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Changes in substrate availability drive carbon cycle response to chronic warming

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ABSTRACT

As earth's climate continues to warm, it is important to understand how the capacity of terrestrial ecosystems to retain carbon (C) will be affected. We combined measurements of microbial activity with the concentration, quality, and physical accessibility of soil carbon to microorganisms to evaluate the mechanisms by which more than two decades of experimental warming has altered the carbon cycle in a Northeast US temperate deciduous forest. We found that concentrations of soil organic matter were reduced in both the organic and mineral soil horizons. The molecular composition of the carbon was altered in the mineral soil with significant reductions in the relative abundance of polysaccharides and lignin, and an increase in lipids. Mineral-associated organic matter was preferentially depleted by warming in the top 3 cm of mineral soil. We found that potential extracellular enzyme activity per gram of soil at a common temperature was generally unaffected by warming treatment. However, by measuring potential extracellular enzyme activities between 4 and 30 °C, we found that activity per unit microbial biomass at *in-situ* temperatures was increased by warming. This was associated with a tendency for microbial biomass to decrease with warming. These results indicate that chronic warming has reduced soil organic matter concentrations, selecting for a smaller but more active microbial community increasingly dependent on mineral-associated organic matter.

1. Introduction

Soils are dynamic components of land ecosystems, storing and regulating the exchange of carbon with the atmosphere. Biological processes in soils are accelerated by warmer temperatures (Lu et al., 2013; Salwa Hamdi, 2013; German et al., 2012), but the net effect of warming on soil carbon stocks over decades to centuries of climate change is less clear (Todd-Brown et al., 2014). At present, gaps in our understanding of how temperature, microbial communities, and soil carbon stocks interact to drive the global carbon cycle prevents better constraint of models of future carbon cycling and climate (Wieder et al., 2013).

Differences between long- and short-term responses to warming remain a major unknown in predicting how soil carbon stocks will be affected by climate change (Bradford et al., 2008; Knorr et al., 2005; Melillo et al., 2002, 2011). Soil microbes both drive and respond to changes in the soil environment by producing extracellular enzymes (Sinsabaugh, 1994) and organic acids (Keiluweit et al., 2015; Clarholm et al., 2015), and metabolizing small soil carbon compounds. In the short run, elevated temperatures are known to augment both resource supply to (German et al., 2012) and maintenance costs on microbes (Frey et al., 2013; Manzoni et al., 2012). In the long term, warming may also indirectly influence the supply and demand of resources by altering the quantity and quality of carbon available to microbes (Lu et al., 2013). Proposed modulators of the long-term soil carbon response to warming include altered vegetation dynamics (Dukes et al., 2005; Sistla and Schimel, 2013; Zhang et al., 2005), shifts in microbial communities (DeAngelis et al., 2015; Luo et al., 2013),

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accelerated microbial turnover (Hagerty et al., 2014), and preferential depletion of specific soil carbon pools (Feng et al., 2008; Pisani et al., 2015; Bradford et al., 2008).

An additional regulator of long-term response of the carbon cycle to warming is physical protection (Davidson and Janssens, 2006; Bach and Hofmockel, 2016). As plant litter decomposes, it goes from being primarily protected by complexity of molecular structure (e.g., aromatic rings of lignin compounds), that are difficult for microbial enzymes to attack (Schmidt et al., 2011; Conant et al., 2011), to being primarily physically protected by virtue of tight associations with minerals (Six et al., 2000; Schmidt et al., 2011). During the transition between these two phases, microbes and invertebrates increasingly fragment the litter (particulate organic matter; "POM"), covering it with binding agents such as glomalin proteins (Wright and Upadhyaya, 1998), extracellular polysaccharides (Schimel and Schaeffer, 2012) and mucus (Bossuyt et al., 2005) that attract minerals to its surface and may eventually preclude access by other microbes (Six et al., 2000; Grandy and Robertson, 2007). Experimental warming has been shown to affect both these stages of the physical protection process, accelerating the physical and chemical fragmentation of litter (Pisani et al., 2015; Feng et al., 2008; German et al., 2012) and reducing the extent and stability of soil aggregate formation (Rillig et al., 2002). Sorption to mineral surfaces can also physically-protect byproducts of microbial metabolism such as small molecules, proteins, and lipids from microbial degradation (Schurig et al., 2013; Kleber et al., 2007; Grandy and Neff, 2008). Warming is expected to shift the sorption-desorption balance for the most strongly-bound mineral-associated organic matter (MAOM) toward desorption (Conant et al., 2011), providing a further mechanism for increased microbially-available carbon under climate warming.

Changes in microbial substrate availability in response to chronic warming may also alter metabolic investment in resource acquisition. Microbes produce and secrete extracellular enzymes to depolymerize soil compounds into monomers and oligomers that can be taken up by the cell (Wallenstein et al., 2010). These enzymes are costly to produce (Allison, 2005; Schimel and Weintraub, 2003; Lever et al., 2015), which means extracellular enzyme activity can be the rate-limiting step in the mineralization of soil organic matter (Bengtson and Bengtsson, 2007; Burns et al., 2013), and that enzymes are generally produced proportionate to microbial nutritional requirements (Sinsabaugh et al., 2009). For instance, increased plant litter inputs to soil in response to 8 years of warming in a grassland ecosystem were accompanied by a substantial decrease in lignolytic enzyme activity and expansion of genes for hemicellulose degradation (Zhou et al., 2012).

Soil warming experiments such as the one at the Harvard Forest in Massachusetts provide an opportunity to evaluate how long- and short-term effects of climate warming may interact to drive changes in the forest soil carbon cycle. Established more than 20 years prior to the present study, the Prospect Hill warming experiment (Peterjohn et al., 1994) enables the study of the decadly-cycling soil carbon pools believed to be the most susceptible to climate change (Hopkins et al., 2012). In this experiment, microbial activity has shown a non-linear response to warming in which soil respiration showed the expected instantaneous increase with warming over the first ten and last six years of the experiment (Melillo et al., 2002; unpublished data), but not the intervening period. During the interval in which soil respiration was similar in heated and control plots, soil carbon stocks (Bradford et al., 2008) and microbial biomass (Bradford et al., 2008; Frey et al., 2008) were noted to be depleted in heated plots, while the communities also showed reduced metabolic potential and lower mass-specific respiration at higher temperatures. These characteristics of a long-term response to warming were attributed to a combination of reduced substrate availability for microbes and adaptation of the microbial community to a lower respiratory state (Bradford et al., 2008). The subsequent secondary increase in soil respiration coincided with a substantial shift in the dominant bacterial community (DeAngelis et al., 2015), and a shift toward classic oligotrophic taxa (Fierer et al., 2007), or microbes suited to slow but steady growth on small, low-quality carbon pools. Meanwhile fungi - considered the major decomposers of fresh litter inputs in forest soils (Boer et al., 2005) - remained less abundant in heated plots (DeAngelis et al., 2015). This may indicate that the microbial communities are accessing carbon pools they did not previously, whether due to novel metabolic capacities within the community (Pold et al., 2015) or due to changes in the substrates available to them.

