



# Long-term warming in a temperate forest accelerates soil organic matter decomposition despite increased plant-derived inputs

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**Abstract** Climate change may alter soil microbial communities and soil organic matter (SOM) composition. Soil carbon (C) cycling takes place over multiple time scales; therefore, long-term studies are essential to better understand the factors influencing C storage and help predict responses to climate change. To investigate this further, soils that were heated by 5 °C above ambient soil temperatures for 18 years were collected from the Barre Woods Soil Warming Study at the Harvard Forest Long-term Ecological Research site. This site consists of large 30 × 30 m plots (control or heated) where entire root systems are exposed to sustained warming conditions. Measurements

included soil C and nitrogen concentrations, microbial biomass, and SOM chemistry using gas chromatography–mass spectrometry and solid-state <sup>13</sup>C nuclear magnetic resonance spectroscopy. These complementary techniques provide a holistic overview of all SOM components and a comprehensive understanding of SOM composition at the molecular-level. Our results showed that soil C concentrations were not significantly altered with warming; however, various molecular-level alterations to SOM chemistry were observed. We found evidence for both enhanced SOM decomposition and increased above-ground plant inputs with long-term warming. We also noted shifts in microbial community composition while microbial biomass remained largely unchanged. These findings suggest that prolonged warming induced increased availability of preferred substrates, leading to shifts in the microbial community and SOM biogeochemistry.

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The observed increase in gram-positive bacteria indicated changes in substrate availability as gram-positive bacteria are often associated with the decomposition of complex organic matter, while gram-negative bacteria preferentially break down simpler organic compounds altering SOM composition over time. Our results also highlight that additional plant inputs do not effectively offset chronic warming-induced SOM decomposition in temperate forests.

**Keywords** Soil warming · Soil organic matter · Cutin · Suberin · Lignin · Nuclear magnetic resonance · Phospholipid fatty acids

## Introduction

Forest ecosystems are integral to the global carbon (C) cycle as they represent one of Earth's largest C reservoirs (Schlesinger and Andrews 2000; Goodale et al. 2002; Pan et al. 2011). Much of this forest C is found in soils in the form of soil organic matter (SOM; Lal 2005; Bonan 2008) and is derived from plant, animal, and microbial detritus at varying stages of degradation (Lehmann and Kleber 2015; Angst et al. 2021). Current stores of SOM-C are two times higher than that of the atmosphere and terrestrial vegetation combined, making it a critical component for climate regulation (Schmidt et al. 2011; Melillo et al. 2017). SOM is essential for several soil processes, such as the biogeochemical cycling of nutrients (Amelung et al. 2008; Dove et al. 2021), as well as maintaining soil fertility (Batjes 1996; Lal 2004). However, anthropogenic activity and global environmental change threaten soil systems and their ability to maintain biogeochemical trajectories (Reichstein et al. 2013; Crowther et al. 2016; Hicks Pries et al. 2017). For example, continuous warming can alter plant species distributions (Walther et al. 2002) and induce changes in both the quality and quantity of plant inputs into forest soils (Liu et al. 2004; Kengdo et al. 2023). Additionally, warming can impact the kinetics of microbial respiration leading to reductions in the amount of C sequestered in forest soils (Wagai et al. 2013; Li et al. 2020; Ofiti et al. 2021) and increased release of CO<sub>2</sub> which have the capacity to further drive warming (Soong et al. 2021). Warming can impact soil microbial communities and plant inputs into the soil, further impacting SOM chemistry

(Schmidt et al. 2011; Yuan et al. 2021; Hernandez et al. 2021). Therefore, continuously rising global temperatures highlight the need for additional studies to determine how long-term soil warming may alter forest SOM biogeochemistry and improve our understanding of the mechanisms governing soil C cycling.

Previous studies have found that soil warming results in an overall loss of soil C (Nottingham et al. 2020; Soong et al. 2021) and enhanced SOM decomposition (Feng et al. 2008; Conant et al. 2011; Pisani et al. 2015; vandenEnden et al. 2021; Stoica et al. 2023). Changes in SOM composition due to rising temperatures have been attributed to shifts in soil microbial biomass, community composition, and substrate utilization, as well as to altered plant productivity (Feng et al. 2008; Frey et al. 2008, 2013; Melillo et al. 2011; Wang et al. 2012). For instance, a short-term (14 month) soil warming study conducted by Feng et al. (2008) showed enhanced lignin degradation as a result of increased fungal biomass. The same study revealed that an accumulation of cutin-derived SOM, arising from leaf waxes, was attributed to increased litter inputs with warming (Feng et al. 2008). Forest soil warming experiments have predominantly utilized small-scale plots ranging from 3 to 6 m<sup>2</sup> in size (Hicks Pries et al. 2017; Melillo et al. 2017; Soong et al. 2021; Stoica et al. 2023) potentially overlooking crucial aspects such as the influence of warming on tree roots, microbial communities, and the quality and quantity of above-ground inputs. Excluding root networks from warming plots may lead to an incomplete understanding of how the entire ecosystem responds to warming. Thus, employing large-scale plots that encompass entire root systems provides a more holistic representation of how trees experience temperature changes in their natural environment and enables comprehensive examination of below and aboveground responses to warming.

While short-term and small-scale studies have contributed valuable insights into the initial responses of SOM to rising temperatures, the complex and dynamic nature of SOM requires a large-scale and long-term perspective to fully unravel the underlying mechanisms and the potential feedback to the climate system. For example, Pec et al. (2021) found that SOM and soil fungal community composition were distinctively altered in short- versus long-term warming experiments. They noted that long-term warming induced significant shifts in soil fungal communities

and a substantial decline in the relative abundance of lignin compared to the control; however, short-term warming did not exhibit compositional shifts in microbial communities and no significant changes in lignin content were observed (Pec et al. 2021). Additionally, long-term warming studies have revealed variations in the degree of SOM degradation over time (Frey et al. 2008; Deslippe et al. 2012; DeAngelis et al. 2015; Romero-Olivares et al. 2017; Pold et al. 2017). This variability has been linked to shifts in microbial biomass and community structure which can impact different SOM compounds to varying extents. Melillo et al. (2017) suggested that there is some level of microbial community reorganization with long-term warming that is linked to the variation in soil respiration. Moreover, the composition of SOM can be altered despite a reduction in microbial biomass (Pold et al. 2017). Furthermore, observed changes in SOM were attributed to altered microbial activity and carbon utilization efficiency (CUE), leading to a decline in preferred microbial substrates, such as simple sugars (Frey et al. 2008, 2013; DeAngelis et al. 2015; Melillo et al. 2017).

Previous molecular-level characterization of SOM composition revealed that the magnitude of perturbations to specific SOM constituents varied over time under warming conditions (Pisani et al. 2015; vandenEnden et al. 2021; Stoica et al. 2023). This highlights the need for further investigation into the impacts of long-term warming on SOM chemistry and cycling at a molecular-level. While the sensitivity of SOM turnover rates to temperature is well-established (Feng et al. 2008; Conant et al. 2011; Pisani et al. 2015; Nottingham et al. 2020; Soong et al. 2021; vandenEnden et al. 2021; Stoica et al. 2023), the intricate molecular-level transformations driving these changes and their implications for soil C sequestration remain a subject of ongoing research.

