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Biogeochemical evolution of soil organic matter composition after a decade of warming and nitrogen addition

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Abstract Forest soils are an important carbon (C) sink and critical component of the global C cycle. Warmer temperatures and increased atmospheric nitrogen (N) deposition are altering the biogeochemistry in forest soils and disrupting the intricate balance between C storage and C respired across the globe. The molecular biogeochemistry of soil organic matter (SOM) with warming, N-addition, and simultaneous warming and N-addition was analyzed in soil samples from the Soil Warming × Nitrogen Addition Study at the Harvard Forest Long-term Ecological Research Site using advanced techniques. The results unequivocally demonstrate that warming and N-addition alter the molecular composition of SOM as individual stressors uniquely and in combination. Warming alone and in combination with N-addition accelerated SOM decomposition while N-addition alone slowed SOM degradation. The two-factor N-addition and warming plots contain SOM more like the warming only plots but exhibited unique changes over time (from 4 to 10 years) that could not be predicted by studying N-addition or warming alone. The specific SOM components and the overall SOM decomposition suggests that N-addition and warming impacts are not additive. N-addition may hinder warming impacts

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antagonistically over time but not to the extent where advanced SOM decomposition from warming is supplanted. As such, the results from warming alone and N-addition alone are not necessarily additive compared to the observed SOM molecular compositional changes when these treatments are applied simultaneously. Marked evolution in the molecular biogeochemistry of SOM demonstrates the sensitivity of SOM trajectories to multiple interactive global environmental changes and the continued need to study long-term impacts more holistically.

Keywords Soil warming · Nitrogen addition · Soil carbon storage · Nuclear magnetic resonance · Targeted soil organic matter analysis

Introduction

Forests are important global carbon (C) sinks and can sequester as much as 2.4 Pg of atmospheric C annually (Pan et al. 2011). Up to 70% of forest C is found in forest soils (Lal 2005); however, global change associated with anthropogenic activities, including burning of fossil fuels and agriculture, may have the potential to change the amount of C stored in these soils (Bond-Lamberty and Thomson 2010; Crowther et al. 2016; Yin et al. 2013). Of particular concern are warmer global soil temperatures and increased inputs of nitrogen (N) to forests through atmospheric N deposition (Bond-Lamberty and Thomson 2010; Crowther et al. 2016; Liu et al. 2016). Warming can alter the quantity and quality of plant inputs to forest soils, but at the same time can enhance the kinetics of microbial degradation, reduce forest soil C (Wagai et al. 2013) and shift microbial contributions to SOM. Release of CO_2 from forests via microbial respiration has the potential to drive further warming (Crowther et al. 2016). Furthermore, there is also evidence that over time some microbial communities may acclimate to warmer temperatures, and enhanced respiration may decline over time with changes in available substrates (Melillo et al. 2017; Rousk et al. 2012; Schindlbacher et al. 2015; Sorensen et al. 2018; Walker et al. 2018; Xu et al. 2015). In temperate forests, additional N may also inhibit microbial degradation (Frey et al. 2014), which may lead to accumulation of soil C (Lu et al. 2011; Savage et al. 2013). These two types of environmental stressors and their contrasting impacts on soil C biogeochemistry emphasizes the need to study soil warming + N in tandem to improve the understanding of biogeochemical mechanisms and perturbations to the C cycle with global environmental changes.

The storage of C in forest soils is dependent on microbial degradation of soil C and the preservation of soil C in the form of soil organic matter (SOM) via protection mechanisms, such as mineral association (Doetterl et al. 2015; Kramer and Chadwick 2018). SOM is a complex mixture of molecules from different sources, which have different molecular sizes and are at different states of degradation (Schmidt et al. 2011). SOM is comprised of a variety of compounds from small molecules, such as plant steroids and sugars, to larger biopolymers including cellulose, lignin, suberin or cutin (Kögel-Knabner 2002; Lehmann and Kleber 2015). Not all components of SOM will respond similarly to environmental change and soil warming has been shown to accelerate SOM decomposition via the preferential use of favored substrates by microbes (Feng et al. 2008; Feng and Simpson 2008, 2009). It was previously hypothesized that this enhanced process was short-lived and that this accelerated decomposition would plateau after preferred substrates were exhausted by soil microbiota (Melillo et al. 2002). However, studies have showed that more complex SOM components are also degraded under simulated warming (Pisani et al. 2015; Feng et al. 2008). For example, the enhanced degradation of lignin with short-term soil warming (Feng et al. 2008) as well as the accelerated decomposition of fine woody debris in field plots (Berbeco et al. 2012) have been reported. Furthermore, these changes in SOM composition with warming may not be directly tied to increased microbial biomass (Contosta et al. 2015) but associated with microbial community reorganization with long-term warming (DeAngelis et al. 2015; Melillo et al. 2017).