In this study, we evaluated whether changes in soil carbon due to warming may feed back and alter the ways in which microbes process soil carbon. We studied the chemical composition and physical location of soil organic matter to determine if substrates available to microbes had been enhanced by warming, and assayed extracellular enzyme activity to determine if changes in substrate availability had altered resource allocation to soil organic matter processing. We show that chronic warming has left its mark on the size and chemical composition of soil organic matter pools, and that thermodynamics are central to explaining continued soil carbon loss.

2. Materials and methods

2.1. Experimental design

2.1.1. Study site

Soils collected for this project were taken from the Prospect Hill warming study at the Harvard Forest Long Term Ecological Research Site in Petersham, Massachusetts (42.54°N, 72.18°W). Here, soils have been heated 5 °C above ambient soil temperatures since 1991 using buried resistance cables controlled by a datalogger which adjusts temperature every 10 min (Peterjohn et al., 1994). The experiment is situated in an area of the forest which was abandoned from agriculture in the late-1800's (Peterjohn et al., 1994), and a plow layer is apparent in the top 10 cm of the mineral soil. The secondary forest regrowth is dominated by red oak (*Quercus rubra*), paper birch (*Betula papyrifera* M.), red and striped maple (*Acer rubrum* L., *A. pennsylvanicum* L.), and white ash (*Fraxinus americana* L.). The understory consists of clubmoss (*Lycopodium obscurum*), wintergreen (*Gaultheria procumbens*), Canada mayflower (*Maianthemum canadense*), and starflower (*Trientalis borealis*) (Farnsworth and Bazzaz, 1993).

Soils are sandy loam Typic Dystrudepts of the Gloucester series (Peterjohn et al., 1994), with approximately 2860 g C m^{-2} soil in the top 10 cm. Atop this mineral soil sits a readily-distinguished mat of organic matter with approximately 2570 g C m^{-2} (DeAngelis et al., 2015). The pH is 3.8 in the organic horizon and 4.4 in the upper mineral soil. Precipitation is distributed approximately evenly throughout the year, with an average of 1180 mm/yr since 1991. Mean monthly temperatures range from −6 °C in January to +20 °C in July (Boose and Gould, 1999; Boose, 2014), with an annual mean of 8 °C. The experimental design consists of eighteen 6 m by 6 m plots in a randomized block design. Each block contains a heated plot, a disturbance control plot where cables have been buried since the beginning of the experiment but not turned on, and a control plot, which has been left undisturbed. We used both organic (O horizon) and upper mineral soil (Ah horizon) samples from all six replicate heated and disturbance control plots unless otherwise noted.

2.1.2. Soil collection

Soils for enzyme assays were collected in on April 28th, June 3rd, June 30th, August 25th, September 22nd, and October 28th 2014 (Day of Year (DOY) 118, 154, 181, 237, 265, and 300, respectively, in the 24th year of the experiment). Two to three 9–10 cm deep soil cores were collected in a 1 × 1m subplot per plot (depending on rock locations and yield) using a 1.27 cm diameter tubular stainless steel soil corer cleaned with 70% ethanol prior to each plot. This depth was chosen to be consistent with previous sampling efforts at our site (Peterjohn et al., 1994; Melillo et al., 2002). A preliminary analysis showed that soil organic matter concentration was similar in the bottom 4 cm of cores from both treatments despite differences in the mean thickness of the organic horizon (25 mm heated vs. 42 mm control, Fig. S1). Pairs of researchers collecting soil cores alternated between heated and control plots to minimize human bias in soil horizon separation. Cores were separated into organic horizon and mineral soil by eye and each horizon was homogenized by hand (unsieved) in the field before being subdivided and put into two Whirl-Paks. One Whirl-Pak was transported to the lab at ambient temperature, and the other which was flash-frozen in a dry ice-ethanol bath (frozen within 10 min of collection) and transported to the lab on dry ice. Enzyme assays and gravimetric percent moisture determination were initiated on fresh soils within 4 h of collection. All soil from the April 28th timepoint (DOY 118) was flash-frozen in the field, so data from enzyme assays are not reported. Gravimetric soil moisture in soil cores collected in 2014 can be found in Fig. S2.

A second round of soil core collection was initiated on July 31st 2015 to (1) determine if warming treatment had affected Q_{10} of extracellular enzyme activity, and (2) evaluate the hypothesis that warming has induced translocation of organic matter from the organic horizon into the mineral soil (Pold et al., 2016). Soils used to assess the quantity of occluded vs. free particulate organic matter (POM), soil organic matter age, and for enzyme Q_{10} determination were collected as a pair of 9–10 cm deep by 1.27 cm diameter cores on July 31st 2015. The organic horizon from both cores was pooled and the mineral soil of one core was used for enzyme assays, enabling us to get temperature response curves on soils collected in a manner similar to the extracellular enzyme activity data in 2014. These soils were transported at ambient temperature to the lab and then immediately assayed for extracellular enzyme activity. While the core was still intact in the field, the mineral soil of the other core was split at 3 cm from the top to be used for physical protection and stable isotope analyses. We elected to look only at the top-most portion of the mineral soil because any increase in translocation of organic matter from the organic horizon with warming should be most apparent in the uppermost mineral soil. Using a consistent depth of mineral soil ensured that when we compared heated and control plot samples for these analyses, the soil samples were of an equivalent depth and horizon. These soils were transported to the lab at ambient conditions and subsequently air-dried.

Soils for total lipid phosphates determination (all dates in 2014) were kept at −80 °C. Soils for stable isotope analysis (DOY 212 2015), C:N analysis (DOY 300 2014) and pyrolysis GC/MS (DOY 300 2014) were dried at 65 °C. Gravimetric moisture content (all dates starting DOY 154 2014) was determined using soils dried to constant mass at 105 °C.

2.2. Soil analyses

2.2.1. Pyrolysis GC/MS

Soils from October 2014 were ground to a fine powder by vortexing horizontally on high for 1hr with five 5 mm stainless steel balls (VWR mini vortexer model #9453000 with MoBio adaptor #13000-V1-15). Samples were packed into quartz tubes with quartz wool, and flash pyrolyzed at 600 °C for 20 s on a CDS Pyroprobe 5150 pyrolyzer connected to a Thermo Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Austin, TX, USA) in line with an ITQ 900 mass spectrometer (Thermo Fisher Scientific). Peaks were analyzed with AMDIS software populated with a compounds library (1000 plant and soil moities) developed with the National Institute of Standards Library (NIST) and published literature (Grandy et al., 2007, 2009; Wickings et al., 2011). We summed the relative abundance of odd-chain alkanes C25-C35 (Abelenda et al., 2011) and n-Dotriacontane (C32) (Baker and Bishop, 2004) to evaluate the relative contribution of plant lipids to our samples; sterols with 4 rings and 27–30 carbon atoms (Abelenda et al., 2011) and C20-C28 dicarboxylic acid methyl esters (Chefetz et al., 2002) have also been suggested as markers of plant lipids in pyrolysis, but were not identified in any of our samples. A summary of the most abundant compounds can be found in Table S1.