Our aim in this study was to identify the extent to which long-term warming enhances SOM degradation and alters SOM chemistry over time. The 18-year duration of our experimental treatment provides a unique opportunity to assess the persistent and cumulative molecular-level impacts of soil warming on SOM biogeochemistry. Moreover, the use of large 30 by 30 m plots exposes entire root systems of trees to warming, which is a novel component of this study. Soil samples were collected from the Barre Woods soil warming study at the Harvard Forest Long-term

Ecological Research site. Our study utilized a combination of advanced targeted and non-targeted molecular-level techniques to provide insights into the factors that regulate SOM composition and degradation. Although long-term warming experiments have demonstrated variability in the magnitude of SOM responses to warming (Romero-Olivares et al. 2017), we hypothesized that SOM decomposition would continue to accelerate in response to long-term warming via preferential use of specific SOM components and that a shift in microbial biomass and community composition would be observed. Overall, this study aims to provide unique molecular-level insights into how SOM chemistry is altered after 18 years of warming and the underlying mechanisms that govern SOM dynamics.

## Materials and methods

### Site description and sampling

Soil samples were collected from the Barre Woods Soil Warming Study at the Harvard Forest Long-term Ecological Research site in Petersham, Massachusetts, USA ( $42^{\circ}28'N$ ,  $72^{\circ}10'W$ ). The site is an even-aged, mixed deciduous forest with a mean weekly air temperature ranging from a high of  $20^{\circ}C$  in July to a low of  $-6^{\circ}C$  in January (Werner et al. 2020). The average annual total precipitation is 108 cm with even distribution throughout the year (Melillo et al. 2011; Werner et al. 2020). The soil type is classified as Canton series soil, characterized by well-drained mineral soil with a coarse-loamy texture (Melillo et al. 2011; Pold et al. 2016; Werner et al. 2020). The site features a distinct forest floor with a soil pH ranging between 4.3 and 5.2, with the underlying mineral soil having a pH range of 4.4–5.5 (Melillo et al. 2011; Werner et al. 2020). The dominant tree species include *Quercus rubra*, *Q. velutina*, *Fraxinus americana*, and *Acer rubrum* (Melillo et al. 2011; DeAngelis et al. 2015; Pold et al. 2016).

The warming experiment began in April 2003 following a one-year period of baseline measurements (Frey and Melillo 2022). The baseline measurements included nitrogen mineralization, trace gas fluxes ( $CO_2$ ,  $CH_4$ ,  $N_2O$ ), tree growth (dendrometer bands), an understory species survey, canopy foliage analysis, and lysimetry (Frey and Melillo 2022). The

Barre Woods plots (30 m × 30 m) encapsulate entire tree root systems and include two treatments, control (ambient) and heated. The temperature in the heated plot is consistently maintained at 5 °C above ambient temperatures using resistance cables buried at a depth of 10 cm and spaced 20 cm apart (Melillo et al. 2011; Werner et al. 2020; Frey and Melillo 2022). These cables span the entire plot, ensuring uniform warming of root systems for trees within the plot. Further information regarding the Barre Woods experimental design may be found in previously published studies (Melillo et al. 2011; Werner et al. 2020).

Soil samples were collected in July 2021, after 18 years of experimental treatment. Each plot is divided into 30 subplots (5 m × 5 m; DeAngelis et al. 2015; Pold et al. 2016). Six of these subplots exhibiting both mature *Q. rubra* and *A. rubrum* trees were randomly selected from the 16 innermost area in both the control and warmed plots, and sampled at three depths (forest floor, and 0–10 and 10–20 cm mineral soil). Two locations within each of these subplots were randomly chosen and two soil cores were taken from each subplot. Samples were acquired using a Giddings slide hammer coring tool (5.7 cm inner diameter; Giddings Machine Co., Windsor, CO) and approximately 200 g of soil were collected per core. Soils were sieved (<2 mm) to remove any plant debris and replicate cores were pooled and homogenized. Soils were frozen, freeze dried (Thermo Savant ModulyoD-115), and composite samples of the core replicates were used for subsequent molecular analyses. Due to the large size of the study plots and the logistical constraints in heating and maintenance, true replication was not feasible, allowing for only one plot per treatment. However, for our analysis, we treated the subplots as field replicates ( $n=6$ ) to account for within-plot variation.

#### Soil carbon and nitrogen measurements

Total soil C and N concentrations were measured on freeze dried, finely ground soil samples via combustion using a Thermo Flash 2000 Elemental Analyzer which uses O<sub>2</sub> gas at a temperature of 950 °C. Each sample from all soil depths (forest floor, and 0–10 and 10–20 cm mineral layer) was analyzed in duplicate. C and N concentrations are reported as a percent based on soil mass.

#### Non-targeted solid-state <sup>13</sup>C nuclear magnetic resonance (NMR) spectroscopy

Solid-state <sup>13</sup>C cross polarization with magic angle spinning (CP-MAS) NMR spectroscopy was used to characterize the overall organic matter composition of the soil samples. For the forest floor, approximately 1 g of each sample was used whereas 5 g from the mineral layer samples (0–10 and 10–20 cm) were repeatedly treated with 10% hydrofluoric acid (HF; ACS Grade) to remove any minerals and paramagnetic materials present in the sample (Schmidt et al. 1997). This treatment also concentrates organic matter which enhances the NMR signal and improves the signal-to-noise ratio (Schmidt et al. 1997; Rumpel et al. 2006). The HF-treated samples were then repeatedly rinsed with deionized water until the electrical conductivity of the supernatant indicated low salt contents. The supernatant was removed after centrifugation and the samples were frozen and freeze-dried prior to analysis. Approximately 250 mg of each sample was packed into a 4 mm zirconium rotor, sealed with a Kel-F cap, and analyzed using a 500 MHz Bruker BioSpin Avance III spectrometer equipped with a 4 mm H-X MAS probe. For the forest floor and mineral samples, data acquisition involved 14,100 and 85,000 scans respectively, under magic angle spinning (54.74°) at a rate of 11 kHz (Dria et al. 2002; Farooq et al. 2013; Sun et al. 2019). The main operating and processing parameters were set as follows: 1 ms ramp-cross-polarization contact time, 1 s recycle delay, and 100 Hz line broadening (Simpson et al. 2008).

The resulting NMR spectra were analyzed using Bruker TopSpin (software version 4.1.4) and integrated into four chemical shift regions corresponding to different functional groups: alkyl (0–50 ppm), *O*-alkyl (50–110 ppm), aromatic and phenolic (110–165 ppm) and carboxyl and carbonyl (165–215 ppm; Baldock et al. 1992; Baldock and Preston 1995). The alkyl C region is mainly derived from cutin, suberin side chains, aliphatic side chains and lipids; the *O*-alkyl C region mainly arises from carbohydrates, peptides and methoxyl carbon in lignin; the aromatic and phenolic carbon region is primarily derived from lignin, and aromatic amino acids observed in peptides; the carboxyl and carbonyl carbon region is mainly made up from fatty acids and amino acids found in peptides (Baldock et al.