In contrast, addition of extra N has been reported to selectively preserve lignin while other components of SOM, such as simple sugars are degraded (Liu et al. 2018; Xia et al. 2017). Nitrogen addition has yielded varying results because extra N can promote plant growth and enhance litterfall (Yue et al. 2016), but may also accelerate or suppress microbial decomposition of soil C (Hobbie et al. 2012; Xia et al. 2017). Studies on the composition of SOM with N addition

have indicated no change (Zak et al. 2017) or significant differences in the molecular-level composition (Feng et al. 2010; Pisani et al. 2015; Wang et al. 2019). For example, Wang et al. (2019) reported that 22 years of added N to a temperate forest reduced microbial biomass and selectively preserved plant steroids, lignin-derived, cutin-derived, and suberin-derived compounds. However, the complexity of soil dynamics necessitates that soil warming and N enrichment be studied simultaneously, especially in long-term field studies. For example, soil warming and N-addition as individual treatments can have varying impacts on soil C but when combined, were reported to accelerate the decomposition of cellulose and peptides more than warming alone in a subalpine coniferous forest (Sun et al. 2019). Other studies have reported that the combined impacts of soil warming and N-addition altered soil microbiota (Xiong et al. 2016) and dissolved organic matter quantity and quality (Yuan et al. 2018) more similar to warming alone. In contrast, Pisani et al. (2015) observed that the molecular-level composition of SOM after warming, N-addition, and warming + N was distinct but that warming + N was more similar to N-addition alone. Consequently, there is uncertainty in how these combined anthropogenic impacts may interact and alter SOM composition, especially at the molecular-level. As such, additional studies are required to determine how sustained soil warming and N-addition may alter forest SOM biogeochemistry, to develop an improved mechanistic understanding of soil C cycle shifts with anthropogenic processes.

To investigate changes in soil C storage and SOM composition with simultaneous warming and N-addition, samples were collected from the Soil Warming × Nitrogen Addition (SWaN) Study at the Harvard Forest Long-term Ecological Research (LTER) site (Petersham, MA USA) after 10 years of experiment and analyzed using integrative molecular biogeochemical methods. Specifically, the following hypotheses were tested based on the current state of knowledge: (1) SOM degradation will be enhanced with chronic soil warming (Berbeco et al. 2012); (2) N-addition will suppress SOM degradation (Frey et al. 2014); and (3) the response of SOM to simultaneous warming and N-addition will be more similar to N-addition alone (Pisani et al. 2015). SOM was characterized using advanced, molecular-level techniques including quantification of small molecules by gas chromatography–mass spectrometry (GC–MS) and determination of the overall C structure of soil samples by solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Additionally, soil C storage, soil C and N contents were measured, and microbial biomass and community composition were determined using phospholipid fatty acid (PLFA) analysis. These results from 10 years of simultaneous soil warming and N-addition are compared to previously reported molecular-level data describing the SOM composition after 4 years (Pisani et al. 2015). This approach allows for simultaneous testing of specific hypotheses but also provides unique insight into SOM compositional and degradation trends that occur with long-term environmental changes over time.

Materials and methods

Field experimental design

Samples were obtained from the Soil Warming x Nitrogen Addition experiment at the Harvard Forest Long Term Ecological Research Site in Petersham, Massachusetts (42° 50′ N, 72° 18′ W; Contosta et al. 2011, 2015) which commenced in 2006. The forest is a transitional mixed hardwood forest, with an average annual temperature of 8.5 °C (Savage et al. 2013) and 100 cm of precipitation annually (Nadelhoffer et al. 1999). Soils at this site are classified as typic dystrochrepts of the Gloucester series with a fine loamy texture (Peterjohn et al. 1994). There are four treatments: control, warming, N-addition and warming + N, which have been applied to 3 m by 3 m plots with six replicates per treatment (Contosta et al. 2011; 2015). In the warming and warming + N treatments, soil temperature is elevated 5 °C above ambient using heating cables buried 10 cm below the surface (Contosta et al. 2011). Heating cables were buried in October of 2005 and turned on in August of 2006. In August of 2006, the N-addition and warming + N treatments began receiving applications of aqueous ammonium nitrate at a rate of 5 g of N m^{-2} $year^{-1}$ equally throughout the growing season (May to October; Contosta et al. 2011). The SWaN plots do not contain canopy trees and canopy inputs are similar across all plots. Soil samples were collected in July of 2016, after 10 years of continuous experiment using the same method applied previously (Pisani et al.

2015; Anthony et al. 2020). Samples were obtained from both the forest floor (O horizon) and the upper 10 cm of the mineral horizon in five replicate plots for each treatment. For the O horizon, a 10 cm × 10 cm block was collected to the depth of the mineral soil, and mineral soil was collected to a depth of 10 cm from below this block using a 5-cm diameter cylindrical core (Anthony et al. 2020). Two random samples per plot were collected, combined into one composite sample per plot, and then stored on frozen ice packs in the field (Anthony et al. 2020). Any large roots or other plant debris (> 2 mm) were removed. Samples were then freeze-dried, sieved, and hand-ground into a fine powder prior to analysis.

Soil C storage and related edaphic analyses

Total soil C and N was measured on dried, finely ground soil samples using a Perkin Elmer 2400 Series II CHN elemental analyzer. Soil C and N concentration results are provided in the Supplementary Material (Fig. S1) and were not statistically significant ($p \leq 0.05$). Soil C storage (Fig. 1) was calculated from soil C values, bulk density, and respective horizon depths (3–5 cm for the O horizons and the 0–10 cm layer of the A horizon; data not shown). Fine roots (≤ 2 mm), soil moisture content, soil pH and inorganic N concentrations were measured as reported by Anthony et al. (2020). There were no statistical differences ($p \leq 0.05$) found for any of these measures (Fig. S2) across horizons and treatments (n = 5).