2.2.2. CN and δ15N analyses

C:N analysis was completed on 2–5 mg organic horizon sample and 5–10 mg mineral soil sample in tin cups on a Costech ECS140 CHN/S/O analyzer against an acetanilide standard. Loss on ignition was completed by measuring mass loss of 105°C-dried soils following 4 h at 550 °C (DOY 154, 181, 237, and 265 2014). Samples collected from the top 3 cm of mineral soil in July 2015 were air-dried, ground in a pestle and mortar, and submitted to the UC Davis Stable Isotope Facility for δ13C and δ15N analysis. Standards used were bovine liver, USGS-41 glutamic acid, nylon 5, and peach leaves. ^{13}C data are presented relative to Vienna PeeDee Belemnite, and ^{15}N data is presented relative to air.

2.2.3. Density fractionation

The top 3 cm of mineral soil from July 31st 2015 was separated into POM and mineral-associated SOM using an adaptation of the microaggregate separation protocol of Six et al. (2000). Air-dried soils were sieved through a 2 mm sieve, and 2 g of this was placed on a 20 cm diameter 250 μm mesh sieve and submerged in 800 ml deionized water. Two hundred 5 mm spherical glass beads were added, and the submerged sieve was shaken at 80 rpm for 5 min to release microaggregates from macroaggregates. The material remaining on the sieve was coarse POM (cPOM), while the flow-through was clay, silt, fine POM and microaggregates. The flow-through was passed through a 53 μm sieve 3 × 1 min by shaking at 50 rpm. The clay fraction was filtered collected on a Whatman #1 filter paper and dried at 65 °C to constant mass. The >250 μm fraction was captured on a Whatman GF/F filter (0.7 μm opening), and then separated into mineral and particulate organic matter with 1.85 g/cm^3 sodium polytungstate (Lajtha et al., 2014) followed by filtering through a GF/F filter. The 53–250 μm fraction was treated similarly, and the resulting pellet was disrupted by shaking with five 5 mm glass beads at 150 rpm overnight. At each step, the density of the supernatant was checked to be within 0.01 g/cm^3 of the desired value, and the sample on each filter was rinsed with distilled water until the density of the eluent reached 1 g/cm^3. All density fractionation steps were repeated twice to ensure complete separation of mineral associated and particulate organic matter. Soil fractions were dried at 65 °C and weighed, and the mass percent of each fraction was calculated as the weight of the soil fraction of interest divided by the total recovered soil mass (mean 95.4 ± 1.74% recovery). All fractions except for free clay were subsequently ground and analyzed for total C and N using elemental analysis. The total amount of carbon in each soil fraction was determined by multiplying the mass percent carbon based on elemental analysis for that fraction by the fraction of total soil mass recovered that was in that fraction. Nitrogen content was calculated similarly.

2.3. Microbial activity

2.3.1. Microbial biomass

Microbial biomass was determined as total lipid phosphate (P). Soil masses of 0.3 g (organic) to 1 g (mineral) were extracted for 12 h in duplicate using a monophasic 2:1:0.8 methanol:-chloroform:citrate buffer (0.15M pH 4) solution (Findlay et al., 1989; Frostegard et al., 1991). Lipids were digested alongside a β-glycerophosphate standard (EMD Millipore 35675) using saturated potassium persulfate at 95 °C for 36 h. Phosphate was quantified with malachite green (D'Angelo et al., 2001), with the digestate kept hot during ammonium molybdate addition in order to minimize rebinding of phosphate to organic compounds (Frostegard et al., 1991).

2.3.2. Enzyme assays

We assayed the potential activity of five hydrolytic and two oxidative enzyme families commonly produced by microbes to break down plant litter. The hydrolytic enzymes β-glucosidase (BG) and cellobiohydrolase (CBH) target bonds in cellulose, the most abundant polymer on earth. β-xylosidase (BX) targets hemicellulose, which is intermingled with and protects cellulose. Acid phosphatase (PHOS) attacks phosphomonoester bonds and unlinks phosphate from biomolecules such as phospholipids and nucleic acids. β-N-acetyl-glucosaminidase (NAG) is responsible for helping break down chitin, which exists in the cell walls of fungi and exoskeletons of arthropods. The two oxidative enzyme families assayed, phenol oxidase (PO) and peroxidase (HPO) non-specifically attack a variety of bonds in lignin and other heterogeneous compounds.

To complete the assays, approximately 1 g fresh soil was suspended in 125 ml 50 mM pH 4.7 sodium acetate buffer to form a slurry using a Waring blender set on high for 1 min. Hydrolytic enzymes were assayed using 200 μl of this slurry and 50 μl of a 100 μm methylumbelliferyl-linked substrate solution (4-Methylumbelliferyl β-D-glucopyranoside for BG, 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide for NAG, 4-Methylumbelliferyl phosphate disodium salt for PHOS, 4-Methylumbelliferyl β-D-cellobioside for CBH, and 4-Methylumbelliferyl-β-D-xylopyranoside for BX, all from Sigma). These plates were read immediately following substrate addition and again after 2, 4, and 6 h of incubation at 25 °C, using an excitation-emission wavelength pair of 350/450 nm on a Molecular Devices Spectramax M2 platereader. We subtracted a substrate only blank and a soil slurry only blank from the fluorescences and then used the difference in fluorescence between the initial and final timepoints to determine potential activity. We used the 2 h timepoint as our T_{final} for NAG and PHOS, and the 4 h timepoint for the remaining hydrolytic enzymes, the time periods over which a preliminary assessment indicated activities were maximal and linear. The oxidative enzyme families phenol oxidase and peroxidase were assayed using a 1:1 ratio of soil slurry:25 mM substrate (L-DOPA for PO and L-DOPA+0.3% hydrogen peroxide for HPO). Because of the read-through colorometric nature of the assay ($\lambda = 460$ nm) and the presence of heterogeneous soil debris in assay plates, we transferred 50 μl aliquots of a spun-down 100 μl aliquot of the assay solution to a clear polystyrene plate for reading after 2, 4, 6, 16, and 24 h. We used the 2 h reading for PO and 6 h reading for HPO, as this is the timeframe over which activity was maximized. Sixteen replicate wells were assayed for all enzymes. An extinction coefficient of 7.9/μmol (DeForest, 2009) was assumed, and HPO activity was calculated as the difference between wells with and wells without hydrogen peroxide addition. Data were standardized per ng lipid phosphates, or per gram dry weight soil where soil was dried to constant mass at 105 °C. C:N, C:P, and N:P potential activities were calculated as the ratio of sums of hydrolytic enzymes (ex: BG+BX+CBH:NAG for C:N ratio.

2.3.3. Predicting activities at in-situ temperatures

In order to predict maximum enzyme activity potential at temperatures observed in the soil at the time of sampling, we incubated soil slurries from July 2015 at 4, 14, 25, and 30 °C. These temperatures cover the range experienced by soils in the field. All processing of samples for slurry preparation was completed at 25 °C, and therefore plates were acclimated to the assay temperature for 1hr prior to taking an initial reading. To predict enzyme activity rates at temperatures in the warming plots ("*in-situ* temperatures"), we assumed temperature sensitivity of V_{max} was stable over the growing season; this appears to hold true for deciduous stands at the Harvard Forest (Drake et al., 2013).