1992; Simpson et al. 2008). Additionally, the alkyl/*O*-alkyl C ratio, calculated by dividing the integrated areas of the alkyl and the *O*-alkyl regions, served as a relative indicator of SOM degradation (Baldock et al. 1992; Simpson et al. 2008; Simpson and Simpson 2012). Higher values of the alkyl/*O*-alkyl C ratio indicate enhanced SOM decomposition as *O*-alkyl C compounds are preferentially degraded over alkyl C compounds (Baldock and Preston 1995).

#### Targeted analysis of SOM compounds

A sequential extraction method targeting various plant- and microbial-derived compounds was applied (Otto et al. 2005). Targeted organic matter compounds were extracted via solvent extraction, base hydrolysis, and copper (II) oxide (CuO) oxidation to yield total solvent extractable, cutin-, suberin-, microbial-, and lignin-derived compounds (Otto and Simpson 2005, 2006a). All soil samples were extracted in duplicate (two analytical replicates). A detailed procedure is described in the Supplementary Materials. Briefly, solvent-extractable compounds were isolated from the soil (1 g for the forest floor, 2 g for mineral soils) using dichloromethane (Optima grade), 1:1 dichloromethane:methanol (v:v; Optima grade), and methanol (Optima grade) sequentially (Otto and Simpson 2005). The soil residues from solvent-extraction were then base hydrolysed (0.5 g for the forest floor, 1 g for mineral soils) with 1 M methanolic (Optima grade) potassium hydroxide (ACS grade) at 100 °C for 3 h in Teflon-lined bombs to obtain ester-bound cutin-, suberin-, and microbial-derived lipids (Góñi and Hedges 1990). After base hydrolysis, soil residues (0.25 g for forest floor samples and 0.5 g for mineral soils) were air dried and oxidized using CuO, ammonium iron (II) sulfate hexahydrate (ACS grade), and 2 M sodium hydroxide (ACS grade). Samples were heated at 170 °C for 2.5 h to extract lignin-derived phenols (Hedges and Mann 1979; Otto and Simpson 2006b). The CuO oxidized extracts were collected via solid-phase extraction (Pinto et al. 2010).

#### Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acids (PLFAs) were extracted to measure microbial biomass and community composition (Quideau et al. 2016). Soil samples (1 g for the

forest floor, 5 g for mineral soil) were extracted using methanol, chloroform, and a 0.15 M citrate buffer (acidified to pH=4). Chloroform, acetone and methanol were then used to elute nonpolar lipids, glycolipids and polar lipids, respectively, from the extracts in the chloroform phase. After fractionation, the polar lipids in the methanol fraction were alkaline methanolized and extracted using a 4:1 hexane:chloroform mixture (v:v) to isolate PLFAs.

#### SOM and PLFA compound identification and quantification

Dried isolated extracts were stored at –20 °C before being quantified via gas chromatography–mass spectrometry (GC–MS). Solvent and CuO extracts were derivatized using *N,O*-bistrifluoroacetamide (>99%) and anhydrous pyridine (>99.8%) at 70 °C for 1 h. Base hydrolysis products were first derivatized with *N,N*-dimethylformamide dimethyl acetal (>97%) for 15 min at 60 °C followed by *N,O*-bistrifluoroacetamide and anhydrous pyridine (Otto and Simpson 2006a). All samples were diluted with hexanes prior to analysis. External standards were prepared and derivatized in the same manner as the extracts for quantification of the targeted compounds. The external standards used included 1-docosanol (>98%), cholesterol (>99%), tetracosane (>99%) and methyl tricosanoate (>99%) for solvent extraction; methyl tricosanoate for base hydrolysis; syringaldehyde (>98%) and syringic acid (>98%) for CuO extracts; and methyl oleate (>99%) for PLFAs. All samples and standards were analyzed using an Agilent 7890B gas chromatograph with a 5977B mass spectrometer operated in electron impact mode with ionization energy of 70 eV. Helium was used as the carrier gas at a flow rate of 1 mL min<sup>−1</sup> and the GC was equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness). The GC oven temperature was maintained at 65 °C for 2 min before being increased to 300 °C at a rate of 6 °C per minute and held for 20 min (7 min for PLFA analysis). An Agilent 7683 autosampler was used to inject a 1 μL aliquot of sample extract with an injector temperature of 280 °C. Data acquisition and processing was done using Agilent Mass Hunter GC–MS Acquisition and the Agilent Enhanced ChemStation software, respectively. Compounds were identified and quantified using the National Institute of

Standards and Technology (NIST) and Wiley 275 mass spectral library. Quantification of targeted SOM compounds and PLFAs was done by integrating peak areas and comparing to the external standards (Otto and Simpson 2005, 2006a, b). Compound concentrations were normalized to the mass of soil used in the extraction and then further normalized to the soil C concentration.

#### Data and statistical analyses

Analyses were performed on samples collected from six subplots from each treatment (heated and control) with two analytical replicates each, following 18 years of warming. A mixed model analysis of variance (ANOVA) was used to compare the concentrations of SOM components between the control and heated samples. For this analysis, subplot samples served as field replicates ( $n=6$ ), and analytical replicates were considered the within-subject factor ( $n=2$ ) to account for variability within each plot, while the treatment (control vs heated) was the between-subject factor. Each soil depth was analyzed separately, and differences were considered statistically significant when  $p \leq 0.05$ . Soil C and N concentration, as well as NMR and PLFA data, were analyzed in the same manner. Statistical tests were carried out using IBM SPSS statistics (software version 28). It is relevant to highlight that the evaluation of SOM compositional shifts was conducted by assessing relative changes

to the control plot. The data from the control plot serves as a benchmark for identifying significant variations in the changes observed in SOM composition. Detailed statistical results ( $p$ -values) are provided in Table S6.

