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Three decades of continuous warming in temperate forests destabilizes persistent forms of soil organic matter

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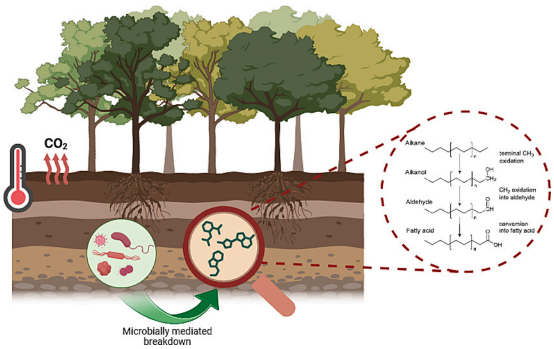
HIGHLIGHTS

- Sustained soil organic matter breakdown after 32 years of warming.
- Persistent plant-derived *n*-alkanes declined under chronic soil warming.
- Shifts in microbial carbon utilization strategies and efficiency.
- Enhanced degradation of chemically stable organic matter.
- Revealed a mechanism capable of destabilizing persistent soil carbon pools.

GRAPHICAL ABSTRACT



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ABSTRACT

Rising temperatures have altered the balance of soil carbon stored versus respired in forest ecosystems worldwide. Yet, the molecular-level mechanisms driving changes to soil biogeochemical processes and the long-term changes to soil organic matter (SOM) dynamics remain unclear. Thus, we leveraged the world's longest soil warming experiment, spanning over three decades in a temperate forest, to investigate changes in SOM chemistry and microbial responses. Using advanced molecular-level techniques, we identified significant perturbations to SOM composition and novel shifts in microbial degradation pathways. Chronic warming enhanced the breakdown of plant-derived lipids typically thought resistant to microbial decomposition. Concomitant shifts in microbial communities indicate altered carbon use strategies, with microbes acclimating to warming and increasingly targeting persistent compounds. We provide new molecular-level evidence that chronic warming disrupts chemically resistant carbon compounds through altered microbial breakdown, revealing mechanisms by which persistent SOM pools may be lost. This further demonstrates that long-term stability is not solely governed

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by intrinsic chemical properties. Our findings underscore the need to fully elucidate long-term microbial functional shifts and their impacts on the stability of persistent SOM pools in a changing world.

1. Introduction

Forest soils are major components of the global carbon (C) cycle, storing up to 47% of total C in temperate forest regions, surpassing stocks from above-ground vegetation and the atmosphere (Goodale et al., 2002; Pan et al., 2011; Scharlemann and Kapos, 2014). Forest soils also play a crucial role in soil surface-atmosphere exchange of greenhouse gases making them a critical buffer against climate change (Grandy and Neff, 2008; Kuzyakov, 2011; Melillo et al., 2017; Schmidt et al., 2011). Much of this C is stored as soil organic matter (SOM), a heterogeneous mixture of plant-, animal-, and microbial-derived compounds that vary in chemical composition, stability, and susceptibility to decomposition (Angst et al., 2021; Bonan, 2008; Lal, 2005; Lehmann and Kleber, 2015). SOM is essential for regulating the biogeochemical cycling of nutrients (Amelung et al., 2008; Dove et al., 2021), and sustaining soil fertility (Batjes, 1996; Lal, 2004). The balance between soil C storage and respiration is mostly governed by above-ground plant inputs and their microbial transformations, which together control the rates of SOM decomposition and persistence (Cotrufo et al., 2015; Prescott, 2010). However, anthropogenic stressors and global environmental change increasingly threaten this balance and the capacity of soils to support key ecological functions (Crowther et al., 2016; Hicks Pries et al., 2017; Reichstein et al., 2013). Although forest soils serve as one of the largest terrestrial C reservoirs (Goodale et al., 2002; Pan et al., 2011; Schlesinger and Andrews, 2000), significant uncertainty remains regarding the long-term persistence and stabilization of SOM in a warmer world (Lehmann and Kleber, 2015; Schmidt et al., 2011).

Climate-driven changes in soil temperature can influence SOM stability by stimulating microbial activity and enzymatic degradation, and through changes in plant productivity, root biomass, and organic matter inputs (Feng et al., 2022; Lal, 2005; Sokol and Bradford, 2019). Various soil warming studies have shown that elevated temperatures can alter microbial activity leading to elevated carbon dioxide (CO_2) emissions from soils that may further amplify warming (Li et al., 2020; Ofiti et al., 2021; Soong et al., 2021; Wagai et al., 2013). Previous studies have reported sustained soil C losses under warming (Nottingham et al., 2020; Soong et al., 2021) and thermally driven changes in SOM composition (Conant et al., 2011; Feng et al., 2008; Pisani et al., 2015; Stoica et al., 2023; vandenEnden et al., 2021). A long-term investigation by Melillo et al. (2017) reported that soil warming induces a pattern of SOM decomposition and CO_2 fluxes to the atmosphere, with phases of substantial soil C loss and periods of stability. With 26 years of warming in a temperate forest, elevated microbial respiration and CO_2 fluxes resulted in cumulative soil C loss that contributed significantly to net ecosystem C emissions, reinforcing the role of soils as a positive feedback to climate warming. These phases were linked to the depletion of labile SOM pools, with microbial acclimation and shifts in carbon use efficiency (CUE) playing a key role in mediating decomposition rates (Melillo et al., 2017). Other studies have found variable soil C responses (DeAngelis et al., 2015; Deslippe et al., 2012; Frey et al., 2008; Pold et al., 2017; Romero-Olivares et al., 2017), likely linked to microbial community reorganization in response to warming. Furthermore, changes in soil C storage and respiration that may be attributed to shifts in microbial CUE have resulted in altered SOM chemistry (DeAngelis et al., 2015; Frey et al., 2013; Frey et al., 2008; Melillo et al., 2017). Microorganisms play a central role in SOM transformation and are themselves sensitive to warming. Thus, warming-induced shifts in microbial community structure or CUE can modify the pathways and rates of SOM turnover, with implications for C storage, CO_2 fluxes, and nutrient cycling (DeAngelis et al., 2015; Frey et al., 2013; Frey et al., 2008; Melillo et al., 2017; Melillo et al., 2011; Wang et al., 2012). This highlights the complexity of SOM responses to prolonged warming and underscores the need for detailed, molecular-level assessments of SOM composition and transformation.