Temperature response curves for the July 2015 samples were completed by fitting the Arrhenius equation to the data for each enzyme. Temperature sensitivity (Q_{10}) was calculated for both 4–14 °C and 14–25 °C. Since extracellular enzyme activity varied seasonally in our data, we determined potential activity at *in-situ* temperatures using a curve of relative change in activity with temperature rather than absolute change. We used soil temperatures collected at 10 cm depth by the datalogger controlling soil heating cables for *in-situ* soil temperatures. Then, starting with the enzyme activity observed at 25 °C for each timepoint, we interpolated potential extracellular enzyme activity at this reference temperature. Finally, we used Q_{10} between 14 and 25 °C, the temperature range at which most of the soil temperatures during the observational period stood, to predict potential activity at *in-situ* temperatures (Wallenstein et al., 2009). These calculations can be summarized as:

$$A_{25DOY\ X} = A_{25DOY\ X-1} + \frac{A_{25DOY\ X+1} - A_{25DOY\ X-1}}{DOY\ X - 1 - DOY\ X + 1} \quad (1)$$

$$A_{\mathrm{TDOYX}} = A_{25\mathrm{DOYX}} - (soiltemperature - 25°C) * A_{25\mathrm{DOYX}} * Q_1 0 \quad (2)$$

where A_{25DOYX} = Activity at 25 °C on DOY X, $A_{25DOYX+1}$ = Activity at 25 °C on DOY first measured after DOY X, $A_{25DOYX-1}$ = Activity at 25 °C on DOY first measured before DOY X, DOYX-1 = DOY enzyme activity was measured on prior to DOY of interest, DOYX+1 = DOY enzyme activity was measured on after to DOY of interest, A_{TDOYX} = Activity at soil temperature on DOY X.

2.4. Data analysis

All data analysis was completed in R v.3.2.2 (R Core Team, 2015) using the RStudio interface v. 0.98.507 (RStudio Team, 2014). The effect of warming treatment and sampling day for each potential extracellular enzyme activity and microbial biomass were assessed using a backwards model selection method (Zuur et al., 2009) using the functions gls and lme in nlme v. 3.1–128 (Pinheiro et al., 2014). This approach enables identification of the simplest model necessary to explain the observed results, and allows for the inclusion of both fixed and random effects (Zuur et al., 2009). Because of large and well-established biological (DeAngelis et al., 2015; Cardenas et al., 2015; Baldrian et al., 2012) and physical (Lajtha et al., 2014) differences, organic horizon and mineral soil samples were analyzed separately. Block was initially included as a random effect, and removed if it did not improve model fit based on a likelihood ratio test of REML estimates (Contosta et al., 2011). Variance structures and autocorrelation were evaluated for inclusion based on corrected Akaike's Information Criterion, which enabled us to

account for lack of independence in data collected from repeatedly sampling the same plots only where doing so improved model fit sufficiently to account for small sample sizes and for the overfitting penalty associated with the additional parameter. Data were adjusted to fit parametric assumptions using log transformation, and model fit was visually confirmed using residual plots. Where residual plots and qqplots showed poor model fit and non-normal data, outliers were identified and removed based on their standardized residuals exceeding the 99^{th} percentile of the standard normal distribution (0–1 datapoints per analysis). The effect of warming treatment on potential enzyme activity at individual time points was subsequently analyzed using Wald tests for generalized linear mixed models in contrast v. 0.19 (Kuhn et al., 2013). Slopes of Arrhenius plots were compared between heated and control plot samples for each soil horizon and enzyme using ANOVA. Since values of percent carbon, nitrogen, loss on ignition, compound classes, and individual compounds from py-GC/MS in soil are constrained on the interval zero to one, these variables were analyzed using a beta regression modified to include repeated measures where appropriate (Cribari-Neto and Zeileis, 2010; Stasinopoulos and Rigby, 2007). Percent differences in percent loss in ignition between heated and control plots, for example, were calculated as the difference between mean values in heated and control plots divided by the mean value in control plots.

3. Results

3.1. Microbial biomass

Microbial biomass averaged over the collection period was reduced by warming treatment, with total lipid phosphates being on average 29.9% lower in heated than control organic horizons ($p = 0.030$), and 21.7% lower in heated mineral than control soils ($p = 0.059$). Warming treatment reduced microbial biomass at individual time-points only in the organic horizon in late September and October (DOY 265 and 300) (Fig. 1).

3.2. Soil chemistry

In the organic horizon, warming treatment led to a non-significant 15.0% reduction in carbon concentration, 11.5% decrease in nitrogen concentration, and a 3.4% decrease in C:N ratio ($p > 0.3$ in all cases). In the full depth of cored mineral soil in late October (DOY 300), warming decreased carbon concentration by 22.9% (Wald test $z = 2.78$, $p = 0.005$) and nitrogen by 26.9% ($z = 2.43$, $p = 0.015$) per gram of soil. There was no effect on the C:N ratio. However when looking at soils collected throughout the sampling period, warming reduced soil organic matter concentration by a third in the organic horizon and by a sixth (14.5%) in the mineral soil (Fig. 1A).

No effect of warming on soil organic matter compound classes (lipids, lignin, proteins, polysaccharides, N-bearing, phenolic, or aromatic compounds) was detected by py-GC/MS in the organic horizon (Fig. 2), although n-Hexadecane, a minor component of the lipid pool, was present at significantly greater relative abundance in heated plots (89.1% greater, Benjamini-Hochberg corrected $p < 0.05$). Likewise, the relative abundance of plant-derived lipids was unaffected by warming treatment ($z = -0.11$, $p = 0.92$). In the mineral soil, warming increased the relative abundance of lipids by 34.3% (beta glm, Wald $z = 2.49$, $p = 0.013$) and decreased the relative abundance of lignin and polysaccharides by 53.3% ($z = 2.17$, $p = 0.030$) and 23.1% ($z = 2.40$, $p = 0.017$), respectively (Fig. 2). Looking at the relative abundance of specific chemical derivatives in the mineral soil, only 1,3-dimethylbutyl benzene (90.7% decrease) and ethenyl oxirane (113% increase) were significantly affected by warming treatment. Plant-derived lipids were not detected in mineral soil samples.

Stable isotope analysis showed the top 3 cm of the mineral soil was less enriched in heavy nitrogen in heated plots (3.1 ± 0.5‰ se) than in control plots (4.4 ± 0.1‰ $t(5.7) = 2.50$ $p = 0.048$). The isotopic signature of carbon was unaffected (−27.7 ± 0.1‰ in heated vs. −27.5 ± 0.2‰ in control, $t(10) = 1.23$ $p = 0.26$).