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

Limited sample masses are available from long-term experiments and this is coupled with constraints on NMR instrument time. As such, we prepared composite samples (one composite per treatment for both the forest floor and mineral samples). This facilitated the analysis of averaged responses across treatments. This approach was also used by Pisani et al. (2015) and as such, applied here for consistency and direct data comparisons. It is also important to note that NMR spectra were not replicated given that each NMR spectrum is an average of thousands of individual replicate scans and solid-state ^{13}C NMR is highly reproducible with measurement errors between 1 and 5% (Sun et al. 2019; Usman and Simpson 2021; Dria et al. 2002). The mineral horizon composites were repeatedly treated with 10% hydrofluoric acid to concentrate organic matter and remove minerals (Rumpel et al. 2006). Samples were analyzed by cross polarization-magic angle spinning NMR, using previously published methods (Conte et al. 2004). The resulting NMR spectra, which arise from the repeated acquisition of thousands of scans (Fig. S3), were integrated into four 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 (165–200 ppm; Preston 2014). Integrated areas were normalized to the total NMR signal and expressed as a

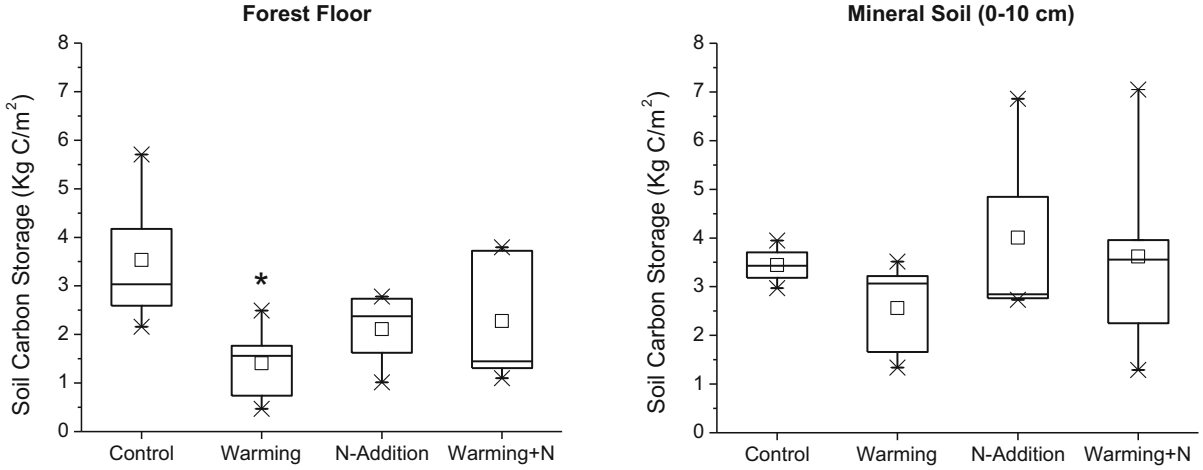

Fig. 1 Soil carbon storage with warming, nitrogen addition, and simultaneous warming + nitrogen for the forest floor and mineral soil (0–10 cm) horizons ($n = 5$). Significant differences relative to the control ($p \leq 0.05$) are indicated with an asterisk

percentage. Alkyl/*O*-alkyl ratios were calculated by dividing the integrated area of the alkyl region by the integrated area of the *O*-alkyl region (Table S1; Baldock et al. 1992). Relative differences were calculated as percentages (based on respective control values) for comparisons (Fig. 2).

Soil organic matter (SOM) compound identification and quantification

Specific molecular components of SOM were extracted sequentially in duplicate from each soil layer and treatment (for a total of $n = 10$). Solvent extraction was used to isolate SOM components including aliphatic and cyclic lipids and simple sugars (Otto and Simpson 2005). Cutin- and suberin-derived compounds were isolated after base hydrolysis which cleaves the side chains of the cutin and suberin biopolymers (Otto and Simpson 2006a). Lignin-derived phenols such as vanillyl, syringyl, and cinnamyl phenols were isolated using copper (II) oxidation methods (Hedges and Ertel 1982; Otto and Simpson 2006b; Rodrigues Pinto et al. 2010). Solvent extracts and copper (II) oxidation products were derivatized using *N,O*-bistrifluoroacetamide and pyridine (Wiesenberg and Gocke 2017), while base hydrolysis products were first derivatized with *N,N*-dimethylformamide dimethyl acetal and then with *N,O*-bistrifluoroacetamide and pyridine. Samples were dissolved in hexane for identification and quantification by gas chromatography–mass spectrometry (GC–MS) with an Agilent 7890B GC with a 5977B MS with electron impact ionization. Compound identification was performed by comparing MS spectra to the Wiley Registry (9th edition), the NIST (2008) and custom mass spectral libraries. Quantification was performed using external standards, with methyl tricosanoate, tetracosane, ergosterol (as TMS ether) and docosanol (as TMS ether) for solvent extractable compound quantification; methyl tricosanoate for cutin- and suberin-derived compound quantification; and syringaldehyde and syringic acid (both as TMS ethers) used lignin-derived compound quantification. The mass of each compound detected was normalized to the soil C content for that treatment plot and results are expressed in mg g^{-1} C (Tables S2–S4). Relative differences were calculated as percentages (based on respective control values) for comparisons (Fig. 3).