As temperature continues to rise, understanding how soil warming impacts SOM dynamics is essential for predicting future C-climate feedbacks. Importantly, the source of organic matter is a key factor. Warming-induced changes in plant inputs can influence SOM dynamics by altering litter production, incorporation, chemistry, and below-ground C allocation, which in turn alter substrate availability. Root inputs are widely recognized as major contributors to soil organic C pools in forest ecosystems as they are deposited directly into the soil matrix (Boyle, 2005; Huang et al., 2020). Compared to above-ground litter, the below-ground pathway is believed to result in a greater proportion of C being incorporated into stable SOM fractions (Huang et al., 2020; Sokol and Bradford, 2019) due to its proximity to soil minerals and aggregates, which facilitate physical protection and organo-mineral associations (Bowden et al., 2024; Poirier et al., 2018; Rasse et al., 2005; Sokol and Bradford, 2019). Particularly, root-derived aliphatic compounds have been found to be more resistant to decomposition than their leaf-derived counterparts (Bowden et al., 2024; Crow et al., 2009). Consequently, root-derived C is believed to be a large contributor to long-term C storage, emphasizing its importance in building long-lived SOM (Boyle, 2005; Yuen et al., 2013). Because warming can alter plant productivity and below-ground allocation, changes in root-derived C inputs may shift the balance between enhanced decomposition and C stabilization, potentially offsetting or amplifying losses of persistent SOM under long-term warming. However, Munday et al. (2025) highlighted that even forest systems previously considered resilient face unavoidable risks under continued warming, with potential loss of critical C pools despite stabilization efforts. Due to the scarcity of multi-decadal experiments, the consequences of warming on more persistent, long-lived SOM components remain poorly understood. Although SOM is composed of compounds that vary in their reactivity and stability, few long-term studies have directly traced how individual SOM components respond to sustained temperature increases and the processes governing their fate. We lack a mechanistic understanding of how prolonged warming influences the retention of compounds suggested as stable, and whether this can alter the stability of SOM as a whole (Li et al., 2020; Melillo et al., 2017; Soong et al., 2021). While traditional models emphasize the inherent persistence of certain biomolecules, recent perspectives increasingly recognize that SOM stability is not an intrinsic property, but one that is shaped by environmental context, microbial accessibility, and energy constraints on decomposition (Lehmann and Kleber, 2015; Melillo et al., 2017; Schmidt et al., 2011). Despite consistent evidence that warming results in significant C losses (Li et al., 2020; Melillo et al., 2017; Soong et al., 2021), the specific SOM components that persist or degrade after more than three decades of continuous warming remain unclear. Additionally, the underlying mechanisms responsible for long-term C losses are largely unresolved, representing a major knowledge gap in our understanding of SOM stability, soil C sequestration, and soil system functioning under climate change. Addressing this uncertainty requires a comprehensive, molecular-level examination of SOM composition and transformation under multi-decadal warming to reveal how chronic temperature increases reshape the stability and turnover of forest soil C.

This study leveraged the unique, long-term soil warming experiment at the Prospect Hill site in Harvard Forest, Massachusetts, where soils have been continuously heated since 1991. As the longest-running warming experiment globally, this multi-decadal experiment provides a rare and unparalleled opportunity to investigate how 32 years of warming impact SOM composition and dynamics. We apply a combination of molecular-level techniques to examine the processes governing SOM composition and stability. We expected that three decades of soil warming would result in continued SOM degradation and shifts in microbial community composition and their C utilization strategies. We further hypothesized that sustained decomposition likely reduces microbial access to easily degradable compounds, ultimately altering the long-term stability of more persistent forms of SOM. To provide a holistic overview of SOM composition and degradation processes, soils were characterized via elemental analysis, microbial biomass and community composition measurements, nuclear magnetic resonance (NMR) spectroscopy, and targeted SOM compound analysis. By integrating chemical and microbial perspectives, we provide novel insights into the mechanisms driving SOM transformation under chronic warming and reveal how persistent forms of C may become vulnerable over time. Collectively, our findings provide a critical multi-decadal perspective on soil C cycling under sustained warming, offering new insights into the vulnerability of SOM and the mechanisms that influence its turnover and long-term persistence in a changing climate.

2. Materials and methods

2.1. Site description and sampling

Soil samples were collected from the Prospect Hill soil warming experiment at Harvard Forest in Petersham, Massachusetts, USA (42.54°N, 72.18°W). The site is an even-aged, mixed hardwood forest with red oak (*Quercus rubra*), paper birch (*Betula papyrifera M.*), red maple (*Acer rubrum L.*), and white ash (*Fraxinus americana L.*) as the dominant tree species (Pold et al., 2017). The warming experiment began in April 1991 and consists of 6 × 6 m plots (Melillo et al., 2017; Pold et al., 2017). The Prospect Hill experiment is arranged in six randomized blocks, each containing a heated and a control plot, resulting in six field replicates per treatment (Melillo et al., 2017; Pold et al., 2017). The temperature in the heated plot is consistently maintained at 5 °C above ambient temperatures using resistance cables buried 10 cm deep, running parallel to each other, spaced 20 cm apart (Melillo et al., 2017; Pold et al., 2017). Further information regarding the Prospect Hill experimental design is described in other studies (Bradford et al., 2008; Melillo et al., 2017; Melillo et al., 2002; Peterjohn et al., 1994).

Soil samples were collected in July 2023 after 32 years of experiment. Sampling was conducted within a 5 × 5 m area at the center of each plot. Samples were taken from two depths (forest floor and 0–10 cm of mineral soil) using a tulip bulb corer. Soils were then freeze-dried (Thermo Savant ModulyoD-115) for subsequent molecular analysis. Control plots were used for comparison with heated samples. Six field replicates per treatment and soil depth were collected and analytical duplicates were used.