3.3. Physical protection of soil organic matter

Using soils collected from the top 3 cm of mineral soil on late July (DOY 213) 2015, we evaluated the hypothesis that warming had reduced the degree to which soil organic matter was protected by separating free and occluded particulate organic matter pools (fPOM and oPOM, respectively). These free and occluded pools are thought to respectively be susceptible and protected from microbial activity. Warming treatment did not affect the relative mass of occluded (physically protected) and free particulate organic matter, but did decrease the relative mass fraction of microaggregates

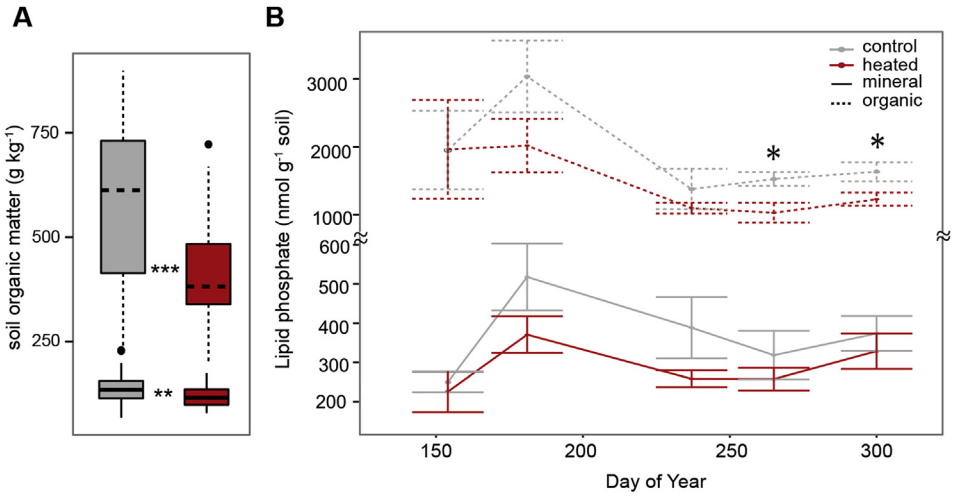

Fig. 1. A. Effect of warming treatment on soil organic matter concentration (percent mass loss on ignition) as evaluated using a beta-GLM for soils collected at all time point. B. Warming effect on microbial biomass (total lipid phosphates) per gram of dry soil. Means are plotted with standard errors. Significance for individual timepoints was evaluated using Wald-tests, while an overall effect was evaluated using a generalized linear mixed model. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

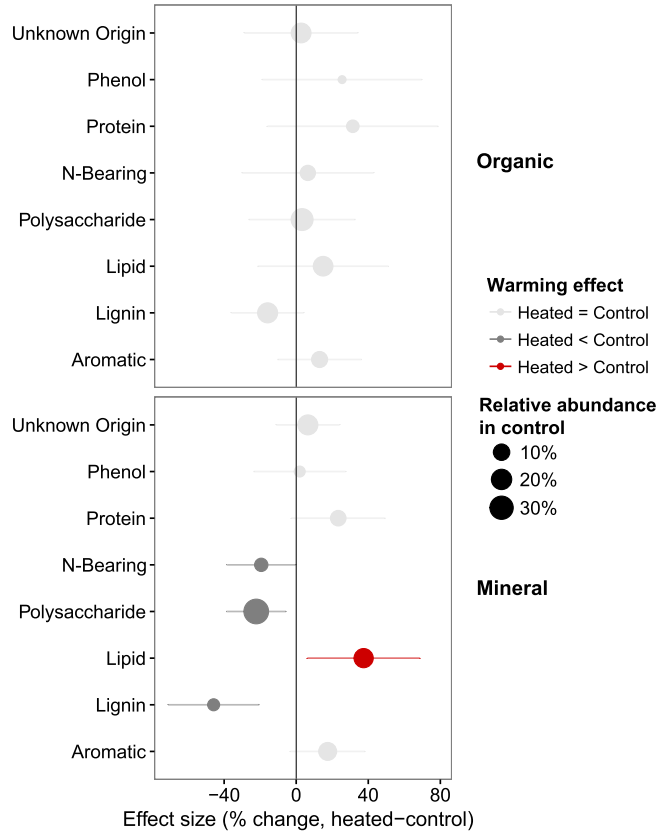

Fig. 2. Change in relative abundance of different compound classes identifiable by pyrolysis GC/MS in soil samples collected in October 2014. Error bars denote 95% bootstrapped confidence intervals on the mean ratio (n = 6). Significance was evaluated on relative percent abundances using a beta regression at $p < 0.05$.

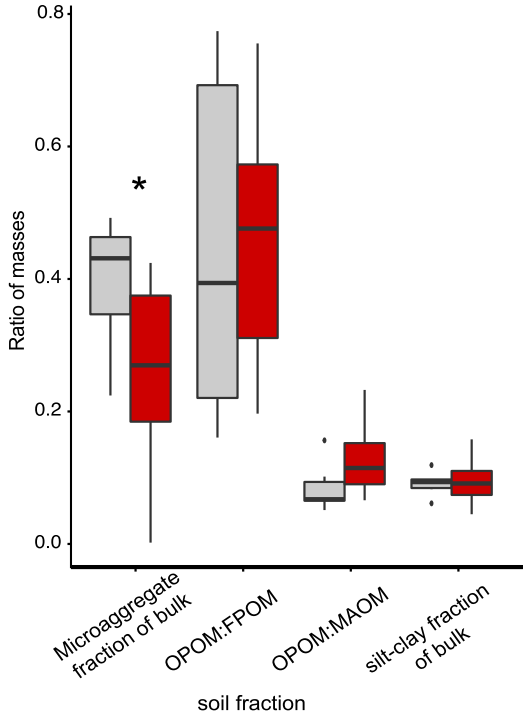

Fig. 3. Relative mass abundance of soil fractions as a fraction of bulk soil or in relation to other soil fractions. FPOM = fine particulate organic matter; MAOM = mineral-associated organic matter; OPOM = microaggregate-occluded particulate organic matter. Significance of ratios was evaluated with a beta regression: *$p < 0.05$.

(Fig. 3). Warming treatment preferentially depleted mineral-associated organic matter, reducing the concentration of carbon and nitrogen (g C or N per g soil) by 36.3% and 27.6%, respectively (Figs. S3B and C). This corresponded to a soil mass fraction corrected decrease of 44.5% for carbon (Fig. S3E; g C per g soil fraction multiplied by g soil fraction per g soil) and 37.5% for nitrogen (Fig. S3F). However, the concentration and relative mass fraction of carbon stored in occluded vs. free particulate organic matter were not affected by warming treatment (Figs. S3B and E). The C:N ratio of organic matter was significantly reduced by warming treatment only in the mineral-associated organic matter (13.7% reduction; Fig. S3A).

3.4. Potential extracellular enzyme activity

Warming treatment reduced NAG activity by 34.1% and PHOS activity by 22.5% in the organic horizon, and HPO by 39.7% in the mineral soil (Fig. 4A, Table 1). There was no effect of warming treatment on the N:P (NAG:PHOS), C:P (BG+BX+CBH:PHOS), or C:N activity ratios (BG+BX+CBH:NAG). Potential activity of all enzymes per gram of soil was greater in organic than mineral soil and showed a strong seasonal trend. Seasonal patterns could be divided into three groups: those for which potential activity decreased over the measurement period (BX, CBH, PHOS), those which peaked in late June (DOY 181 - BG, HPO), and those which showed two seasonal peaks (NAG). C:N and C:P activity ratios peaked in late June (DOY 237). Phenol oxidase activity was very low and could not be detected in some samples, particularly in late September (DOY 265). Therefore, we did not include it in subsequent analyses.