Phospholipid fatty acid (PLFA) analysis

Microbial biomass and community structure were assessed via PLFA analysis (Bligh and Dyer 1959; Frostegård et al. 1991). Lipids were extracted from freeze-dried soil (1 g) using phosphate buffer, chloroform, and methanol (0.8:1:2; v:v:v). The polar (phospholipids) and neutral lipids were isolated

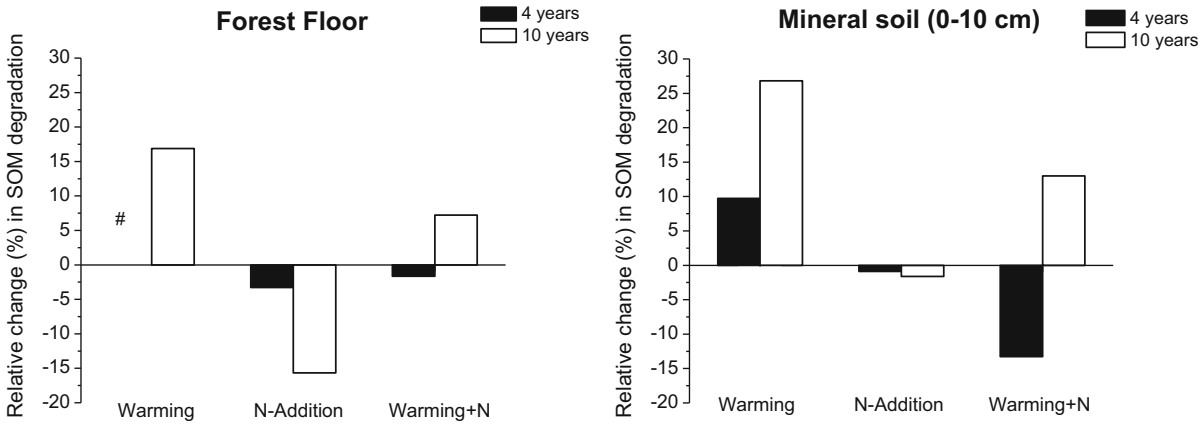

Fig. 2 Relative percent differences (expressed in percentage change relative to the control) in SOM degradation (measured using solid-state ^{13}C NMR alkyl/*O*-alkyl carbon ratios from composited samples; Table S1). Positive differences reflect enhanced SOM degradation relative to respective control plots (years 4 versus year 10). Negative differences are indicative of suppressed SOM degradation (or SOM preservation) relative to the control. # represents a difference of zero for this treatment. Data from 4 years of experiment was taken from Pisani et al. (2015). Solid-state ^{13}C NMR spectra and integration data are provided in Table S1 and Fig. S3