2.2. Soil carbon and nitrogen measurements

Total soil C and nitrogen concentrations were measured on freeze dried, finely ground soil samples by combusting samples at 950 °C under a stream of oxygen gas using a Thermo Flash 2000 Elemental Analyzer. Each sample from both soil depths (forest floor, and 0–10 cm mineral layer) was analyzed in duplicate. C and nitrogen concentrations are reported as a percent based on soil mass.

2.3. Targeted analysis of SOM compounds and microbial extracts

Targeted compounds were extracted using solvent extraction, base hydrolysis, and copper (II) oxide (CuO) oxidation to quantify total solvent-extractable, cutin-, suberin-, microbial-, and lignin-derived compounds (detailed procedure is provided in the Supplementary Information; Hedges and Ertel, 1982; Otto and Simpson, 2005, 2006a). Briefly, small molecules were first isolated from soils (0.8 g forest floor; 2 g mineral) via solvent extraction using dichloromethane, 1:1 dichloromethane:methanol (v:v), and methanol sequentially (Otto and Simpson, 2005). Solvent-extracted soil residues (0.4 g forest floor; 1 g mineral) were then base hydrolysed in Teflon-lined bombs for 3 h at 100 °C using 1 M methanolic potassium hydroxide to isolate ester-bound lipids associated with cutin and suberin (Goñi and Hedges, 1990). Half of the soil residues from base hydrolysis were used for CuO oxidation by heating at 170 °C for 2.5 h with CuO, ammonium iron (II) sulfate hexahydrate and 2 M sodium hydroxide in Teflon-lined bombs to isolate lignin-derived phenols (Fig. S1; Hedges and Mann, 1979; Otto and Simpson, 2006b). The lignin-derived phenol monomers and dimers released through copper oxidation were then isolated and purified via solid-phase extraction (Pinto et al., 2010).

Phospholipid fatty acids (PLFAs) were extracted using a modified Bligh-Dyer method to measure microbial biomass and community composition (Bligh and Dyer, 1959; Quideau et al., 2016). Membrane lipids were extracted from soil samples (1 g forest floor; 5 g mineral) with methanol, chloroform, and sodium citrate buffer (acidified to pH 4). A silicic acid chromatography column was used to fractionate the extracts into nonpolar lipids, glycolipids and polar lipids by elution with chloroform, acetone and methanol, respectively. 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. Total microbial biomass was calculated by summing together all the PLFA concentrations that are attributed to gram-positive, gram-negative bacteria, actinobacteria, fungi, and arbuscular mycorrhizal fungi (Harwood and Russell, 1984). The full extraction details can be found in the supplementary methods (Text S1).

2.4. Compound identification and quantification

Dried isolated extracts were stored at −20 °C before quantification via gas chromatography–mass spectrometry (GC–MS) using external standards. All targeted SOM extracts were derivatized with *N,O*-bistrifluoroacetamide (≥ 99%) and pyridine (> 99.8%) and using *N,N*-dimethylformamide dimethyl acetal (> 97%) for base hydrolysed extracts only (Otto and Simpson, 2006a). Samples were diluted with hexanes prior to analysis and external standards were prepared and derivatized in the same manner as the extracts for quantification of the targeted compounds. Compound analysis was performed using an Agilent 7890B gas chromatograph with a 5977B mass spectrometer operated in electron impact mode with ionization energy of 70 eV. Samples (1 μL) were injected with an injector temperature of 280 °C onto a HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) using a split injector with helium as the carrier gas at a flow rate of 1 mL min^{-1}. The gas chromatograph operation program was set as follows: 65 °C for 2 min before being increased to 300 °C at a rate of 6 °C min^{-1} and held for 20 min (7 min for PLFA analysis). Data was acquired and processed using Agilent Mass Hunter GC–MS Acquisition (version B.07.03.2129) and the Agilent Enhanced ChemStation software (version F.01.03.2357), respectively. Compound identification was performed using the National Institute of Standards and Technology, Wiley 275, and an in-house mass spectral library. Targeted SOM compounds and PLFAs were quantified based on peak areas from external standards (Otto and Simpson, 2005, 2006b; Otto and Simpson, 2006a). Compound concentrations were normalized to the mass of soil extracted and then further normalized to the soil C concentration to assess changes in SOM compounds relative to the overall soil C pool across treatments. Although these compounds constitute a small proportion of total SOC (Otto and Simpson, 2007), their quantification allows detection of selective losses within chemically distinct C pools that may not be apparent from bulk SOC measurements alone.

2.5. Solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy

Overall SOM composition was characterized using solid-state ^{13}C cross polarization with magic angle spinning (CP-MAS) NMR spectroscopy. Mineral soil samples (8 g) were treated with 10% hydrofluoric acid (HF) to enhance the NMR signal and improve the signal-to-noise ratio by concentrating the organic matter and reducing minerals and paramagnetic materials that may interfere with acquisition (Rumpel et al., 2006; Schmidt et al., 1997). The HF-extracted samples were then repeatedly rinsed with deionized water to remove excess salts and then freeze-dried. All samples were subsequently ground into a fine powder and approximately 250 mg of each sample was packed into a 4 mm zirconium rotor and sealed with a Kel-F cap. Samples were 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 71,000 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 50 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 including alkyl C (0–50 ppm), *O*-alkyl C (50–110 ppm), aromatic and phenolic C (110–165 ppm) and carboxyl and carbonyl C (165–215 ppm; Fig. S2 and S3; Baldock et al., 1992; Baldock and Preston, 1995). The alkyl C region is mainly derived from terminal methyl groups from cutin, suberin side chains and other extractable lipids (Baldock et al., 1992; Simpson et al., 2008). Carbohydrates, peptides and methoxyl C in lignin predominantly resonate in the *O*-alkyl C region, while the aromatic and phenolic C resonances primarily arise from lignin, and aromatic amino acids observed in peptides (Baldock et al., 1992; Simpson et al., 2008). The carboxyl and carbonyl C region originates from fatty acid and amino acid side chains (Baldock et al., 1992; Simpson et al., 2008). After integration, the signal for each chemical shift region was normalized to the total signal and expressed as a percentage, allowing for the comparison of relative abundances across resonance regions. Additionally, the proportion of alkyl to *O*-alkyl C (alkyl/*O*-alkyl C ratio) was used to assess the relative stage of SOM decomposition (Baldock et al., 1992; Simpson et al., 2008; Simpson and Simpson, 2012). This ratio increased with SOM degradation as *O*-alkyl C compounds are typically preferentially degraded over alkyl C compounds (Baldock and Preston, 1995; Simpson and Simpson, 2012).

