry-weight equivalent) were placed in 250 mL open glass jars, which were placed within sealed, pint-sized jars with rubber septa sealed in their lids. Approximately 20 mL of water was placed in the bottom of each jar to maintain a humid headspace and 50% water filled pore space. Throughout the incubation, air samples from the headspace of the jars were collected as described above for the 707 day incubation, with a similar frequency (daily for five days and every 2-3 days thereafter). Respiration of the added substrate was

calculated by subtracting the amount of respiration in the control (non-amended) soil samples from the substrate-amended soil respiration, paired by laboratory replicates. Headspace gas samples were also collected at Days 7, 21, 28 and 42 and measured for $\delta^{13}\text{C}$ using a Micromass VG isochrome-EA mass spectrometer (Micromass UK Ltd., Manchester, UK).

After 21 days of incubation, half of the samples were harvested (“Day 21”) and analyzed for extractable carbon with and without a chloroform amendment (we used the difference in extractable carbon between the two as a proxy for microbial biomass) and extra-cellular enzyme activity. We measured chloroform-extractable carbon using a 24 hour chloroform fumigation-extraction method with 0.5M K_2SO_4 (based on methods used by Wu et al., 1990) and potential soil extra-cellular enzyme activity using a fluorescence microplate assay method developed by Steinweg and McMahon (2012). These were measured as indices of microbial biomass and enzyme activity, respectively.

We added a second pulse of substrate ($600 \mu\text{g C g}^{-1}$ dry soil of dried and finely ground aboveground wheat or corn tissue) to the amended samples at Day 21 to assess if a regenerating microbial biomass pool was respiring substrate. Alternatively, if the microbial biomass pool was not regenerating, this assay would allow us to evaluate whether the incubation-depleted extra-cellular enzyme and biomass pools were sufficient to depolymerize and respire an additional round of added substrate. At Day 21, all remaining soils (amended and unamended) were well stirred and returned to the incubation chambers for another 21 days. We continued to measure CO_2 in the headspace of the incubation jars, daily for the first five days (Day 21-25) and then every two to three days for the remainder of the incubation (Day 26-40). At the end of the incubation,

208 samples were analyzed for microbial biomass and extra-cellular enzyme activity (“Day
209 42”).

210 *Investigating the soil extra-cellular enzyme pool using enzyme assays*

211 We investigated the activity of three hydrolytic, carbon-acquisition extra-cellular
212 enzymes: α -glucosidase (AG), β -glucosidase (BG), β -D-cellobiohydrolase (CB); and one
213 nitrogen-acquisition extra-cellular enzyme: N-acetyl- β -d-glucosaminide (NAG) to
214 determine whether decomposition of SOM and the added substrate was controlled by the
215 potential activity of the soil extra-cellular enzyme pool, and whether the size of the extra-
216 cellular enzyme pool was coupled to biomass or substrate availability.

217 Following a protocol developed by Steinweg and McMahon (2012), 1.375 g of
218 soil was blended with 45 mL of 50 mM sodium acetate buffer with a pH of 5.5 in a soil
219 blender (Waring 8011G) on high for 60 seconds. The soil solution was then pipetted into
220 deep-well plates and fluorescently-labeled substrate was added in excess. The deep-well
221 plates containing the soil sample solution and substrate were covered and incubated at
222 30°C for 2 hr 15 min. The fluorescent tags on the substrates only absorb electromagnetic
223 radiation and fluoresce when separated from the substrate, which occurs when extra-
224 cellular enzymes catalyze the depolymerization of the fluorescent tag from the substrate.
225 Using a plate reader, the amount of fluorescence emitted by the incubated slurries was
226 analyzed as an analog for enzyme activity.