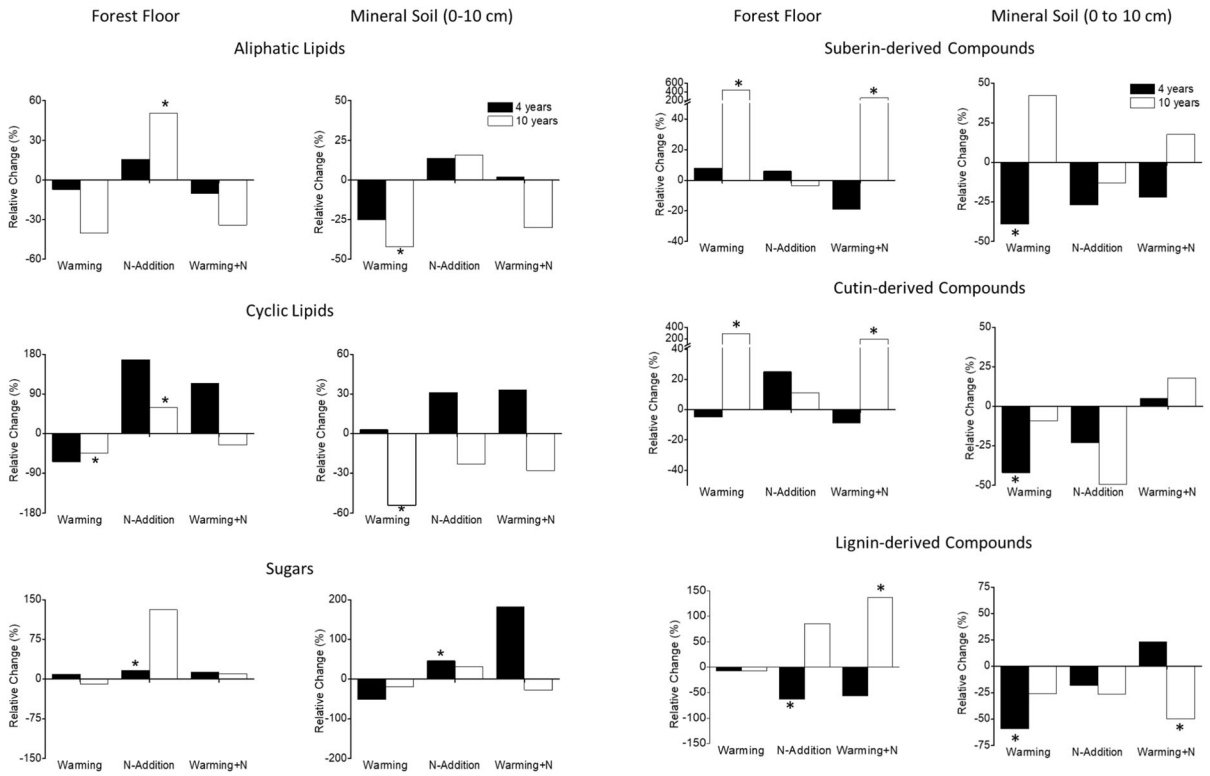

Fig. 3 Relative percent differences (expressed in percentage change relative to the control) in average concentrations of various SOM compound classes measured by GC–MS in the forest floor and mineral horizons after 4 and 10 years of experiment (data from 4 years of experiment was obtained from Pisani et al. (2015) and calculated relative to control data after 4 years of experiment). Significant differences ($p \leq 0.05$) of compound classes ($n = 10$) are marked with an asterisk. Several individual SOM compounds within these classes exhibited statistical significance ($p \leq 0.05$) are reported in detail within the Supplementary Material (Tables S2–S4) after 10 years of experiment and in Pisani et al. (2015) after four years of experiment

separately using silicic acid chromatography and methylated using 0.2 M methanolic potassium hydroxide (1 mL) at 60 °C for 30 min to form fatty acid methyl esters (FAMEs) that were quantified on a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA). FAME peaks were compared against a standard library of FAMEs specific to fungi (18:2ω6,9c, 18:1ω9c; Frostegård and Bååth 1996; Vestal and White 1989), bacteria (i15:0, a15:0, c15:0, i16:0, 16:1ω7c,t, i17:0, a17:0, 18:1ω7c, and cy19:0; Guckert et al. 1985; Tunlid and White 1992; Vestal and White 1989; Zelles 1997) and arbuscular mycorrhizal fungi (16:1ω5c; Olsson et al. 1995). Standards for each marker were used to convert measured peak areas to PLFA concentrations and then normalized to the mass of soil extracted. All raw data for PLFAs is provided in Tables S5–S7.

Data and statistical analyses

To account for replication through field treatment (fixed factor, n = 5) and analytical replication (random factor, n = 2) a univariate general linear model was used with warming and N addition as fixed effects. Bonferroni post-hoc tests were used to compare the concentrations of SOM components across treatment levels after 10 years. Differences were considered significant when $p \leq 0.05$. For soil C storage, C, N and PLFA data, a one-way ANOVA with a Bonferroni post-hoc test ($n = 5$) was used, and differences were considered significant when $p \leq 0.05$. Statistical tests were performed using IBM SPSS Statistics (version 20). The data collected in this study were compared to previously published results from the same site after 4 years of treatment (Pisani et al. 2015) who employed the same statistical approach to compare

changes in SOM composition relative to the control plots. Significance (p) values are provided in the Supplementary Information (Tables S8–S13). It is important to note that SOM compositional shifts were assessed via relative changes to the control plot. The control plot data is used as a reference point to determine significant differences for the observed changes in the SOM composition and this approach was consistently applied for both year 4 (Pisani et al. 2015) and year 10 datasets.

Results

Soil C, N, and soil C storage

Average soil C and N contents varied in both the forest floor and mineral soil (0–10 cm) with warming, N-addition, and warming + N but not significantly (Fig. S1). Soil C storage declined with warming in the forest floor ($p \leq 0.05$; Fig. 1). Warming also reduced soil C storage in the mineral soil but not significantly, relative to the control. The addition of N increased soil C storage in the mineral soil horizon but not significantly. Warming + N did not significantly alter soil C storage relative to the control.

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

The solid-state ^{13}C NMR spectra for all samples (Fig. S3) show differences in the relative proportions of the main SOM categories of alkyl, *O*-alkyl, aromatic and phenolic, and carboxylic and carbonyl C (Table S1). *O*-alkyl C compounds, such as cellulose, are preferred substrates by microbes and relative increases in the alkyl/*O*-alkyl C ratio are indicative of enhanced SOM degradation (Baldock et al. 1992).With soil warming, the alkyl/*O*-alkyl C ratio increased in both the forest floor and mineral soil horizons relative to the control (Fig. 2). The extent of degradation in the mineral soil horizon was more advanced after 10 years than reported after 4 years of soil warming (Pisani et al. 2015). With N-addition, the alkyl/*O*-alkyl ratio did not change after 4 years relative to the control, but after 10 years the ratio was lower in the forest floor, suggesting more preservation of SOM. With simultaneous warming and N-addition, the degradation appeared to be suppressed after 4 years in the mineral soil; however, after 10 years the SOM in the forest floor and mineral soil horizons was more degraded than the control (Fig. 2). This observation suggests a fundamental shift in the biogeochemical cycling of SOM between 4 and 10 years with simultaneous warming and N-addition.