To predict *in-situ* potential rates, we generated temperature sensitivity curves for extracellular enzyme activity measured over a range of temperatures that occur in warmed and control plots in the field. The coefficient of variation for the slopes of these curves were between 5.9 and 8.4% in all cases. Using t-tests to compare slopes of an Arrhenius equation fit to our data, we found no change in the temperature sensitivity of enzyme activity with chronic soil warming, with activation energies averaging 36 kJ/mol over all enzymes in the organic horizon and 43 kJ/mol in the mineral soil (Fig. S6). We observed no effect of warming treatment on activation energy, which was confirmed with soils collected on April 28th (DOY 118 2014) and stored at −80 °C for six months prior to assay (data not shown). Predicted potential β-xylosidase activity per gram of soil at *in-situ* temperatures was estimated to be 29.3% higher in heated mineral than control mineral soils for those days we measured it, while cellobiohydrolase activities were projected 34.0% higher. Phosphatase was similarly increased 23% by warming treatment in the mineral horizon, driven by an increase in late June and October (DOY 181, 300). Peroxidase activity was reduced by 49.7% in the mineral soil, with the warming effect apparent on all sampling days except early June (DOY 154) (Fig. 4B and Fig. S5C). β-glucosidase activities were greater in heated than control mineral soils only in early June (DOY 154; Fig. S5C). Based on integrating the area under the curve in Fig. 4B over the course of the assayed period, potential BG, BX, CBH and PHOS activities are expected to be 0.87, 1.12, 1.08, and 0.95 times as high in heated than control plot organic soils, and 1.12, 1.19, 1.16, and 1.19x as high in heated than control plot mineral soils.

To evaluate whether microbial communities may allocate more resources to biopolymer degradation in the chronically warmed plots, we also evaluated potential extracellular enzyme activity per unit microbial biomass ("specific activity", Fig. S5B, Table 1). Warming increased specific activity of β-xylosidase by an average of

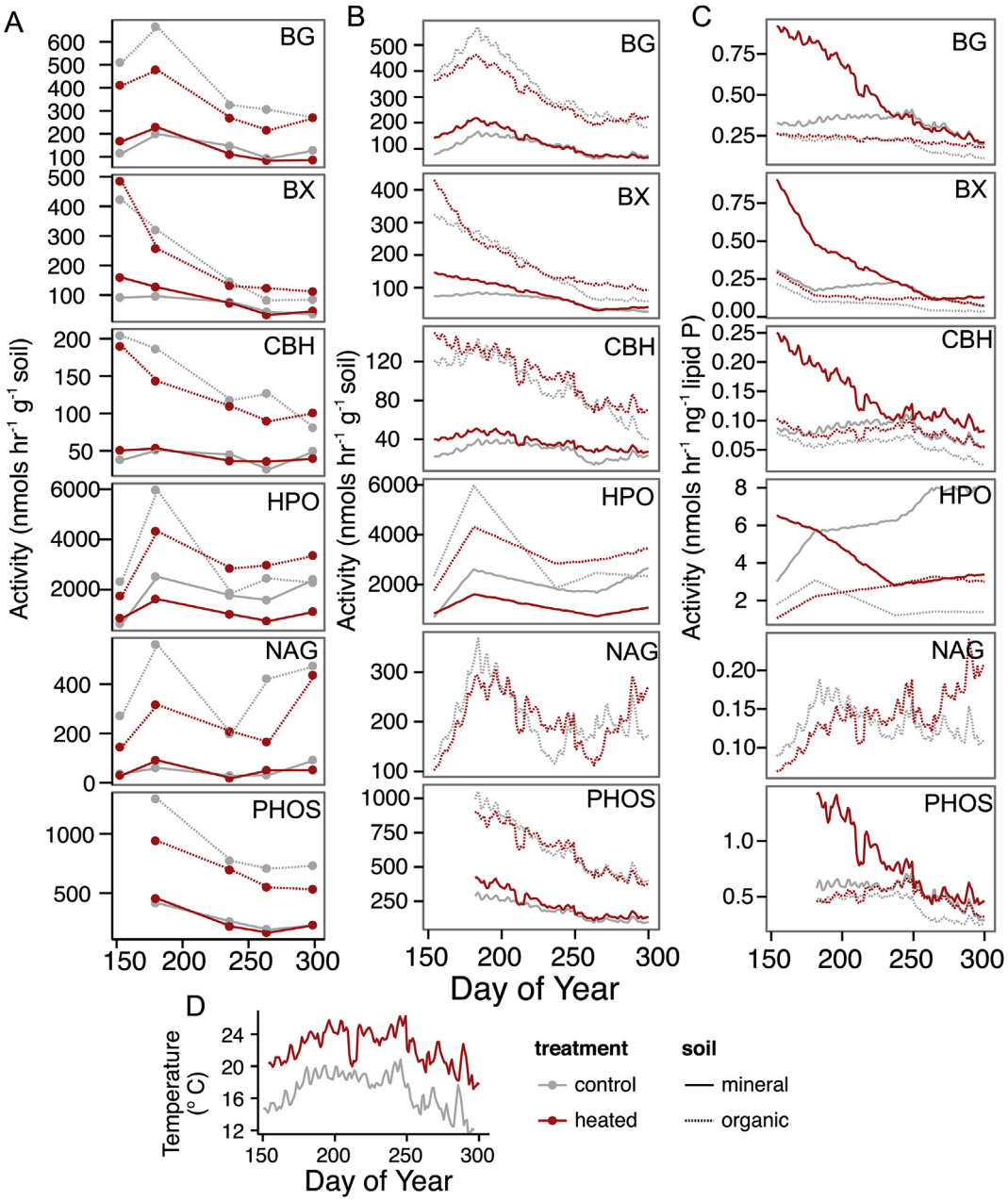

Fig. 4. Observed and predicted *in-situ* enzyme activities. Observed potential extracellular enzyme activities per gram of soil (A) assayed at 25° C. Predicted extracellular enzyme potential per gram of soil (B) and per unit microbial biomass (C) at *in-situ* soil temperatures over the course of the 2014 growing season. Soil temperatures (D) are measured at 10 cm depth. The statistics associated with the data can be found in Table 1, and figures with error bars can be found in Fig. S5. BG = β-glucosidase; CBH = cellobiohydrolase, BX = β-xylosidase, PHOS = acid phosphatase, and HPO = peroxidase.

30.3% in the organic horizon and 77.6% in the mineral soil. Potential specific cellobiohydrolase activity was increased by an average of 38.9% in the mineral soil.