227 *Statistical analyses*

228 Four field replicates were collected from the cultivated site and three from the
229 forested site at both sampling dates (2007 and 2010). In the lab, each replicate was split
230 into four equal sub-samples, two of which were given a substrate addition. Half of the

samples were harvested at Day 21, and the other half at Day 42. We used analysis of variance (ANOVA) and repeated measures/within subjects analysis of variance to test the effects of depletion (soil carbon and biomass) and substrate addition on respiration, microbial biomass and extra-cellular enzyme activity. Differences were considered statistically significant for $P < 0.05$.

RESULTS

Cumulative respiration throughout the 42 day incubation was significantly greater in the amended versus unamended soils, and in the fresh versus incubation-depleted soils from both the forested (Fig. 1a.) and cultivated (Fig. 1b.) sites. An exception to this pattern was the forested, amended, incubation-depleted soil, which respired as much as the forested, amended, fresh soil. Respiration from the forested soil in all treatments was much more variable than respiration from the cultivated soil. Respiration was approximately six times higher in the cultivated fresh soil versus the cultivated incubation-depleted soil without a substrate amendment. With a substrate amendment, respiration was roughly twice as high in the fresh soil. In the forested soil this pattern also occurred, but to a lesser degree, with respiration three and 1.2 times higher in the fresh versus incubation depleted soil without and with a substrate amendment, respectively.

Respiration attributed to the added substrate – calculated by subtracting the control (non-amended) soil respiration from the substrate-amended soil respiration – increased between day 21 and 42, but was not significantly different among fresh or incubation-depleted soils from either site at Day 21 or Day 42 (Fig. 2). At Day 21, roughly 62% of the amount of carbon from the added substrate had been respired from

both the incubation-depleted forested soils and the fresh cultivated soils, with 28% and 49% respired from the fresh forested and incubation-depleted cultivated soils, respectively. At Day 42, roughly 65% and 60% of the amount of carbon from the added substrate was respired from the incubation-depleted cultivated and fresh forested soils, respectively, with 50% and 40% respired from the incubation-depleted forested and fresh cultivated soils, respectively.

Chloroform-extractable organic carbon rather than total biomass was used as a surrogate for microbial biomass because we didn't know whether published k values determined for field fresh soils were applicable to the incubation depleted soils. These decreased from field levels over the course of both the 707 and 42 day incubation by an average 60-70% (Fig. 3). Substrate amendments had no effect on the microbial biomass pool size of the incubation-depleted soils, which was not significantly different between Days 21 and 42 and between amended and unamended samples. By the end of the 42 day incubation, microbial biomass in the cultivated soils (Fig. 3b.) was statistically similar between the fresh and incubation-depleted soils. In the forested (Fig. 3a.) fresh soils, microbial biomass was only slightly –but statistically significantly –reduced overall. In the forested incubation-depleted soils, biomass was significantly reduced. There was no significant effect of substrate amendment on the decline in microbial biomass over the 42 day incubation in the fresh soils from either site.

Activity of the three carbon cycling extra-cellular enzymes (α -glucosidase (AG), β - glucosidase (BG), and β -D-cellobiohydrolase (CB)) in both the cultivated and forested soils was sometimes significantly greater in the fresh soils than the incubation-depleted soils, though substrate amendment had no impact on the potential extra-cellular enzyme

activity in the fresh or depleted soils at either Day 21 or Day 42 (Fig. 4). Land use (i.e., cultivated versus native) was the strongest determinant of changes in potential extra-cellular enzyme activity, with much higher NAG activity and generally lower overall activity of the other three extra-cellular enzymes in soil from the forested plots (Fig. 4a.) versus that from the cultivated plots (Fig. 4b.). Variability was also greater in soil from the forested plots. The activity of nitrogen-acquisition extra-cellular enzyme we measured (N-acetyl- β -d-glucosaminide) was slightly greater in the forested plots compared to other studies, but was not affected by any of the other experimental treatments. The type of substrate added (wheat versus corn) had no effect on potential soil extra-cellular enzyme activity, respiration, or microbial biomass.