2.6. *Data and statistical analyses*

Statistical tests were carried out using IBM SPSS statistics software (version 28). Analyses were performed on samples collected from six field plots per treatment (heated and control), and each plot sample was analyzed in duplicate (two analytical replicates), resulting in 12 measurements per treatment per soil depth ($n = 12$). Shapiro-Wilk and Levene's tests were used to assess normality and homogeneity of variance, respectively. A mixed model analysis of variance (ANOVA) was used to determine the differences in SOM and microbial indices between the control and heated samples. For this analysis, field replicates ($n = 6$) and analytical replicates ($n = 2$) were considered the within-subject factor to account for variability, 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 nitrogen concentration, as well as NMR data were analyzed in the same manner. Shifts in SOM composition were evaluated based on relative changes to the control data, which served as a reference for identifying significant variations. Additionally, principal component analysis (PCA) was conducted to explore multivariate relationships between SOM molecular components and microbial indices across treatments. PCA biplots were generated separately for the forest floor and mineral soil to visualize patterns of co-variation among variables and treatments (Fig. S4).

3. Results

3.1. *Warming substantively alters SOM composition and microbial communities*

Soil C and nitrogen concentrations exhibited a downward trend in response to warming in the forest floor and mineral soils (Fig. 1A, B, Table S1) but were not significantly different relative to the control. However, several significant changes were observed in the molecular-level composition of SOM. The relative degree of SOM decomposition, as measured by the NMR alkyl/*O*-alkyl C ratio, significantly increased ($p \leq 0.05$) in the mineral layer with warming (Fig. 1C, Table S2; Baldock et al., 1992; Simpson et al., 2008). In contrast, chronic warming did not significantly impact SOM degradation in the forest floor (Fig. 1C, Table S2). Consistent with these results, PCA biplots show distinct treatment-associated shifts in SOM components between the forest floor and mineral layer, highlighting their diverse responses to warming (Fig. S4).

Warming resulted in a significant reduction in total microbial biomass in the forest floor, whereas no significant difference was detected in the mineral layer (Fig. 2A, Table S3, S4). Along with total biomass, the concentration of saturated, monoenoic, gram-positive, gram-negative and total bacterial PLFAs in the forest floor all decreased with warming ($p \leq 0.05$; Table S3). However, microbial community composition (evaluated using ratios of fungal-to-bacterial and gram-negative-to-gram-positive bacterial PLFAs) did not differ significantly between treatments at either depth (Table S3, S4). Further, PLFA-based stress indicators (*cy*17:0/16:1ω7c and *cy*19:0/18:1ω7c) did not suggest elevated microbial stress with warming (Table S3, S4). Despite evidence of accelerated SOM degradation in the mineral soil, the *cy*17:0/16:1ω7c ratio significantly decreased ($p \leq 0.05$) indicating a lack of any microbial stress with changes in SOM chemistry (Table S4). Additionally, short-chain ($< C_{20}$) acyclic lipids (*n*-alkanols and *n*-alkanoic acids) were detected and are predominantly of microbial origin (Lichtfouse et al., 1995; Otto et al., 2005). While the abundance of these lipids declined with warming at both soil depths, they did not significantly differ when compared to the control (Fig. 2B; Table S3, S4). In addition, microbial-derived lipids, consisting of C_{14}-C_{18} branched alkanoic acids from cellular and extracellular microbial sources (Jia et al., 2019; Otto and Simpson, 2007) increased ($p \leq 0.05$) in abundance in the mineral layer with warming (Fig. 2C, Table S4). Contrastingly, no significant differences were observed for microbial-derived lipid concentrations in the forest floor between treatments (Fig. 2C, Table S3).

3.2. *Warming enhanced decomposition of plant-derived compounds including persistent forms*

Detected SOM compounds of plant origin included long-chain ($\geq C_{20}$) aliphatic lipids (*n*-alkanes, *n*-alkanols, *n*-alkanoic acids, and *n*-alkane-α,ω-dioic acids), cyclic lipids (terpenoids and plant-derived steroids), and simple sugars (Lichtfouse et al., 1995; Otto et al., 2005). In the forest floor, concentrations of long-chain lipids generally declined following warming, with significant decreases detected for total lipids, *n*-alkane-*α,ω*-dioic acids, and long-chain *n*-alkanes (Fig. 3A, Table S3). Notably, long-chain *n*-alkane concentrations were reduced by more than 50% in heated soils relative to control (Fig. 3A, Table S3). The 0–10 cm mineral layer exhibited a similar trend, with a significant reduction ($p \leq 0.05$) in the abundance of long-chain *n*-alkanes with warming (Fig. 3A, Table S4). Particularly, the concentration of C_{27} and C_{29} *n*-alkanes, which can be traced back to roots and other plant waxes, decreased ($p \leq 0.05$) in the heated soils compared to the control (Fig. 4A, Table S5). In contrast, long-chain *n*-alkanol concentrations increased in the mineral soils following warming (Fig. 4A, Table S4). Notably, C_{26} and C_{30} *n*-alkanol concentrations exhibited the most pronounced increases ($p \leq 0.05$) with warming (Fig. 4A, Table S5). Furthermore, the *n*-alkane/*n*-alkanol ratio decreased ($p \leq 0.05$) by more than 50% in the heated