Targeted soil organic matter (SOM) compound identification and quantification via GC–MS analysis

Lipids and solvent extractable small molecules

A variety of solvent extractable small molecules from various plant and microbial sources were detected in the forest floor and mineral soil horizons (Fig. 3 and Table S2). Ten years of warming decreased the concentration of aliphatic lipids, cyclic lipids, and sugars in the both the forest floor and mineral soil horizons (Fig. 3). This included significant ($p \leq 0.05$) reductions of plant-derived SOM components, such as long-chained alkanoic acids and terpenoids (Table S2). The relative decrease in aliphatic lipids is more pronounced after 10 years than after 4 years (Fig. 3). In contrast, the addition of N significantly increased the concentrations of aliphatic lipids, cyclic lipids, and sugars in the forest floor (Fig. 3). Increased concentrations of long-chained alkanoic acids and plant-derived steroids were also observed (Table S2) and suggests the accumulation of plant-derived SOM in the forest floor with added N. In the mineral soil, aliphatic lipids and sugars increased with N-addition after 10 years (Fig. 3 and Table S2). This pattern of accumulated SOM components with N-addition at 10 years is consistent with the results observed after 4 years of treatment (Pisani et al. 2015). With simultaneous warming and N-addition, the concentrations of aliphatic lipids and cyclic lipids decreased in the forest floor after 10 years (Fig. 3), similar to observations with warming alone. The concentrations of sugars in the forest floor increased with warming + N after 10 years, but not to the same extent as that observed in the N-addition treatment (Fig. 3). Aliphatic lipids, cyclic lipids and sugars decreased in the mineral soil with warming + N, which was again like the response of warming alone (Fig. 3). The observed increase in aliphatic lipid, cyclic lipid and sugar concentrations after 10 years is markedly different from observations made after 4 years (Pisani

et al. 2015), where these compounds decreased (relative to the control). This distinction in the SOM composition from 4 to 10 years suggests a shift from accumulation to a reduction in SOM components over time with simultaneous warming and N-addition.

Suberin- and cutin- derived compounds

Suberin- and cutin-derived compounds, which arise from plant roots and leaf wax coatings, respectively, were significantly more abundant in the warming and warming + N plots in the forest floor ($p \leq 0.05$) after 10 years (Fig. 3). This is consistent with the observations made after 4 years of warming (Pisani et al. 2015), however, with continued experiment time (10 years) this relative change was more pronounced (Fig. 3). The observed increase in suberin-derived compounds with warming + N after 10 years of experiment showed a change in trajectory compared to the 4 year observation (relative decrease). Similarly, the response in the forest floor with N-addition differed between 4 and 10 years; however, the decrease in suberin at year 10 was not statistically different from the control (Table S3). In the mineral soil samples, suberin-derived compounds decreased with N-addition after both 4 and 10 years (Fig. 3). With warming and warming + N, the concentrations of suberin-derived compounds in the mineral horizon increased after 10 years, however these changes were not statistically significant.

The concentration of cutin-derived compounds increased significantly after 10 years of warming in the forest floor but decreased in the mineral soil (Fig. 3). The response of cutin-derived compounds with N-addition was consistent between 4 and 10 years, with increased concentrations in the forest floor and decreased concentrations in the mineral soil (Fig. 3). Simultaneous warming and N-addition increased the concentrations of cutin-derived compounds in the forest floor and the mineral soil. The extent of cutin degradation can be estimated by the relative abundance of C_{16} or C_{18} ω-hydroxyalkanoic acids, and ratios of these compounds ($\omega\text{-}C_{16}/\sum C_{16}$ or $\omega\text{-}C_{18}/\sum C_{18}$) increase with progressive cutin degradation (Goñi and Hedges 1990; Otto and Simpson 2006a). Despite observed decreases in cutin-derived compounds in the mineral horizon of the warming and N-addition plots, no significant changes in the cutin degradation ratios were observed after 10 years (Table S3).

Lignin-derived compounds

Warming decreased lignin-derived compounds in both the forest floor and mineral soil samples but not significantly (Fig. 3; Table S4). Lignin oxidation ratios (acid to aldehyde ratios) significantly increased in the forest floor (Table S4) with warming, suggesting the enhanced degradation of lignin. N-addition increased lignin-derived compounds in the forest floor after 10 years (Fig. 3). Lignin-derived compounds decreased with N-addition in the mineral soil, which was also observed after 4 years (Pisani et al. 2015). Warming and N-addition resulted in a significant ($p \leq 0.05$) increase in lignin-derived compounds in the forest floor and a decrease in lignin-derived compounds in the mineral soil (Fig. 3 and Table S4). This result is markedly different than observations made after 4 years of experiment (Pisani et al. 2015). Interestingly, lignin oxidation ratios show progressive lignin degradation after 10 years (as compared to 4 years) in the warming + N-addition plots relative to the control (Table S4).

Phospholipid fatty acid (PLFA) concentrations

Microbial biomass was assessed using phospholipid fatty acid (PLFA) analysis, but concentrations were not significantly different ($p \leq 0.05$) with warming, N-addition, or their interaction. Specifically, there were no changes in total microbial biomass in any of the treatments after 10 years (Fig. S4; Tables S5 and S6). For most treatments, neither total bacterial biomass nor that of the actinomycetes was affected by warming, nitrogen, or their combination (PLFAs from actinomycetes did significantly increase with warming + N in the mineral soil horizon; Table S5). Total fungal biomass was also unchanged across the treatments. The ratio of fungi to bacteria, the proportion of Gram + to Gram- bacteria, monoenoic/saturated PLFAs, and cyclic/monenoic precursor PLFAs were also not statistically different (Table S7).