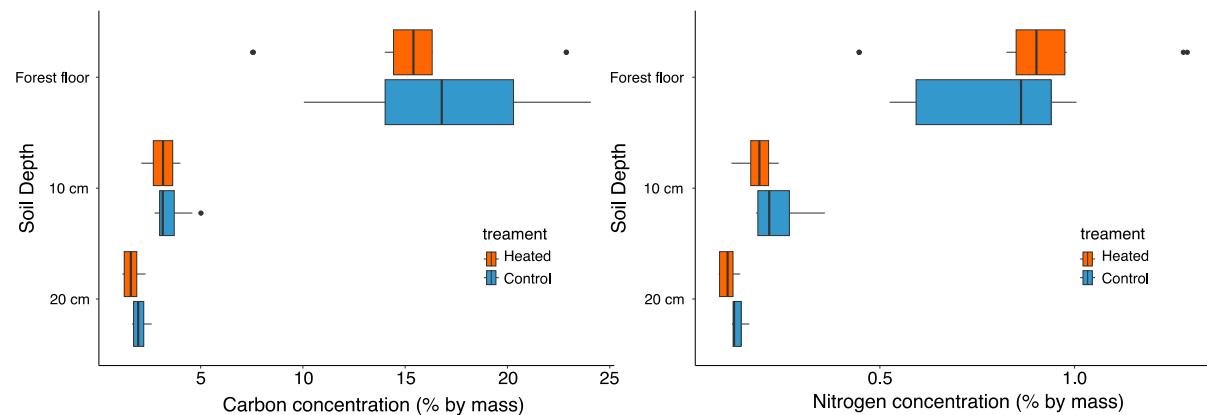
## Results

### Soil carbon and nitrogen concentrations

Soil C and N concentrations were not significantly influenced by heat at any of the soil depths analyzed ( $p \leq 0.05$ ; Table S1 and S6). We noted that there were slight decreases in both C and N concentrations in the mineral soils (0–10 and 10–20 cm) relative to the control after 18 years of treatment, but these were not significant (Fig. 1, Table S6).

### Non-targeted solid-state $^{13}\text{C}$ nuclear magnetic resonance (NMR) spectroscopy

Solid-state  $^{13}\text{C}$  NMR spectroscopy was used to observe the differences in the relative proportions of the regions representing the main SOM categories. The chemical shift regions integrated included the alkyl C region (0–50 ppm), *O*-alkyl C (50–110 ppm), aromatic and phenolic (110–165 ppm), and carboxyl and carbonyl region (165–215 ppm; Table S2). Warming increased alkyl

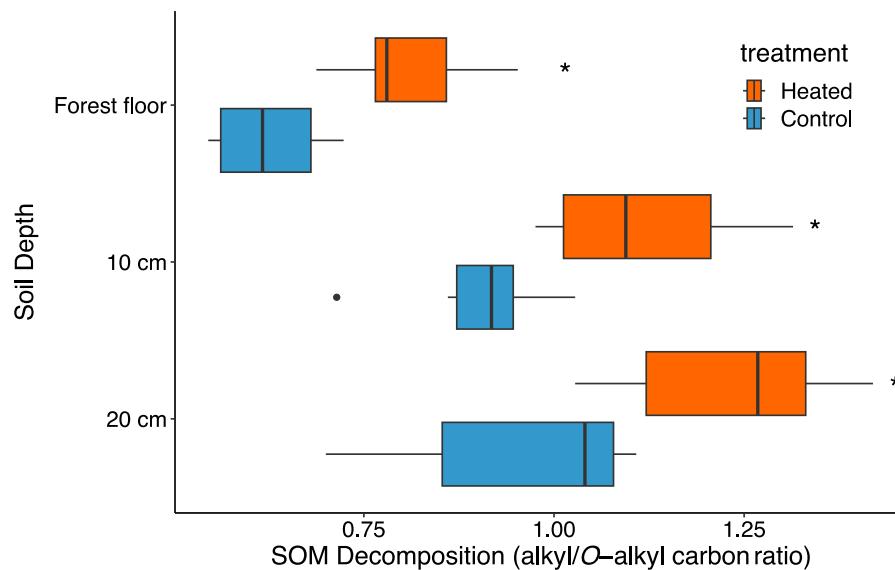


**Fig. 1** Distribution of total carbon (C) and nitrogen (N) concentrations (% by mass) as measured on the forest floor and mineral soils (0–10 and 10–20 cm) from the Harvard Forest Barre Woods Soil Warming Study after 18 years of treatment.

Differences between the heated and control plots were not statistically significant ( $p \leq 0.05$ ). Detailed C and N concentration data is provided in Table S1

C and decreased *O*-alkyl C at all three soil depths relative to the control (Table S2). Moreover, soil warming significantly increased the alkyl/*O*-alkyl C ratios at all three soil depths relative to the control. This is indicative of enhanced SOM decomposition in response to warming (Fig. 2) as *O*-alkyl compounds are labile and more easily degraded compared to alkyl compounds (Baldock and Preston 1995; Simpson and Simpson 2012). Furthermore, *O*-alkyl C stemming from oxygen and nitrogen-substituted aliphatic constituents found in peptides, carbohydrates, and methoxyl C in lignin dominated the forest floor chemistry (Table S2, Figure S2 and S3; Baldock et al. 1992; Simpson et al. 2008). Similarly, the control treatment from both depths of the mineral horizon (0–10 and 10–20 cm) was dominated by *O*-alkyl C (Table S2, Figure S4 and S6); however, this was not the case for the heated treatment which had higher proportions of alkyl C arising from cutin, suberin, and aliphatic side-chains and lipids (Table S2, Figure S5 and S7; Baldock et al. 1992; Clemente et al. 2012). After warming, the aromatic and phenolic region originating from lignin and aromatic amino acids showed a slight decrease on the forest floor only, while the carboxyl and carbonyl region stemming from fatty acids and amino acids found in peptides revealed no significant changes at any soil depth relative to the control (Table S2; Baldock et al. 1992; Simpson et al. 2008).

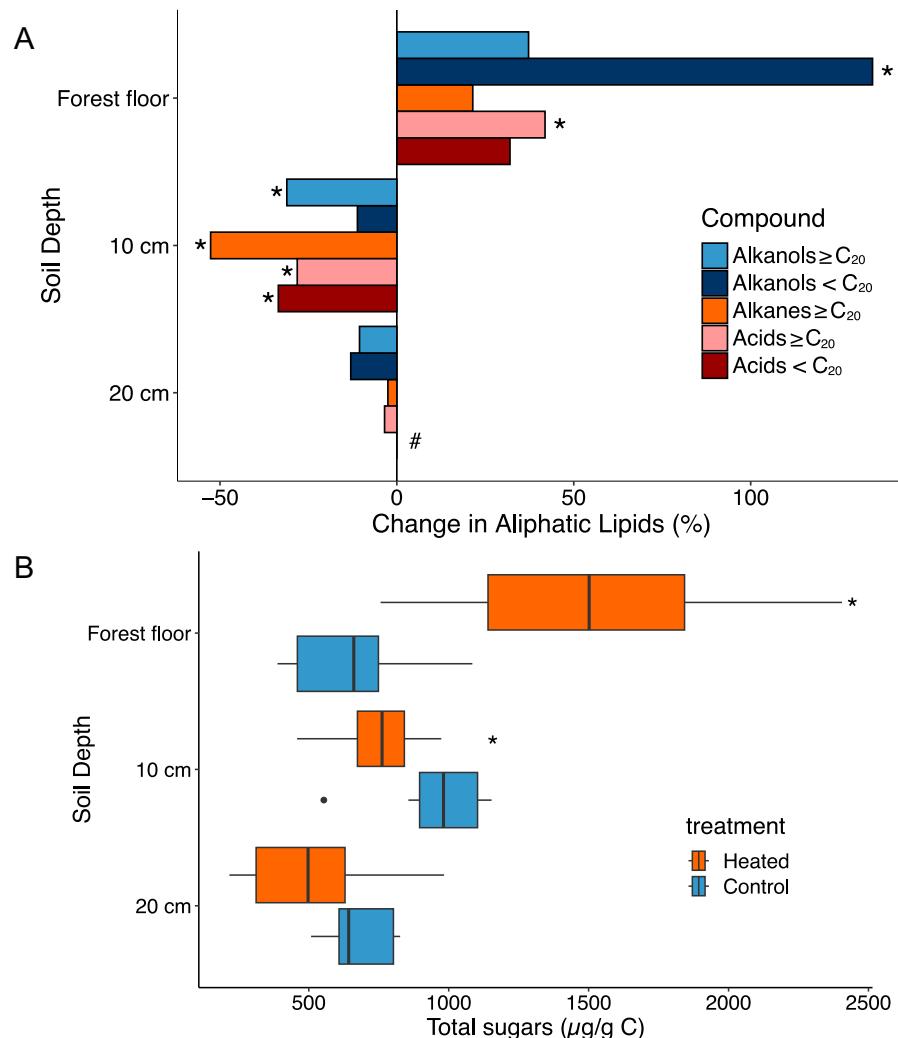
**Fig. 2** SOM decomposition based on the alkyl/*O*-alkyl carbon ratio from solid-state  $^{13}\text{C}$  NMR spectra for the forest floor, 0–10, and 10–20 cm layer samples. Asterisks (\*) indicate statistically significant ( $p \leq 0.05$ ) differences relative to the control plots. Detailed NMR data is provided in Table S2