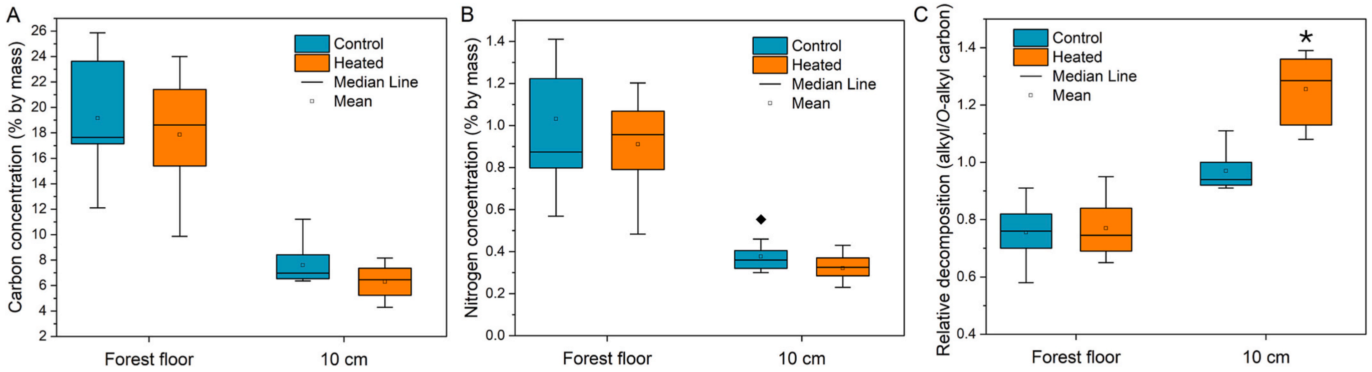

Fig. 1. A) Distribution of total carbon concentration (% by mass); B) total nitrogen concentration (% by mass); C) NMR ratio (alkyl/*O*-alkyl C) used to assess the relative state of SOM decomposition. Values were determined by solid-state ^{13}C cross polarization with magic angle spinning as measured on the forest floor and mineral soils (0–10 cm). Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) and diamond symbols (◆) denote data points that fall outside the boxplot whisker range. Detailed data is provided in Tables S1 and S2.

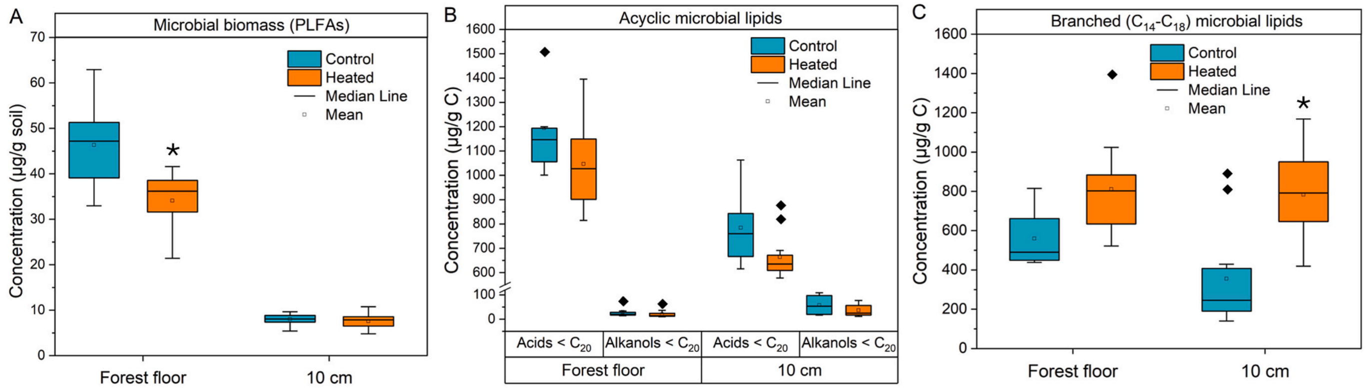

Fig. 2. A) Total microbial biomass (PLFA concentrations); B) microbial-derived short-chain ($< C_{20}$) acyclic lipid (*n*-alkanols and *n*-alkanoic acids) concentrations; C) microbial-derived branched lipid concentrations (consisting of $C_{14} - C_{18}$ alkanoic acids) measured within the forest floor and 0–10 cm mineral layer samples. Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) and diamond symbols (◆) denote data points that fall outside the boxplot whisker range. Detailed data are provided in Tables S3 and S4.

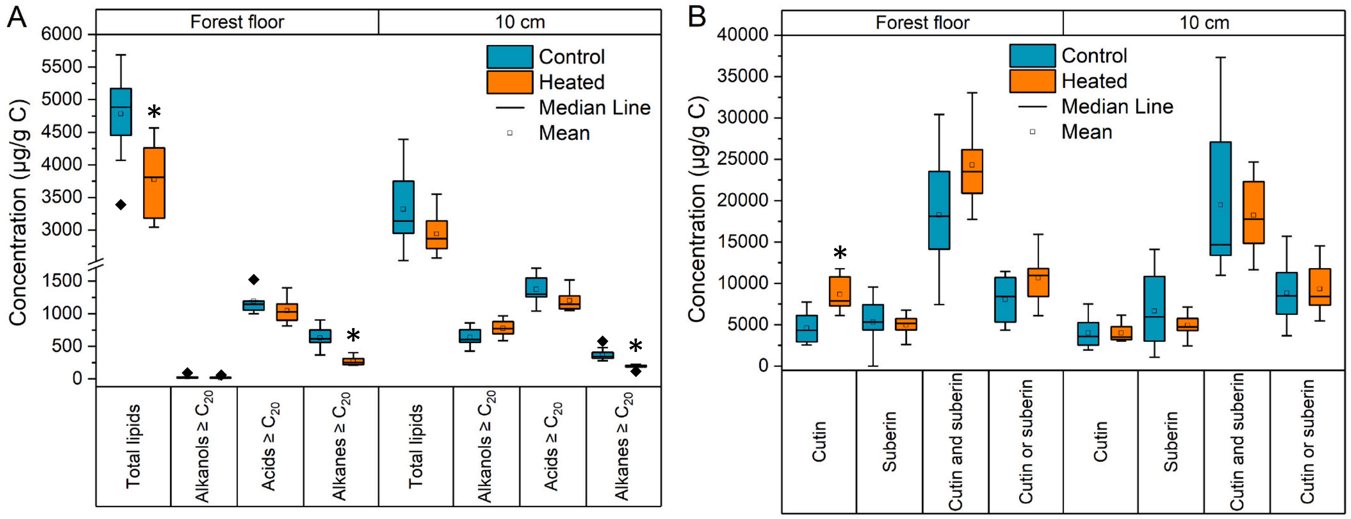

Fig. 3. A) Concentration of total (sum of all *n*-alkanes + *n*-alkanols + *n*-alkanoic acids + *ω*-hydroxyalkanoic acids + *n*-alkane-*α*,*ω*-dioic acids) and long-chain ($\geq C_{20}$) aliphatic lipids (compounds consisting *n*-alkanes, *n*-alkanols and *n*-alkanoic acids) normalized to carbon concentration; B) Total cutin-, and suberin-derived compound concentrations measured within the forest floor and 0–10 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. Asterisks (*) indicate statistically significant ($p \leq 0.05$) differences relative to the control and diamond symbols (◆) denote data points that fall outside the boxplot whisker range. Detailed data are provided in Tables S3 and S4.