Discussion

Warming enhances the decomposition of SOM

Soil warming is hypothesized to reduce soil C storage through acceleration of microbially-mediated SOM degradation, either by changing microbial community composition or substrate utilization efficiency (Classen et al. 2015; Crowther et al. 2016; Kardol et al. 2010; Kirschbaum 1995). We observed a decline in soil C storage (Fig. 1) with warming in the forest floor only which suggests that warming likely accelerated microbial degradation of SOM. We also observed increased alkyl/*O*-alkyl ratios in both the forest floor and mineral soil, measured by NMR spectroscopy (Fig. 2), which is indicative of enhanced SOM degradation (Baldock et al. 1992) and supports our first hypothesis which proposed sustained SOM degradation with continuous warming. A higher alkyl/*O*-alkyl ratio after both 4 and 10 years of warming indicates that the relative SOM degradation state is more advanced relative to the control. Additionally, the difference between the alkyl/*O*-alkyl ratios in the control and warmed plots is markedly higher after 10 years as compared to 4 years in both the forest floor and mineral soil horizons (Fig. 2). For example, 10 years of soil warming enhanced SOM degradation by $> 16\%$ in the forest floor and $> 26\%$ in the mineral soil. This suggests that not only did SOM degradation continue to increase with prolonged warming but that the extent of degradation increased from 4 to 10 years in both the forest floor and mineral soil horizons.

Further evidence for advanced SOM degradation with warming is illustrated by the decreased concentration of aliphatic lipids, cyclic lipids, and sugars in both the forest floor and the mineral soil horizons (Fig. 3). Several of these compounds, especially simple sugars, are preferred substrates for soil microbiota (Jones and Murphy 2007) and their decreased concentration indicates preferential degradation of these SOM components. Interestingly, after both 4 and 10 years of warming, suberin-derived compounds, which are derived from roots, increased in the forest floor (significantly) and mineral soil (insignificantly; Fig. 3). Since there were no differences in fine root biomass across the treatments (Fig. S2), the increased suberin-derived compounds likely reflect reduced substrate utilization by soil microbes and live root turnover processes indirectly related to the measured pool of root biomass. Compounds from leaf waxes (cutin-derived compounds), which are less preferred substrates, were higher in the forest floor after 10 years suggesting the potential accumulation of these compounds with continued warming. However, the concentration of cutin-derived compounds in the mineral soil horizon was approaching ambient conditions (control) with sustained warming suggesting less preservation of these slow cycling SOM compounds with time (Fig. 3). Because the experimental plots do not receive direct inputs from the forest canopy, and we did not observe differences in fine root biomass, the observed changes in specific SOM compounds may be attributed to changes in trajectories of root turnover and/or microbial substrate use preferences due to soil warming.

The observed decrease in lignin-derived compounds (Fig. 3; Table S4) in both soil layers and significant increase in syringyl oxidation in the forest floor (Table S4) can also be attributed to an increase in SOM degradation with soil warming. Other temperate forest warming studies have reported increased lignin degradation with warming and shifts in microbial community structure (Feng et al. 2008; Schnecker et al. 2016). For instance, 20 years of warming at Harvard Forest increased the diversity of lignin-degrading bacteria (Pold et al. 2015). Furthermore, it was reported that the size of the microbial community did not change, but rather C utilization efficiency did (Pold et al. 2017). In this study, fungal to bacterial biomass decreased with warming in the mineral horizon but not significantly (Table S7). Microbial community structure changes may alter microbial substrate utilization efficiency with soil warming. Another study on these same soil samples found that soil warming dramatically altered fungal community composition (Anthony et al. 2020). Turnover among ectomycorrhizal fungi which comprised $\sim 70\%$ of the total fungal community (Anthony et al. 2020), may be indirect drivers of SOM degradation as they mine for organic N. The relative abundance of saprotrophic fungi, including white rot fungi which decompose lignin, was not affected by warming (Anthony et al. 2020). Thus, decreases in extractable lignin compounds under warming may not be related to increases in the proportion of free-living fungi, but rather which species are present and how they grow. Dominant taxa included ectomycorrhizal members of the Russulaceae which are widely capable of modifying lignin

(Looney et al. 2018). Fungal rRNA genes, an indicator of fungal growth, were also higher under soil warming without a concomitant increase in fungal biomass, which lends support to the idea that fungal activity is greater under warming but does not lead to higher biomass production (Anthony et al. 2020). Changes in fungal community composition and physiology may enhance SOM degradation with implications for lowering soil organic C via reduced microbial biomass production and stabilization (Cotrufo et al. 2013).

N-addition suppresses SOM decomposition

Long-term N-addition has been shown to increase soil C accumulation by suppressing microbial degradation of SOM (Frey et al. 2014; Wang et al. 2019), in particular, via the inhibition of lignin-degrading enzymes (Carreiro et al. 2000). After 10 years of N-addition, significant changes in soil C, N or soil C storage were not observed in the forest floor, except when in combination with warming (Fig. 1; Fig. S1). This finding is consistent with observations that nine years of N-addition at Harvard Forest did not significantly change soil C or N pools (Magill and Aber 2000) and studies that have reported increases in C storage with N-addition in temperate forests in the northern United States only after 15 to 20 years (Nave et al. 2009; Frey et al. 2014). Several notable SOM changes were observed that support our second hypothesis that 10 years of added N suppresses SOM decomposition. For example, the alkyl/*O*-alkyl ratios in the N-addition plots are lower than the control plots (Fig. 2 and Table S1) which suggests that SOM degradation was suppressed. This observed suppression in SOM degradation in the N-addition plots (where N is added at about 8 times the background rate) is consistent with observations that N deposition at between 2 and 20 times the ambient rate inhibits litter decomposition (Knorr et al. 2005). Furthermore, the concentrations of aliphatic lipids and sugars were higher in both horizons, and increases in cyclic lipids in the forest floor also support that the SOM turnover was slower and N-addition perturbed the ambient biogeochemical cycle (Fig. 3 and Table S2).