## Targeted analysis of SOM compounds

**Solvent-extractable compounds.** The solvent extracts from the forest floor and 0–10 and 10–20 cm mineral soils contained various lipids arising from plant and microbial sources. Several aliphatic lipids (*n*-alkanes, *n*-alkanols, *n*-alkanoic acids, and *n*-alkane- $\alpha$ ,  $\omega$ -dioic acids), cyclic lipids (terpenoids and plant-derived steroids), and sugars were detected on the forest floor and mineral soils. Aliphatic lipids were classified into short-chain ( $<\text{C}_{20}$ ) and long-chain ( $\geq\text{C}_{20}$ ) which are characteristic of SOM derived from microbial biomass and plant waxes, respectively (Lichtfouse et al. 1995; Otto et al. 2005). Long-chain aliphatic lipids were the most abundant solvent-extractable compounds at all three soil depths while short-chain lipids were minor components (Fig. 3A). In the forest floor, warming increased the concentration of aliphatic lipids (Fig. 3A), cyclic lipids, and sugars (Fig. 3B) relative to the control (Table S3). This included significant ( $p \leq 0.05$ ) increases in the overall concentration of aliphatic lipids (Table S3) and sugars (Fig. 3B), as well as in short-chain alkanols and long-chain alkanoic acids (Fig. 3A). The 0–10 cm mineral layer had significant reductions in plant-derived SOM including lower concentrations of long-chain alkanes, alkanols and alkanoic acids, relative to the control (Fig. 3A, Table S4). We also noted a significant decrease in the total concentration of aliphatic lipids, cyclic lipids (Table S4) and sugars (Fig. 3B) in the

**Fig. 3** **A** Percent change relative to the control in the concentration of aliphatic lipids (compounds consisting of *n*-alkanes, *n*-alkanols and *n*-alkanoic acids) normalized to carbon within the forest floor, 0–10 cm, and 10–20 cm mineral layer samples; **B** Total sugar concentrations measured within the forest floor, 0–10 cm, and 10–20 cm mineral layer samples. Asterisks (\*) indicate statistically significant ( $p \leq 0.05$ ) differences relative to the control plots and “#” indicates no measured difference. Data for solvent-extractable compound results and sugar concentrations are provided in Tables S3, S4 and S5

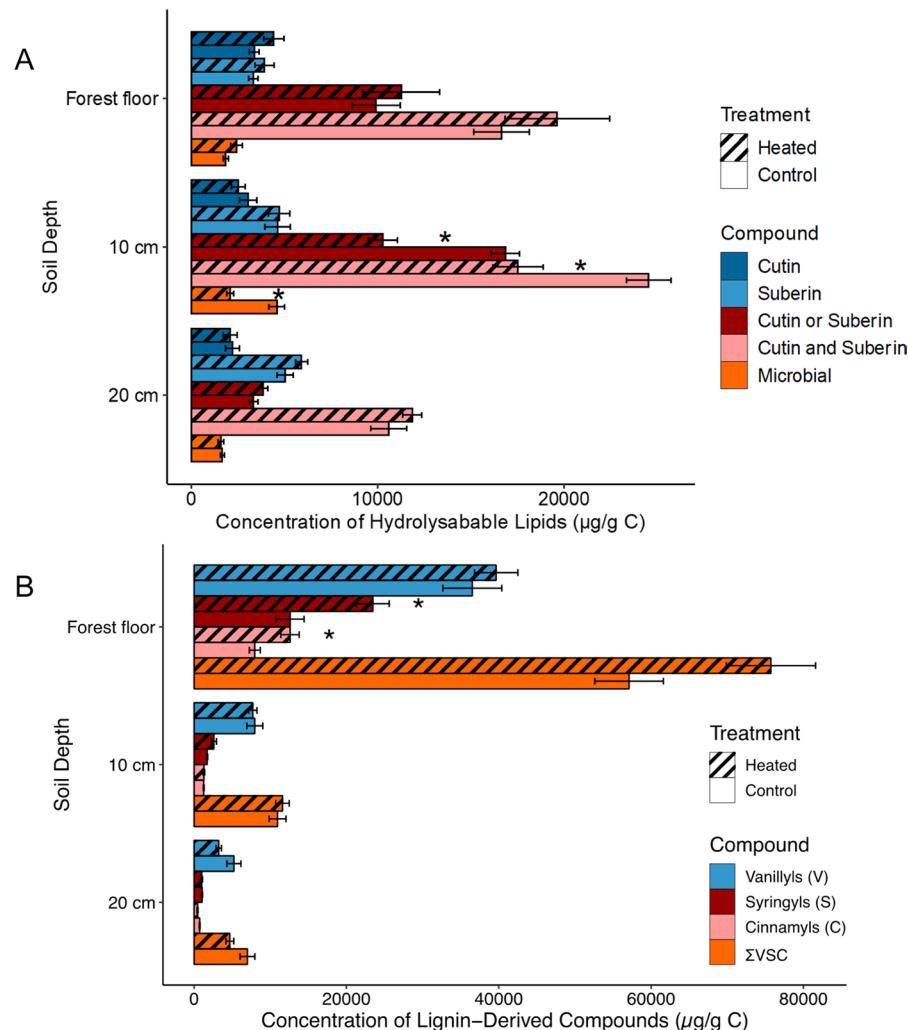


0–10 cm soil depth with warming. Moreover, warming significantly decreased the concentration of plant-derived steroids, total steroids, and total cyclic lipids (Table S5) in the 10–20 cm mineral layer when compared to the control samples. However, there were no significant changes observed for short-chain alkanols and long-chain alkanes, alkanoic acids and alkanols, as well as no measured differences for short-chain alkanoic acids with warming in the 10–20 cm soil depth (Fig. 3A).