Physically disturbing the soil through mixing contributed significantly to cumulative soil respiration and increased respiration rates in incubation-depleted soils by about three-fold. After 707 days of incubation we observed a spike in respiration following the mixing and substrate addition to amended soils at Days 0 and 21 (Table 2). The largest relative spike in respiration following disturbance occurred during Days 22-25 in the unamended, incubation-depleted cultivated soil and forested soils ($31.2 \pm 1.2\%$ and $22.6 \pm 5.5\%$ of the cumulative 42 day respiration, respectively). Substrate addition dampened this disturbance effect in the cultivated depleted and fresh soils, where only 15% of total respiration occurred during Days 22-25.

DISCUSSION

Our results show that increased respiration was prompted by substrate addition, with no observable response in microbial biomass. Also, while the long-term incubation

depleted both labile substrate and microbial biomass pool sizes, it did not affect the ability of the remaining microbial biomass to respire a new substrate addition. While respiration was greater in the fresh and the amended soils than in the depleted soils and unamended soils, alleviating substrate limitation through added substrate in the depleted soils yielded respiration rates similar to or even greater than those from the fresh, unamended soils from both the cultivated and forested soils. If microbial biomass limited respiration in our investigation, the depleted biomass pool would have limited respiration of the added substrate. This suggests that SOM decomposition in the two soils we investigated was not limited by the depleted microbial biomass or enzyme pool sizes.

Chloroform-extractable carbon (the index of microbial biomass that we used) and extra-cellular enzyme pools were diminished after 707 days of incubation, but neither was related to the decomposition rates of the added substrate. A similar or greater proportion of the carbon substrate amendment was respired from the incubation-depleted soil in comparison to the fresh soil. Additionally, substrate amendment affected neither the size of the microbial biomass (Fig. 3) nor extra-cellular enzyme pools (Fig. 4). This result shows that the diminished microbial biomass and extra-cellular enzyme pools remaining after 707 days of incubation were sufficient to process added substrate – which was added at levels similar to that available over the course of 42 days under field conditions, without concurrent regrowth in pool sizes. This result is noteworthy because it suggests that a significant decrease of the microbial biomass over the course of long-term incubation did not limit soil respiration. In addition, although the mechanisms driving biomass declines during incubation are not well understood, sample processing and the controlled incubation setting may have changed the variation and quantity of

microhabitats in the soil. This potential loss of microhabitats could explain the significant loss of microbial biomass we observed in the fresh soils over the 42 day incubation.

The ability of the microbial biomass to rapidly decompose substrate added at fairly low but field equivalent levels without growth is not a novel finding. A twelve month study by Calbrix et al. (2007) found that while substrate amendments influenced microbial genomic shifts, there was no meaningful increase in microbial biomass pool size. Additionally, following the 2010 British Petroleum oil spill into the Gulf of Mexico, high microbial respiration of the hydrocarbons was not accompanied by a microbial biomass growth response (Raloff, 2011). This shows that microbial respiratory response to carbon additions is not necessarily linked to microbial biomass growth response.

The wrong substrate type was inadvertently added to three of the four subsets of the forested soil (corn instead of wheat). Because substrate was added on a g C g^{-1} dry soil basis, this had no impact on the overall experiment, but disallowed us from utilizing the $\delta^{13}\text{CO}_2$ to determine priming. This meant we were unable to identify the source of the respired SOM, or detect a potential priming response in the forested soil. In the cultivated soil, we were also unable to differentiate between the $\delta^{13}\text{C}$ signatures of the amended wheat substrate from the pre-1993 alfalfa-derived SOM. This rendered the use of mixing models or an investigation of priming impossible for the cultivated soils. However, we observed no significant effect of substrate type on any response variable we measured, which is likely a reflection of both the general nature of microbial respiration and the similar biochemistry and stoichiometry of the two types of plant material we used.