At *in-situ* temperatures for the five timepoints used for enzyme assays in this study, specific extracellular enzyme activity was projected to generally be lower in the organic horizon than the mineral soil (Fig. 4C). Specific BX, BG, and PHOS activities are expected to be 47.8%, 39.4%, and 23.1% greater in the organic horizon of heated plots than control plots on the days we sampled. In the mineral soil, specific BG, BX, CBH, and PHOS activities are expected to be 70.4%, 105%, 89.3%, and 53.7% higher in heated than control soils, while specific HPO activity is expected to be 33.3% lower (Fig. 4C, Table 1). This effect was most apparent for BG, BX, and CBH at our first sampling point in early June (DOY 154), and for HPO at the last two samplings (DOY 265, 300) in late September and October. After accounting for soil temperatures over the entire growing season, specific potential BG, BX, CBH and PHOS activities are expected to be 1.12, 1.36, 1.27, and 1.17x as high in heated than control organic horizon soils, and 1.36, 1.45, 1.41, and 1.32x as high in heated than control plot mineral soils. C:N ratio of potential specific extracellular activity at *in-situ* temperatures is expected to be unaffected by warming treatment (Table 1).

4. Discussion

Climate warming is expected to accelerate the carbon cycle (Lu et al., 2013), with the long-term fate of soil a substantial and poorly-defined feedback to climate change (Todd-Brown et al., 2013). In

Table 1
Effects of sampling timepoint at warming treatment on potential extracellular enzyme activity using mixed models. Values in table are F statistics for models fit to data (see methods) followed by symbols for p-values. Values are bolded if significant at $p < 0.05$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. - indicates the effect was not in the final model. NA indicates a model was not fit due to inadequate or missing data.

	Soil	Comparison	BG	BX	CBH	NAG	PHOS	HPO	PO	C:N	N:P	C:P
Activity (25 °C, g^{-1} soil)	Organic	Warming	3.97	0.19	0.27	**6.09***	**5.28***	0.14	2.89	2.66	2.53	0.06
		Timepoint	**7.87*****	**28.20*****	**4.59****	**2.80***	**7.16*****	**9.85*****	**14.74*****	7.71***	1.85	1.57
		Warming*timepoint	0.17	–	–	–	0.32	2.50	2.31	–	–	–
	Mineral	Warming	0.01	2.35	0.09	0.29	1.43	**16.99*****	0.05	0.47	0.22	0.51
		Timepoint	**11.34*****	**15.54*****	**4.77****	**8.11*****	**22.82*****	**9.58*****	**12.68*****	**9.56*****	2.21	**7.40*****
		Warming*timepoint	1.63	1.49	1.71	–	0.85	–	–	1.32	–	–
Specific Activity (25 °C, g^{-1} lipid phosphate)	Organic	Warming	0.01	**10.84****	1.09	1.21	1.77	2.81	3.10			
		Timepoint	2.09	**16.90*****	2.55	1.93	2.72	1.39	**15.82*****			
		Warming*timepoint	–	1.59	0.54	–	–	2.03	1.64			
	Mineral	Warming	2.24	**10.50****	3.35	2.42	1.34	2.61	0.56			
		Timepoint	**5.74*****	**24.09*****	**5.00****	**2.58***	**3.26***	0.92	**3.69***			
		Warming*timepoint	1.94	1.59	1.40	0.72	–	–	NA			
Activity (in-situ temperature, g^{-1} soil)	Organic	Warming	0.48	3.77	1.52	0.19	0.02	0.48	NA			
		Timepoint	**8.25*****	**23.39*****	**4.58****	**3.13***	**14.53*****	**5.10****	NA			
		Warming*timepoint	–	–	–	0.27	–	1.68	NA			
	Mineral	Warming	3.87	**5.54***	**5.98***	NA	**6.95***	**32.32*****	NA			
		Timepoint	**17.55*****	**18.78*****	**8.50*****	NA	**37.75*****	**11.43*****	NA			
		Warming*timepoint	2.15	1.74	**2.67***	NA	1.00	–	NA			
Specific Activity (in-situ temperature g^{-1} lipid phosphate)	Organic	Warming	1.28	**14.94****	**5.14***	1.89	**12.05****	1.71	NA	0.22	0.31	0.02
		Timepoint	1.46	**14.16*****	2.23	1.67	**6.60****	1.47	NA	**3.04***	1.20	1.05
		Warming*timepoint	–	1.30	–	–	–	1.95	NA	–	1.15	–
	Mineral	Warming	**9.45****	**13.47*****	**7.68****	NA	**9.75****	**5.27***	NA	NA	NA	0.88
		Timepoint	**8.83*****	**21.60*****	**3.03***	NA	**6.86*****	0.53	NA	NA	NA	0.16
		Warming*timepoint	1.69	2.49	0.97	NA	–	1.63	NA	NA	–	–

this study, we evaluated how more than two decades of warming has affected the physical, chemical, and biological drivers of soil carbon stability in a temperate forest ecosystem. We found warming-induced reductions in SOM concentrations and microbial biomass in the organic horizon, but little warming effect on enzyme activity. In the mineral soil, we found higher specific extracellular enzyme activity at field temperatures and depletion of lignocellulose, which is consistent with a more active microbial community targeting specific SOM compounds in heated plot soils.

4.1. Changes in the availability and structure of soil organic matter

A quarter century of warming has substantially reduced soil organic matter concentration at our study site. Although soil respiration was similar between heated and control plots when noticeable soil organic matter loss was first reported a decade ago (Bradford et al., 2008; Melillo et al., 2002), respiration was higher in heated than control plots in the years leading up to sampling for the present study (Melillo, unpublished). We have hypothesized that this increase in respiration despite an overall depletion of soil organic matter pools could be attributed to microbes in heated plots accessing carbon previously physically or chemically unavailable to them. We evaluated this hypothesis by looking at the coarse chemical composition of the top 10 cm of mineral and organic soil after 24 years of warming in October 2014, and at physical protection of soil organic matter in the top 3 cm of mineral soil after 25 years of warming in July 2015.

We did not find clear evidence that reduced physical protection had facilitated microbial access to previously-occluded particulate organic matter pools in the upper mineral soil. Since particulate organic matter does not need to desorb from mineral surfaces in order to be attacked by microbes, it is generally not considered to be physically protected unless it is tightly associated with minerals in microaggregates (Six et al., 2000). Therefore, if warming treatment had reduced the degree to which soil organic matter was protected, we expected to find: 1) a reduction in the relative mass abundance of microaggregates; and 2) a decrease in occluded fine particulate organic matter (FPOM) carbon within microaggregates from heated plots (Six et al., 2000). Mean relative mass of microaggregates was lower in heated than control plots, but there was no effect of warming on the amount of carbon occluded as FPOM in microaggregates (Fig. S3E). Since minerals are more dense than particulate organic matter, a lower mineral:particulate organic matter mass ratio could explain the reduction in microaggregate mass despite no change in occluded POM in heated plots. However, we did not evaluate bulk density in the present study to infer what this would mean for total carbon stocks.