**Cutin- and suberin-derived compounds.** The ester-bound hydrolysable lipids from the forest floor and mineral layer consisted of various compounds including *n*-alkanols, *n*-alkanoic acids, branched alkanoic acids, *n*-alkane dioic acids,  $\omega$ -hydroxyalkanoic acids, di- and tri-hydroxyalkanoic and epoxy acids and

$\alpha$ -hydroxyalkanoic acids (Otto and Simpson 2006a). These compounds are characteristic of SOM derived from soil microbes, cutin, suberin or both cutin and suberin. Suberin-derived compounds are indicative of below-ground inputs (plant roots) while cutin-derived compounds arise from above-ground inputs (leaf litter; Kögel-Knabner 2002; Otto and Simpson 2006a). All three soil depths were dominated by lipids derived from both cutin and suberin (Fig. 4A). Warming on the forest floor resulted in higher concentrations of cutin- and suberin-derived lipids compared to the control (Fig. 4A, Table S3), but these were not statistically significant. Additionally, there were no significant differences measured in specific cutin- or suberin-derived compounds at any of the soil depths after warming (Fig. 4A; Table S4). However, the

**Fig. 4** **A** Total microbial-, cutin-, and suberin-derived compound concentrations measured within the forest floor, 0–10 cm, and 10–20 cm mineral layer samples. Cutin or suberin refers to compounds derived from both biopolymers while cutin and suberin indicates the sum of all cutin-, suberin- and cutin or suberin-derived compounds; **B** Total lignin-derived compound concentrations measured within the forest floor, 0–10 cm, and 10–20 cm mineral layer samples. Asterisks (\*) indicate statistically significant differences ( $p \leq 0.05$ ). Details of hydrolysable lipid and lignin-derived phenol concentrations are provided in Tables S3, S4 and S5



total sum of compounds originating from either cutin or suberin, as well as from both cutin and suberin ( $\Sigma$ SC) was significantly lower in the 0–10 cm mineral soil depth relative to the control (Fig. 4A). In the mineral soils, warming increased the concentration of suberin-derived compounds, but decreased the concentration of lipids originating from cutin (Fig. 4A, Tables S3 and S4). The degradation of cutin can be assessed by comparing the relative abundance of  $\omega$ -hydroxyalkanoic acids to total hydrolysable acids, expressed as  $\omega\text{-C}_{16}/\sum\text{C}_{16}$  (Otto et al. 2005; Otto and Simpson 2005). Progressive cutin degradation has been shown to yield higher values of this ratio (Otto and Simpson 2006a). Additionally, cutin and suberin degradation can be evaluated using the ratio between mid-chain-substituted acids and total suberin

and cutin acids, expressed as  $\sum\text{Mid}/\sum\text{SC}$ . For this ratio, decreasing values are indicative of enhanced decomposition (Otto and Simpson 2006a). There were no significant differences in the  $\omega\text{-C}_{16}/\sum\text{C}_{16}$  or  $\sum\text{Mid}/\sum\text{SC}$  ratios between the control and heated treatments at any soil depth (Tables S3, S4 and S5).

**Microbial-derived lipids.** Lipids measured in the forest floor and mineral layer included long-chain ( $\text{C}_{14}\text{--C}_{18}$ ) branched alkanoic acids stemming from cellular and extracellular microbial sources (Otto and Simpson 2007; Jia et al. 2019). However, since these are not membrane lipids, they are distinct from PLFAs. The concentration of microbial-derived lipids significantly decreased after warming in the 0–10 cm soil depth (Fig. 4A, Table S4). In contrast, there were no significant differences measured in the forest floor

and 10–20 cm mineral soils (Fig. 4A, Tables S3 and S5).

**Lignin-derived compounds.** CuO oxidation products included vanillyl, syringyl and cinnamyl phenols which are characteristic of lignin derived from major plant groups (Hedges and Mann 1979). The concentration of syringyl and cinnamyl phenols increased ( $p \leq 0.05$ ) with warming in the forest floor (Fig. 4B). However, warming did not significantly change vanillyl or total lignin-derived compound ( $\Sigma$ VSC) concentrations in the forest floor (Fig. 4B, Table S3). In the mineral horizon (0–10 and 10–20 cm), there were no significant variations in individual or total lignin-derived compound concentrations with warming (Fig. 4B, Tables S4 and S5). Moreover, ratios of syringyl to vanillyl (S/V) and cinnamyl to vanillyl were used to investigate any changes in the overall lignin chemistry with warming. The cinnamyl/vanillyl ratio increased ( $p \leq 0.05$ ) in the forest floor after warming (Table S3) but was not significantly altered in the mineral horizon (Figure S1, Tables S4 and S5). With warming, the S/V ratio slightly increased at all three soil depths, but these changes were not significant (Figure S1, Tables S3, S4 and S5). Additionally, progressive lignin degradation was assessed using acid-to-aldehyde ratios for vanillyl ( $Ad_v/Al_v$ ) and syringyl ( $Ad_s/Al_s$ ) compounds. However, no differences were observed at any soil depth with warming (Tables S3, S4 and S5).

**Phospholipid fatty acids.** With heat, total microbial biomass (total PLFAs) decreased in the forest floor and 0–10 cm mineral layer; but not significantly (Fig. 5A). Total PLFA concentrations significantly decreased with warming in the 10–20 cm mineral soil depth relative to the control (Fig. 5A). Moreover, increased ( $p \leq 0.05$ ) concentrations of PLFAs from actinomycetes were observed with warming in the forest floor (Table S3) while decreased ( $p \leq 0.05$ ) concentrations of fungal PLFAs were noted in the 0–10 cm mineral layer (Table S4). Similarly, bacterial and gram-negative bacteria PLFAs both decreased ( $p \leq 0.05$ ) after warming in the 10–20 cm mineral horizon relative to the control (Table S5). No fungal PLFAs were detected in the 10–20 cm mineral layer in both, the control and heated soil samples (Table S5).