The levels of potential extra-cellular enzyme activity we observed in both the forested and cultivated soil were comparable to other studies (Bailey et al., 2010;

Trasarcepeda et al., 2007; German et al., 2011; Waldrop and Firestone, 2006). Interestingly, nitrogen acquisition enzyme activity was consistently higher in the forested soils, which could reflect differences in nitrogen demand and availability between the two sites. However, we found no evidence that the extra-cellular enzyme pool had any meaningful effect on respiration in our study. While extra-cellular enzyme pools were sometimes smaller in the depleted soils than in the fresh soils, they did not significantly change in the fresh soils over the course of the 42 days. This pattern of extra-cellular enzyme activity appears to be decoupled from microbial biomass and respiration, and could be explained by extra-cellular enzyme having longer residence times than microbial biomass. Alternatively, the diminished microbial biomass might have been sufficient enough to generate and maintain a large enough pool of carbon-cycling extra-cellular enzymes to ensure that depolymerization of available SOM did not limit decomposition. In this case, the examination of extra-cellular enzymes added little to our understanding of how soil respiration is limited during long-term incubations.

That there was no microbial growth response to substrate addition in the incubation-depleted soil is an interesting result. The size of the microbial biomass pool decreased over the course of both incubation periods, regardless of substrate availability, but remained sufficient to respire the added substrate carbon. This could result from plant residue decomposition being a general microbial process rarely lost from a microbial community even when a significant proportion of that community dies, shrinks, or becomes inactive. It could also be attributable to a shift towards a smaller, old microbial community with an altered life strategy to survive starvation periods by turning over slowly and promoting low substrate utilization efficiency, i.e. substrate is preferentially

used for maintenance and energy (more carbon is respired as CO₂ than stored in biomass) over anabolism and reproduction (when more carbon is fixed as biomass than respired as CO₂) (Steinweg et al., 2008).

The “Exomet Pathway” might be another explanation of our results. In this respiration pathway, intracellular oxidative enzymes released to the soil matrix following cell lysis persist in the soil independently of the microbial biomass, oxidizing SOM directly to CO₂, and bypassing assimilation and respiration by the microbial biomass (Maire et al., 2012).

The large effect of physical mixing on respiration (Gregorich et al., 1989; Rovira and Greacen, 1957) suggest that while available SOM was considerably depleted, spatial isolation protected remaining, older, available SOM stores from microbial attack until physical mixing brought these stores into proximity with extra cellular enzyme and microbiota during the 42 day incubation. This is evidence that spatial proximity/accessibility of the older, available SOM to microbial decomposers and extra-cellular enzymes is an important control on soil respiration limits (Dungait et al., 2012). By mixing the soil, we increased the accessibility of the existing available SOM. Further, the degree of respiration response to mixing reflected the level of substrate depletion and not the size of the microbial biomass. This offers more support for the idea that SOM availability –and not the size of the microbial biomass –limits respiration during laboratory incubations. This also has implications for decomposition kinetics, because the characterization of older SOM as resistant is often attributed to its biochemical complexity. Here, our results show that accessibility played a significant role in preserving SOM during the course of the 707 day incubation.

Decreases in microbial biomass and extra-cellular enzyme pool size occurred in concert with a depletion of labile carbon, despite a lack of evidence that microbial biomass and extra-cellular enzymes were reduced enough to limit respiration of added carbon. Although microbial limitation of respiration will occur at some minimum value of biomass and extra-cellular enzyme pools, we did not appear to reach these minima during our study. Our work points to a reduction in available SOM pools (especially those affected by accessibility) –rather than a depletion of the microbial biomass and extra-cellular enzyme pools –as the rate-limiting step in soil respiration observed during laboratory incubations.

Interdisciplinary biogeochemistry models that include information about the soil system its biota and its soil organic matter dynamics across scales can provide relevant, valuable information to policymakers (Hinckley et al., 2014). Our results show that decomposition of both wheat and corn straw residues did not change after major changes in the soil biomass during extended incubation. Our results support the omission of biomass values in biogeochemical models relevant at policy-level scales. The effect of disturbance on decomposition rates that appeared to have reached steady state after 707 days highlights the need to better understand the role spatial complexity of both labile and more resistant materials (Paul, 2015). Carbon pools that turn over slowly are indeed protected by spatial accessibility under conditions where other factors were held constant.