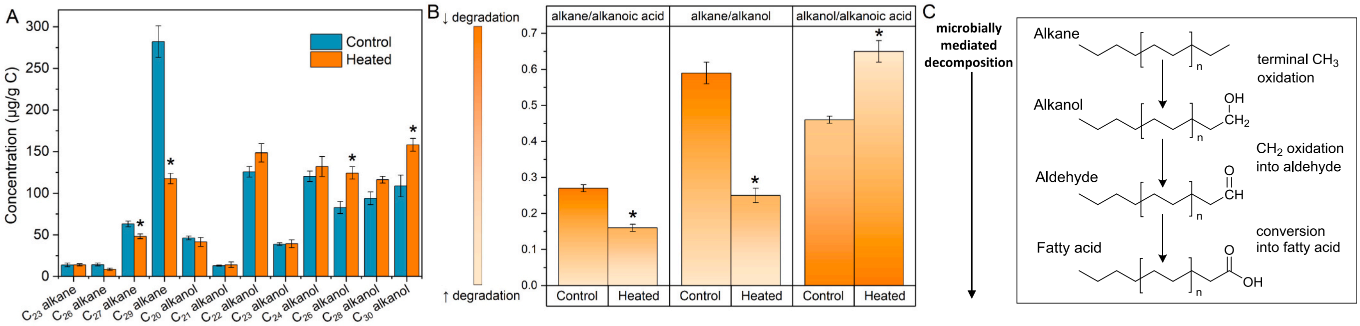

Fig. 4. A) Concentration of specific chain-lengths for *n*-alkanes and *n*-alkanols in the 0–10 cm mineral layer; B) Aliphatic lipid ratios consisting of *n*-alkane/*n*-alkanoic acid, *n*-alkane/*n*-alkanol and *n*-alkanol/*n*-alkanoic acid in the 0–10 cm mineral layer; C) Proposed pathway for the aerobic degradation of alkanes by microbes. Asterisks (*) indicate statistically significant ($p \leq 0.05$) differences relative to the control plots. Detailed data are provided in Tables S3, S4 and S5.

samples compared to the control (Fig. 4B, Table S5). Similarly, the *n*-alkane/*n*-alkanoic acid ratio significantly decreased with warming, while the *n*-alkanol/*n*-alkanoic acid ratio increased ($p \leq 0.05$) in the 0–10 cm layer after warming (Fig. 4B, Table S5).

Compounds characteristic of SOM derived from above-ground inputs (cutin from leaf litter), below-ground inputs (suberin from roots), or a combination of both cutin and suberin biopolymers were detected (Kögel-Knabner, 2002; Otto and Simpson, 2006a). After warming, the forest floor exhibited a general increase in plant-derived compound concentrations, however, only the abundance of cutin-derived components increased significantly (Fig. 3B, Table S3). In contrast, cutin-derived compounds were not significantly impacted with heat in the 0–10 cm mineral layer. Moreover, the concentration of lignin-derived compounds showed no significant variations in total or individual compound classes (vanillyl, syringyl, and cinnamyl phenols) between treatments in either the forest floor or mineral layer (Table S3 and S4). However, the forest floor exhibited a significant ($p \leq 0.05$) increase in the acid-to-aldehyde ratio for vanillyl ($(Ad/Al)_v$), indicating enhanced oxidative transformation without detectable net losses of lignin-derived phenols under warming (Table S3).

4. Discussion

Soil organic C and nitrogen concentrations trended downward in the forest floor and mineral soil following thirty-two years of warming aligning with previous studies that have documented losses in total soil C and nitrogen stocks at the Prospect Hill site (Bradford et al., 2008; Melillo et al., 2017). This is also consistent with increased CO_2 emissions reported by Melillo et al. (2017), where sustained warming triggered intermittent phases of elevated decomposition and C releases to the atmosphere. These fluxes reflect cumulative losses of soil C over time, consistent with the molecular-level shifts in SOM chemistry observed in our study and in other warming studies reporting altered SOM composition alongside changes in C inputs (Pisani et al., 2015; Sistla et al., 2013; vandenEnden et al., 2021). Moreover, evidence of enhanced SOM degradation in the forest floor and mineral layer supports our hypothesis regarding the sustained breakdown of SOM components with 32 years of warming. The relative stage of SOM decomposition (alkyl/*O*-alkyl C ratio) was less responsive in the forest floor than in the mineral layer, likely reflecting warming-induced increases in plant inputs to the forest floor (Baldock et al., 1992; Simpson and Simpson, 2012). Several studies have indicated that above-ground inputs can increase with soil warming (Cramer et al., 2001; Feng et al., 2008; Kengdo et al., 2023; Liu et al., 2004). Thus, the increased abundance of cutin-derived compounds (associated with leaf litter) in the forest floor following warming, is likely an indicator of enhanced surface litter inputs that offset compositional differences from accelerated SOM degradation, thereby masking the overall impacts. Similarly, the lack of significant changes in lignin phenol concentrations does not necessarily imply persistence. Instead, elevated lignin oxidation indices ($(Ad/Al)_v$ ratio) in the forest floor indicate enhanced oxidative processing under warming, suggesting that increased degradation may be offset by above-ground inputs.