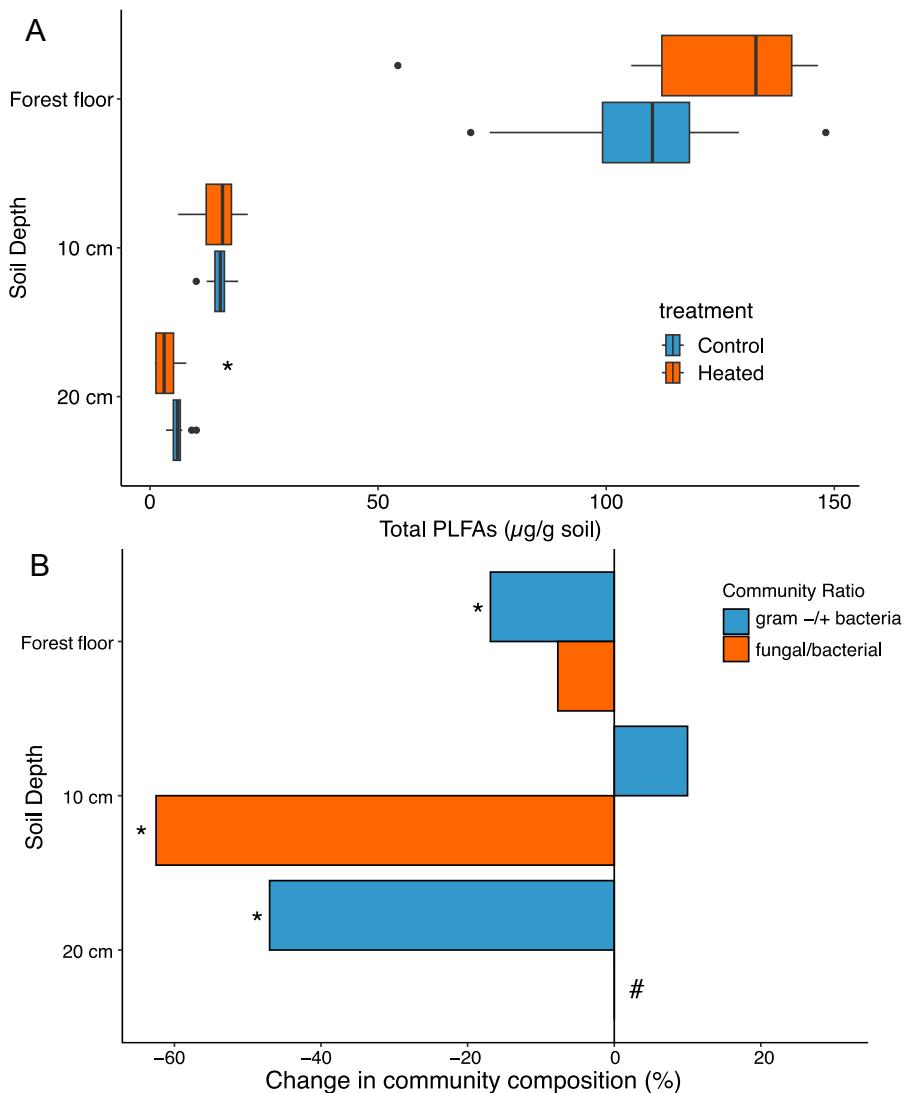
Shifts in community composition were also assessed using ratios of fungal to bacterial PLFAs, as well as gram-negative to gram-positive bacteria.

The ratio of fungi to bacteria significantly decreased relative to the control in the 0–10 cm mineral horizon (Fig. 5B). The ratio of gram-negative to gram-positive bacteria decreased ( $p \leq 0.05$ ) in the forest floor and 10–20 cm soil depth but the fungal/bacterial ratio was not significantly altered (Fig. 5B). The cy17:0/16:1 $\omega$ 7c, cy19:0/18:1 $\omega$ 7c, and monoenoic/saturated PLFA ratios are indicative of microbial stress and have been shown to increase with substrate limitation, shifts in community composition, and changes in biomass size (Willers et al. 2015). The ratio of monoenoic to saturated PLFAs significantly increased in the 0–10 cm mineral soil depth (Table S4) but decreased ( $p \leq 0.05$ ) in the 10–20 cm depth (Table S5). In addition, the cy19:0/18:1 $\omega$ 7c ratio decreased ( $p \leq 0.05$ ) with warming in the 10–20 cm relative to the control (Table S5). No significant differences were observed in the forest floor for any of the stress ratios (Table S3).

## Discussion

Overall, we observed several significant changes to the SOM molecular-level chemistry after 18 years of warming. These findings are consistent with other studies that reported changes to SOM chemistry with concomitant shifts in C inputs and microbial CUE (Pisani et al. 2015; DeAngelis et al. 2015; vandenEnden et al. 2021). For example, Stoica et al. (2023) reported distinct SOM compositional changes as well as differences in microbial community composition that accelerated SOM degradation in the forest floor after 14 years of warming. Similarly, Pisani et al. (2015) observed enhanced SOM decomposition but no notable changes in organic C concentrations. In our study, we obtained evidence of enhanced SOM degradation that occurred with chronic soil warming. For instance, an increase in overall SOM decomposition (alkyl/O-alkyl C ratio) was noted with warming, in both the forest floor and mineral layer, via analysis of the C composition using solid-state  $^{13}\text{C}$  NMR (Fig. 2; Baldock et al. 1992; Simpson and Simpson 2012). This observation is consistent with the first part of our hypothesis as well as previous studies which showed that continued warming accelerates SOM degradation and alters C cycling (Conant et al. 2011; Ofiti et al. 2021; Stoica et al. 2023). The

**Fig. 5** **A** Total microbial biomass (PLFA concentrations) measured in the forest floor, 0–10 cm, and 10–20 cm layer samples; **B** Percent change relative to the control in microbial community composition as measured in the forest floor, 0–10 cm, and 10–20 cm layer samples. Asterisk (\*) indicates a change which is statistically significant ( $p \leq 0.05$ ) and “#” indicates no measured difference. Full details of total PLFA concentrations are provided in Tables S3, S4 and S5



enhanced decomposition is mainly attributed to the relative decline observed in *O*-alkyl C components (Table S2), arising mainly from carbohydrates and peptides (Clemente et al. 2012). This reduction suggests an ongoing process of microbial decomposition, particularly of preferred substrates such as cellulose (Hobara et al. 2014). Increased sugar concentrations in the forest floor were also observed and are indicative of active cellulose breakdown into smaller molecules by microbes (Fig. 3B; Jones and Murphy 2007; Bao et al. 2019). The concentration of sugars (Fig. 3B) in the mineral horizons declined, demonstrating the lack of persistence of these components, which were likely used by the

soil microbiota as substrates (Karhu et al. 2010; Pisani et al. 2015; Bao et al. 2019).

We also observed increased lignin-derived compounds (Fig. 4B), and long-chain ( $\geq C_{20}$ ) *n*-alkanoic acids (Fig. 3A) in the forest floor (Table S3) which is further evidence for increased above-ground plant inputs with warming (Hedges and Mann 1979; Kögel-Knabner 2002; Simoneit 2005; Otto and Simpson 2007). This accumulation of plant-derived aliphatic lipids (Fig. 3A; Table S3) is indicative of increased C inputs from the overlaying vegetation, which has been previously observed with soil warming (Cramer et al. 2001; Liu et al. 2004). In addition, there was an increase ( $p \leq 0.05$ ) in alkyl C compounds (Table S2)

arising from aliphatic lipids and plant biopolymers, such as cutin, which are common in leaf cuticles from various plants (Goñi and Hedges 1990; Simpson et al. 2008; Simpson and Simpson 2012). Moreover, the cinnamyl/vanillyl ratio increased ( $p \leq 0.05$ ; Table S3) in the forest floor after warming. As suggested by Feng et al. (2008), this increase is likely attributed to a higher input of cinnamyl compounds (associated with leaves) rather than the degradation of more persistent vanillyl phenols (Hedges et al. 1988; Opsahl and Benner 1995). Although there were notable differences observed for some lignin-derived compounds in the forest floor (Fig. 4B; Table S3), NMR analysis did not reveal any significant changes in the aromatic and phenolic region (Table S2) suggesting that lignin-derived SOM was relatively insensitive to warming which is in contrast to other studies (Feng et al. 2008; Pold et al. 2015; Schnecker et al. 2016; vandenEnden et al. 2021).