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LIST OF FIGURES

Fig. 1. Cumulative soil respiration over the 42 day incubation from soils from forested (panel a.) and cultivated (panel b.) land use plots at the Kellogg Biological Station, with varying levels of labile substrate depletion: fresh amended (solid squares), fresh unamended (solid circles), depleted amended (unfilled squares), and depleted unamended (unfilled circles). Values are means $\pm$ standard error.

Fig. 2. Day 42 cumulative soil respiration from the substrate amended soil minus that from the control soils for both incubation-depleted and fresh soils collected from cultivated and forested plots from Kellogg Biological Station. The level of substrate amendment –600 μ g C gram soil at Day 21, and an additional 600 μ g C at Day 42 –is represented by the broken lines. Values are means $\pm$ standard error, and the same letters denote statistically significant similar values.

Fig. 3. Chloroform-extractable organic carbon (here a surrogate for microbial biomass) at Day 21 and 42 from the substrate amended and unamended, fresh and incubation-depleted soils harvested from forested (panel a.) and cultivated (panel b.) plots at the Kellogg Biological Station. Values are means $\pm$ standard error, and the same letters denote statistically significant similar values

Fig. 4. Potential extra-cellular enzyme activity of carbon-acquisition enzymes α -glucosidase ("AG"), β -glucosidase ("BG"), β -D-cellobiohydrolase ("CB"); and one nitrogen-acquisition extra-cellular enzyme: N-acetyl- β -d-glucosaminide ("NAG") in the depleted and fresh amended ("Add") and unamended ("No Add") soil from forested (panel a.) and cultivated (panel b.) plots at the Kellogg Biological Station at Days 21 and 42. Values are means $\pm$ standard error, and the same letters denote statistically significant similar values.

Table 1. Kellogg Biological Station site descriptions, including total % carbon (C) respired after 707 days of incubation at 30°C. Percent carbon values are means $\pm$ standard error. Kellogg Biological Station's mean annual temperature and mean annual precipitation are 9.7°C and 890mm, respectively.

Site	Vegetation	Total C (%)	C:N	C respired after 707d (% of total soil C)
Cultivated	Corn crop	0.76 ($\pm$ 0.03)	12.8	20.34 ($\pm$ 0.64)
Forested	Deciduous forest	1.22 ($\pm$ 0.05)	8.5	18.02 ($\pm$ 1.61)

1 Table 2. The contribution of mixing and substrate amendment to soil respiration rates
2 ($\mu\text{C/g soil/day}$) from the incubation-depleted cultivated and forested soils collected from
3 Kellogg Biological Station following an initial disturbance (following 707 days of
4 incubation) at day 0, and a second disturbance (and second substrate amendment) at day
5 21. Days -35 and -1 are time points during the 707-day respiration prior to the start of the
6 42-day incubation. Values are means $\pm$ standard error.

Treatment	Day -35	Day -1	Day 2	Day 6	Day 20	Day 22	Day 24
Cultivated Unamended	1.06 (± 0.31)	1.22 (± 0.10)	5.14 (± 1.20)	4.16 (± 0.83)	1.38 (± 0.61)	4.67 (± 1.36)	5.73 (± 2.01)
Cultivated Amended	--	--	24.5 (± 11.30)	37.85 (± 2.44)	5.57 (± 1.01)	37.33 (± 3.43)	48.16 (± 1.83)
Forested Unamended	2.51 (± 0.52)	2.42 (± 0.48)	6.92 (± 1.53)	38.69 (± 17.79)	2.35 (± 0.91)	5.88 (± 1.36)	11.5 (± 2.16)
Forested Amended	--	--	22.89 (± 6.86)	71.80 (± 22.76)	2.38 (± 1.23)	35.20 (± 6.82)	48.38 (± 4.96)

7

8

Day 21

Day 42