In the mineral layer, SOM degradation was notably enhanced as shown by the significant decline in cellulose and other readily decomposable microbial substrates (*O*-alkyl C; Baldock and Preston, 1995; Clemente et al., 2012; Simpson and Simpson, 2012) suggesting microbial reorganization in response to different substrate availability. This illustrates that responses to warming are depth-dependent, likely reflecting the balance between fresh plant inputs, substrate composition, and microbial processing dynamics that differ fundamentally between the forest floor and mineral soil. These depth-dependent relationships are also reflected in the PCA distribution of SOM components and microbial indices (Fig. S4). Moreover, plant-derived lipids, which are typically degradation products of plant biopolymers (cutin and suberin), decreased significantly in the mineral layer, despite being widely considered as persistent and chemically stable in soil (Kögel-Knabner, 2002; Otto and Simpson, 2006a; Schmidt et al., 2011). These findings align with previous studies demonstrating that sustained warming accelerates SOM degradation and disrupts C cycling (Conant et al., 2011; Knorr et al., 2024; Ofiti et al., 2021; Stoica et al., 2023). However, the patterns of perturbations to SOM composition reflect microbial C use strategies that have not been identified previously with respect to persistent forms of SOM.

Microbial responses to changes in the SOM composition were evident, further supporting our hypothesis regarding shifts in microbial communities and reinforcing earlier observations at this site (Bradford et al., 2008; Frey et al., 2008; Melillo et al., 2017; Pold et al., 2017). The reduction in microbial biomass as well as the specific decline in gram-positive and gram-negative bacteria in the forest floor were likely driven by the accelerated decomposition of easily degradable organic matter during the early phases of this experiment (Melillo et al., 2017). This shift in SOM composition may have favoured microbes capable of degrading complex compounds, such as fungi, whose abundance was not impacted by heat (Feng and Simpson, 2009). Prior research at this site has reported warming-induced shifts in microbial biomass, community composition, and substrate utilization (Bradford et al., 2008; DeAngelis et al., 2015; Frey et al., 2008; Melillo et al., 2017), likely driven by decreased access to labile C pools (Allison et al., 2010; Bradford et al., 2008). Thus, the significant accumulation of microbial-derived lipids in the mineral soil suggests changes in substrate utilization resulting in increased turnover of plant-derived organic matter (DeAngelis et al., 2015; Frey et al., 2008; vandenEnden et al., 2021). Observed correlations among microbial indices and SOM components further support this interpretation, with the PCA showing alignment between microbial-derived lipids and SOM degradation indicators under warming (Fig. S4). The increased abundance of microbial lipids is consistent with findings by Ding et al. (2019), who reported significantly increased total microbial residues across soil depths, particularly in surface soils (0–10 and 10–20 cm), under warming conditions. Additionally, the accumulation of microbial-derived lipids in the mineral soil likely reflects enhanced microbial breakdown of plant material which could explain why, despite increased above-ground inputs, cutin-derived compounds are not persisting at lower soil depths. This could indicate altered CUE of decomposed plant material due to warming (Frey et al., 2008; Melillo et al., 2017; Schindlbacher et al., 2011), potentially driven by enhanced extracellular enzyme function capable of degrading both labile and more resistant organic matter (Ma et al., 2023). This interpretation is supported by findings from Santos et al. (2019), who showed that elevated temperatures can stimulate microbial metabolism, leading to increased nutrient turnover and higher microbial respiration rates. Consequently, increased CUE could result in greater production and deposition of microbial-derived lipids into the soil. Moreover, studies have reported that microbial stress may occur in response to sustained changes in overall microbial community growth and composition (Frostegård et al., 2011; Wixon and Balser, 2013) or as a result of substrate limitations (Bossio and Scow, 1998; Feng and Simpson, 2009). Here, we did not observe significant changes in microbial growth or community composition in the mineral soil, suggesting that any stress responses would be more likely linked to limitations in substrate availability. The decline in microbial indices commonly associated with substrate availability (monoenoic PLFAs; Feng and Simpson, 2009; Kieft et al., 1994; Zelles et al., 1992) further supports reduced access to easily degradable organic matter. However, despite evidence for reduced access to labile substrates, the microbial stress indices ($cy17{:}0/16{:}1\omega7c$ and $cy19{:}0/18{:}1\omega7c$) did not indicate elevated microbial stress under warming, suggesting that microbes may have adjusted their C utilization strategies without experiencing any strain. The apparent disconnect between reduced availability of labile C and the absence of microbial stress suggests that microbes reorganized by shifting their C utilization strategies toward the decomposition of more persistent SOM pools.

The observed molecular-level changes in SOM chemistry and microbial C utilization strategies indicate that persistent substrates may have become increasingly targeted for microbial degradation under sustained warming. One of the most prominent and novel findings was the significant decline in long-chain *n*-alkane concentrations in the forest floor and mineral layer under chronic warming. Long-chain *n*-alkanes are generally considered to be long-lived and resistant to degradation due to their chemical structure, relative inertness, and hydrophobic nature making them less accessible to soil microorganisms (Baldock et al., 1992; Bush and McInerney, 2013; Huang et al., 1999; Labinger and Bercaw, 2002; Lorenz et al., 2007; Schmidt et al., 2011). These lipids have also been observed to accumulate in various soil types (Feng et al., 2008; Lorenz et al., 2007; Lützow et al., 2006; Nierop, 1998) and be large contributors to older soil organic C pools (Angst et al., 2016). Therefore, contrasting previous studies, our results suggest that prolonged warming has accelerated the decomposition of these plant-derived lipids, supporting our hypothesis that the long-term stability of more persistent compounds would be impacted. Particularly, the abundance of C_{29} *n*-alkanes, derived from plant roots and other waxes, significantly decreased in the mineral layer with warming challenging previous assumptions regarding the persistence of these plant inputs. This enhanced degradation could highlight potential long-term reductions in C storage capacity under warming conditions. Interestingly, although the abundance of most aliphatic lipids declined, the significant increase in long-chain *n*-alkanol concentrations in the mineral soils after warming, coupled with the marked decline in *n*-alkanes, likely reflects a degradation process driven by altered microbial activity. Typically, *n*-alkanes are not preferred growth substrates for microorganism that would rather utilize other compounds before turning to alkanes (Rojo, 2009). However, heat has been shown to alter the C utilization patterns of microbes and reduce their capacity to utilize simple C substrates (Frey et al., 2008; Melillo et al., 2017). Therefore, our results suggest that microbes may be re-directing their C usage from preferred substrates, such as cellulose, to these persistent *n*-alkanes. Previous studies have shown that microbes are capable of aerobically degrading alkanes using enzymes (Fig. 4C; Hussain et al., 2024; Rojo, 2009; Watkinson and Morgan, 1990; Wentzel et al., 2007). Aerobic degradation typically begins with oxidation of the terminal methyl group by alkane hydroxylase, forming a primary alcohol (alkanol). The alcohol is then oxidized by alcohol dehydrogenase into the corresponding aldehyde which is subsequently converted by aldehyde dehydrogenase into a fatty acid (alkanoic acid; Hussain et al., 2024; Rojo, 2009). Thus, the accumulation of long-chain *n*-alkanols suggests a partial progression of this degradation pathway, while the decrease in *n*-alkanoic acids may reflect a bottleneck in downstream oxidation steps or rapid turnover by microbes. Shifts in the ratios between these compounds (declining *n*-alkane/*n*-alkanol and *n*-alkanoic acid ratios) further indicate altered SOM composition and enhanced microbial oxidation of more persistent components. Together, these results suggest altered microbial degradation strategies toward enhanced breakdown of chemically persistent substrates following three decades of warming, leading to the destabilization of SOM components traditionally considered as being more long-lived in soils.