Our findings collectively suggest that despite the increase in the concentration of several plant-derived compounds, overall enhanced SOM decomposition was observed with depth, suggesting enhanced microbial processing with warming that was not offset by additional plant C inputs. Interestingly, we did not observe any increases in microbial biomass (Fig. 5A) with warming, in partial contrast to our hypothesis, but changes to microbial community composition (Fig. 5B) were observed. Alterations in microbial community composition align with the second part of our hypothesis and are likely indicative of potential shifts in CUE and substrate utilization (Frey et al. 2008; DeAngelis et al. 2015; vandenEnden et al. 2021). In a warming study conducted at Harvard Forest by Pold et al. (2017), the size of the microbial community was not altered; however, there were notable changes in CUE which resulted in reductions in the concentration of different SOM components. Moreover, we identified a shift in the ratio of gram-negative to gram-positive bacteria (Fig. 5B), further indicating a potential response to changes in substrate availability (Fanin et al. 2019; Stoica et al. 2023). The alteration in bacterial community composition may be driven by the breakdown and utilization of different SOM components, as gram-positive bacteria are often associated with more efficient decomposition of complex organic matter such as cellulose (Griffiths et al. 1999; Feng and Simpson 2009). This is especially relevant in the forest floor, where microbes

may have acclimated to a continuous supply of plant-derived organic matter with warming. As previously mentioned, the overall accumulation of total sugars in the forest floor and the increased degradation of cellulose (*O*-alkyl C region) suggests altered substrate utilization patterns, with microbes showing a preference for these additional C sources. These findings support the observations made by Anthony et al. (2021) who reported changes in community composition with long-term soil warming, potentially influencing substrate utilization patterns. In addition, a long-term warming study carried out by Melillo et al. (2017) hypothesized that fluctuations in the rate of soil C emissions may be associated with microbial community reorganization and acclimation to warmer temperatures. Therefore, the observed shifts in microbial community composition, coupled with the lack of changes in microbial biomass and accelerated SOM decomposition, suggest microbial reorganization in response to a greater availability of preferred substrates, leading to an overall change in soil biogeochemistry (Frey et al. 2008, 2013).

The impacts of long-term warming on soil dynamics also showed a complex interplay between microbial communities and SOM chemistry, revealing unique trends in soil C biogeochemistry with depth. The mineral soils showed evidence of advanced SOM degradation with long-term warming, but the biochemical processes differed in comparison to the forest floor. For example, plant-derived SOM components, such as sugars (Fig. 3B), steroids (Table S4), long-chain aliphatic lipids (Fig. 3A), and compounds derived from cutin (Fig. 4A) decreased in the mineral soils. These decreases were most intense in the 0–10 cm layer, contrasting with the trends observed in the forest floor, and suggests that these SOM components are not accumulating in the mineral soil and may be preferentially degraded by microbes (Otto and Simpson 2006a, b; Lagomarsino et al. 2006). Interestingly, microbial-derived lipids in the 0–10 cm soil depth (Fig. 4A; Table S4) exhibited decreased concentrations ( $p \leq 0.05$ ), in contrast to Stoica et al. (2023) who reported an increase in these compounds for a different soil warming experiment at Harvard Forest. Although not much is known about the stability of these compounds, our results suggest that they may undergo utilization and recycling processes, potentially indicating their lack of long-term persistence; however, further research is required to gain

insight into the stability of these microbial-derived compounds.

In contrast, warming impacted SOM chemistry in the 10–20 cm soil layer uniquely compared to the upper layers, possibly reflecting reduced biological activity at this depth. Overall, enhanced SOM degradation was observed for the 10–20 cm mineral layer, along with a significant decrease in microbial biomass (Fig. 5A), which uniquely altered specific SOM chemical indices compared to other soil layers (Figs. 3 and 4). Most chemical changes were observed in the 0–10 cm mineral layer and the forest floor, where microbial communities may be actively responding to a continuous influx of organic matter without altering their biomass. The absence of observed microbial responses in the upper layers are in agreement with prior research and observations made for other soil warming experiments at the Harvard Forest LTER site (Frey et al. 2008; Stoica et al. 2023). For instance, vandenEnden et al. (2021) reported a lack of variation in overall microbial biomass in response to warming, suggesting that increased SOM degradation may be attributed to shifts in substrate utilization. This implies some aspect of microbial reorganization in response to long-term warming that enables enhanced SOM degradation without a corresponding rise in microbial biomass (Bradford et al. 2008). It is also evident that the degree of acclimation to prolonged soil warming allowed microbes to display a preference for typical substrates rather than diversifying their resources. Thus, our findings suggest that the overall composition of SOM represents a state where despite the increase in organic matter inputs and the absence of significant alterations in microbial biomass following 18 years of warming, there is a clear augmentation in overall SOM decomposition rates. This highlights the intricacies of the ecological responses of SOM chemistry to prolonged warming.

## Conclusion

Overall, this research stands as a pioneering biogeochemical study, representing a comprehensive molecular-level analysis of a long-term warming experiment that encapsulates entire tree root systems. We found evidence of increased above-ground plant inputs which did not offset SOM decomposition at the Barre Woods site during the extended

warming period. This challenges assumptions that a greater influx of organic matter inputs could contribute to the mitigation of SOM degradation. Moreover, our molecular-level analysis revealed significant alterations in SOM chemistry, including enhanced degradation and shifts in microbial community composition. These findings suggest that microbial community changes in response to chronic warming result in perturbations to the natural biogeochemical cycles that microbes mediate within soils. As microbial communities adjust their composition and activity to efficiently decompose SOM components, they play a significant role in influencing the overall dynamics of soil C cycling. Notably, our findings suggest that warming actively accelerates SOM decomposition, prompting substantial shifts in its chemistry which will further alter the long-term biogeochemical trajectory of soil C. We also noted that the overall treatment-induced changes to the molecular-level SOM composition exceeded any variability from subplot samples within the Barre Woods large experimental plots which include entire root systems and trees. Our study introduces a novel perspective and crucial insights into the impacts of extended warming on soil dynamics and highlights the importance of long-term monitoring to accurately predict future changes in soil C cycling and storage.

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**Data availability** All soil organic matter compound concentrations and solid-state  $^{13}\text{C}$  NMR spectra are listed in the supplementary materials. Raw data files can be obtained by contacting the corresponding author.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

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