Reduced physical protection of soil organic matter may also be observed as increased desorption from minerals at elevated temperatures. We found evidence for this in the present study as a depletion of mineral-associated organic matter in the top 3 cm of mineral soil. MAOM consists primarily of small labile byproducts of microbial activity which are believed to be protected from decomposition by sorption and bonding to mineral surfaces (Kleber et al., 2007; Grandy and Neff, 2008). Elevated temperatures are expected to shift the sorption-desorption balance for strongly-bound organic matter on mineral surfaces towards desorption (Conant et al., 2011), thereby making more carbon available for microbial consumption. Since these microbial byproducts have been preferentially consumed in the upper mineral soil of heated plots (Figs. S3B and C), this indicates the remaining soil organic matter should show markers of being less processed.

We found warming treatment has affected the chemistry and age of bulk soil organic matter in the upper mineral soil. Coarse chemical analysis of the upper 7–9 cm of mineral soil showed that warming treatment increased the relative abundance of lipids and decreased the relative abundance of lignin and polysaccharides in late fall. Lipids have previously been observed to increase in absolute abundance with short-term (1–5 year) warming (Feng et al., 2008; Pisani et al., 2015), and in particular the accumulation of plant-derived lipids such as cutins. However, we were unable to distinguish between plant- and microbially-derived lipids with the methods used here. Warming also decreased potential peroxidase enzyme activity in the mineral soil throughout much of the growing season in the present study, indicating a shift away from degradation of complex heteropolymeric plant matter. This is

consistent with warmer temperatures accelerating the conversion of litter structural polymers to soil organic matter (Schurig et al., 2013) such that microbes in heated plots now increasingly feed on late-stage decomposition byproducts such as microbial residues.

However, we did not observe other signatures of increased microbial processing such as decreased C:N ratio of the remaining soil organic matter or an enrichment in heavy nitrogen (Melillo et al., 1989). Instead the $\delta 15N$ for the top-most 3 cm of mineral soil was lower in the heated plots than the control plots. This could be the result of warming treatment reducing the degree to which "old" mineral-associated organic matter is fractionated against during microbial assimilation, which has previously been observed in subarctic peatlands and temperate forests (Hopkins et al., 2012; Dorrepaal et al., 2009). The lower $\delta 15N$ signature - which suggests reduced age of soil organic matter with warming - could also be due to a reduction in microbial biomass (Dijkstra et al., 2006) or mycorrhizal fungal abundance (Hobbie and Hobbie, 2008). Alternatively, the younger nitrogen ratio present in the upper mineral soil of heated plots may be due to increased translocation of less-processed particulate organic matter from the organic horizon into the upper mineral soil (Haddix et al., 2016). Such increased translocation of soil organic matter into the mineral soil could be due to increased freeze-thaw cycle frequency (Edwards et al., 2007) or invertebrate activity (Blankinship et al., 2011), both of which have been noted to occur with warming at other sites. This is consistent with the enrichment of genes for polysaccharide degradation we previously observed with warming in the top 10 cm of mineral soil at this site (Pold et al., 2016), but contrasts with the observed preferential depletion of lignocellulose from the upper mineral soil. This suggests that any increased translocation of POM from the organic horizon to upper mineral soil would have to be cutin-rich. Furthermore, since we observed a decrease in nitrogen concentration in the upper 8–9 cm of mineral soil, sufficient mineral-associated organic matter would have to be lost from the deeper mineral soils in order to explain the disparity between nitrogen age in the upper 3 cm and the quantity of nitrogen in the upper 8–9 cm of mineral soil. Therefore, we did not find substantiative evidence that increased translocation of partially-degraded organic matter from the organic horizon is driving the changes we observed in the physical and chemical nature of organic matter remaining in the mineral soil.

4.2. Effects of chronic warming on soil microbial activity

In light of these changes in the quantity and quality of soil carbon that we observed, microbial communities in heated plots appear to be working harder to access carbon. As has previously been observed at our site (Frey et al., 2008; Bradford et al., 2008), warming-induced depletion of soil organic matter led to a seasonal reduction in microbial biomass. Warming is expected to inflate cellular carbon demand by increasing the costs of membrane charge maintenance, lipid turnover (Hall et al., 2010), and the heat stress response (Manzoni et al., 2012). This diverts resources away from growth (Tucker et al., 2012; Allison, 2014; Frey et al., 2013). In order to avoid a reduction in microbial biomass, the rate of substrate supply must keep pace with demand. This requires the production of enzymes and transporters with considerable energy (Lever et al., 2015) and nitrogen (Schimel and Weintraub, 2003) costs to the cell. Since we observed a reduction in microbial biomass despite a predicted increase in extracellular enzyme activity per unit microbial biomass at *in-situ* temperatures, this indicates that the rate of substrate supply in heated plots was inadequate to keep pace with demand. As such, elevated respiration in heated plots is likely a consequence of substantially increased per-capita respiration offsetting a smaller microbial pool, rather than being caused by a larger resource supply facilitating net growth of the microbial biomass.

However, we also recognize that while incubating soil slurries with non-limiting substrate concentrations may provide an estimate of potential rates of extracellular enzyme activity, they do not accurately represent the enzyme-substrate dynamics of soil. Of particular note is the well-known tradeoff between the Michaelis Menten constant (K_m - substrate affinity) and the maximum catalytic rate of enzymes (V_{max}), which we did not assess in the present study. Temperature increases are expected to increase both V_{max} and K_m, leading to a partial cancellation of the positive effect of warming on enzyme activity (German et al., 2012). The microbial community may overcome this increase in K_m with warming by generating more rigid isoenzymes capable of maintaining substrate affinity at higher temperatures (Wallenstein et al., 2010), which would be expected to lead to reduced V_{max} compared to enzymes from unheated soils at low but not high temperatures. However, we did not find evidence for this in the present study as the slopes of the Arrhenius curves were similar for soils from heated and control plots. Previous studies at the Harvard Forest have confirmed a positive correlation between V_{max} and K_m for a range of extracellular enzymes independent of season (Drake et al., 2013). Rather, reduced substrate availability is likely to be limiting the response of extracellular enzyme activity to warming *in-situ* (Brzostek and Finzi, 2012). This means that although warming may increase potential activity of enzymes responsible for targeting soil organic matter, this need not translate into greater C availability for cell growth.

5. Conclusion

We found that accelerated soil respiration resulting from increased temperatures is associated with a reduction in soil organic matter concentration and living microbial biomass. Warming has fundamentally changed the carbon accessed by microbial communities in the mineral soil. We suggest that the remaining particulate organic matter is enriched in plant-derived lipids such as cutins, which is consistent with a younger ^{15}N signal appearing in the top 3 cm of mineral soil despite a reduction of total carbon and nitrogen in the top 8–9 cm of mineral soil. The smaller microbial community present appears able to continue to access these soil carbon stocks without producing additional enzymes due to: 1) the direct thermodynamic acceleration of extracellular enzyme activity with warming; and 2) the utilization of chemically-simple mineral associated organic matter, which more readily desorbs from mineral surfaces as the soil warms. This is consistent with previous reports (Luo et al., 2013; Bradford et al., 2008) that both adaptation of the microbial community and changes in substrate availability determine long-term carbon cycle response to warming. The present research supports the use of biogeochemical measurements from long-term climate manipulation experiments to link the activities of microbial communities to ecosystem-level processes such as climate change.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.03.002.

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