Chronic warming may drive microbes to shift their C use strategies toward less preferred and more persistent compounds through a combination of substrate availability changes, thermal acclimation, altered soil conditions, and changes in microbial communities. Warming can accelerate microbial activity, leading to faster decomposition of labile C (cellulose and other simple sugars; Fanin et al., 2022; Melillo et al., 2017; Schindlbacher et al., 2011). As those preferred substrates become less available, microbes may increasingly rely on chemically stable compounds like *n*-alkanes, not necessarily due to a change in substrate preference but due to altered availability. Warming can also alter soil environments which can impact the accessibility and bioavailability of different C substrates (Pallandt et al., 2025). This may limit diffusion of soluble C, favoring microbes better at breaking down hydrophobic substrates like *n*-alkanes. Additionally, over time, microbial communities may thermally acclimate by adjusting gene expression to favor the production of enzymes capable of degrading complex hydrophobic compounds (monooxygenases and peroxidases; Donhauser et al., 2021, Donhauser et al., 2020). These functional shifts have been observed as increases in genes for complex C degradation and reductions in those for labile substrates use under warming (Domeignoz-Horta et al., 2023; Liu et al., 2024; Melillo et al., 2017). This may shift substrate use toward more persistent compounds even when more labile C is still present. In parallel, warming may result in reorganization toward microbes that are inherently better at breaking down persistent compounds, further shifting community-level substrate use, even if labile substrates are still available (Feng et al., 2008; Jiang et al., 2023; Liu et al., 2024). This aligns with the findings by Frey et al. (2013) who noted increased degradation of C sources more resistant to degradation in a temperate forest following 20 years of soil warming. Moreover, elevated temperatures may activate microbial stress-resilience pathways, including hydrocarbon degradation pathways (Fanin et al., 2022) which incidentally promote *n*-alkane breakdown even if it was not previously a dominant metabolic route. Together, our findings indicate that chronic warming destabilizes persistent plant-derived SOM and reinforce the concept that warming can fundamentally alter microbial processing of plant-derived C (Conant et al., 2011).

5. Conclusions

Our findings indicate that long-term soil warming destabilizes SOM pools often regarded as persistent and long-lasting (Schmidt et al., 2011). The loss of long-chain *n*-alkanes raises questions regarding the hypothesized stability of persistent compounds in future climate change scenarios. While root-derived inputs are typically viewed as a more stable source of soil C relative to above-ground inputs (Angst et al., 2016; Schmidt et al., 2011), our results suggest that under warmer climates, root C may be more vulnerable to degradation than traditionally thought. The results from this study challenge long-standing assumptions that plant waxes and root inputs are inherently stabilized forms of soil C and support the idea that warming not only impacts labile C, but also persistent C pools (Conant et al., 2011; Knorr et al., 2024; Ofiti et al., 2021). The enhanced processing of stable compounds noted in our study may point toward increased turnover of previously stored C and contests the view that chemical stability ensures long-term SOM persistence. Instead, SOM stability appears to depend not only on compound chemistry, but also on environmental conditions, substrate availability, and microbial processing capacity. This also suggests that if persistent SOM compounds can be degraded under warming, then soil C storage capacity, and thus the role of soils as long-term C sinks, may be overestimated in current models. While our results do not determine whether persistent SOM degradation will continue indefinitely or eventually stabilize under prolonged warming, they highlight the importance of multi-decadal research. The vulnerability of compounds considered chemically persistent only became apparent after more than three decades of warming, suggesting that short-term studies may overestimate long-term soil C stability. Moreover, the preferential destabilization of specific SOM components observed here may provide mechanistic context for previously reported intermittent phases of C loss at this site, reinforcing the need for continued monitoring to accurately project soil C-climate feedbacks. Therefore, projections of future soil C-climate feedback should be revised to account for the vulnerability of stable C pools and the reorganization of microbial decomposition strategies under prolonged warming.

CRediT authorship contribution statement

Atzín X. San Román: Writing – original draft, Methodology, Investigation, Formal analysis. **Serita D. Frey:** Writing – review & editing, Resources, Project administration, Investigation. **Melissa A. Knorr:** Writing – review & editing, Resources, Project administration. **Huan Tong:** Writing – review & editing, Software. **Jerry M. Melillo:** Writing – review & editing, Conceptualization. **Myrna J. Simpson:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online and provide details on site characteristics, sample collection, targeted compound extractions, and instrumental analyses. Detailed C and N, NMR, PLFA and targeted compound data are provided in Tables S1-S5, and statistical results are listed in Table S6. Fig. S1 shows total lignin-derived compound concentrations measured within the forest floor and 0–10 cm mineral layer samples. Figs. S2 and S3 show the solid-state average relative signal intensity from ^{13}C nuclear magnetic resonance spectra of all samples. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2026.181777.

Data availability

Data will be made available on request.

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