At other temperate forests, increases in cutin-, lignin-, and suberin-derived compounds have been observed after 10 (vandenEnden et al. 2018) and 22 (Wang et al. 2019) years of N-addition. Similarly, the accumulation of lignin was observed after 20 years of N-addition at Harvard Forest (Frey et al. 2014). Our data reflect differences in the sequestration of cutin, lignin and suberin in the forest floor and mineral soil with N-addition as well as differences with time (Fig. 3). The NMR data (Table S1) highlight the accumulation of components such as cellulose and peptides (*O*-alkyl region) as well as differences in the regions where other plant biopolymers (cutin, lignin and suberin) would be detected. These differences coupled with changes in extractable cutin-, lignin- and suberin-derived compounds (Fig. 3) likely reflect not only an overall reduction in SOM degradation but also increased sequestration of these components in soil. The turnover of plant biopolymers in soil under N-addition can be impacted by the presence of other, more abundant SOM components (e.g. "Onion layering" model; Sollins et al. 2006). Previous studies have indicated that the extractability of cutin-, lignin- and suberin-derived compounds is dependent on the degradation state of the biopolymer (more intact, less degraded biopolymers do not equate to high yields of their extractable side chains; Pisani et al. 2015) or due to strong associations with minerals (Lin and Simpson 2016). Thus, the accumulation of cellulose could alter the stabilization of other plant-derived compounds with N-addition through preferential interactions with minerals or mineral-stabilized SOM. This may then further reduce microbial biomass and diversity due to a decline in SOM chemical diversity and resource partitioning for microbes. Consequently, there is likely a cascade of interconnected alterations to SOM composition and soil C biogeochemistry with N-addition.

Warming and N-addition alters SOM composition divergently with time

After 4 years of warming + N addition, the observed changes in SOM biogeochemistry mimicked the response of N-addition alone more than warming alone (Pisani et al. 2015). For example, the measured alkyl/*O*-alkyl ratios suggested that the overall relative state of SOM degradation in the forest floor and mineral soil of warming + N plots were more similar to the SOM in the N-addition plots after 4 years (Fig. 2). Furthermore, suppressed degradation was also evidenced by increased concentrations of cyclic lipids and sugars and decreased concentrations of extractable lignin-derived compounds (Pisani et al.

2015). Also, mineral soil layers exhibited increased concentrations of lipids, cyclic lipids and sugars (Pisani et al. 2015). These observations after 4 years in the N-addition and warming + N suggested that changes in the warming + N were controlled most strongly by the addition of N than warming alone (Pisani et al. 2015). Between 4 and 10 years, SOM degradation patterns and molecular biogeochemistry have evolved markedly and are now mirroring the response of the warming treatment (Fig. 2). This is supported by the increased alkyl/*O*-alkyl ratios (Fig. 2) and shows that after 10 years, SOM degradation has advanced and is now more consistent with trends observed with warming alone, in contrast to our hypothesis. While warming and N additions may have antagonistic impacts on C cycling (Yue et al. 2017), our results are consistent with the concept that warming and N-additions in combination increase SOM degradation (Sun et al. 2019) but there is evidence that these impacts are not additive. A comparison of the relative change in SOM degradation (Fig. 2) shows that after 10 years, the trajectory of SOM degradation shifted but the extent of SOM degradation with warming + N is still lower than warming alone. This trend is consistent with observations for several SOM components (Fig. 3) which exhibited an overall shift in SOM composition that is also more consistent with the warming treatment than N-addition (Fig. 3). The fungal community composition with warming + N was also more similar to warming than the N-addition treatment (Anthony et al. 2020). These results contradict our third hypothesis because after 4 years, aliphatic lipids and sugars increased with N-addition and warming + N, suggesting a decline in the microbial processing of SOM. In contrast, the concentration of these compounds decreased with warming and is indicative of accelerated SOM turnover. However, after 10 years, aliphatic lipid concentrations have decreased with warming + N (and warming) but continued to increase with N-addition (Fig. 3). Similar observations were made for cyclic lipids and the proportion of suberin-derived compounds. Sugars with warming and warming + N were similar, likely due to the fact that these compounds are preferred microbial substrates and exhibit a heightened sensitivity to environmental change. Collectively, these changes illustrate that with time, SOM composition in the warming + N plots has shifted distinctly to become more like warming alone than N-addition alone.

It is essential to note that the response of forest SOM to simultaneous warming and N-addition is dynamic and cannot be predicted from either treatment individually or as a simple additive combination of warming and N-addition. It is likely that excess N in combination with warming still resulted in some antagonistic impacts when compared to warming and N-addition alone because there is evidence for unique molecular-level responses when warming and N-addition are combined. For example, in the mineral soil horizon, sugars and lignin-derived compounds exhibited different trends with N-addition and warming + N (Fig. 3). Similarly in the forest floor, suberin-derived compounds were not significantly different with N-addition but increased significantly with warming + N (Fig. 3). Additionally, the concentration of the cutin-derived compounds increased in the mineral soil with warming + N but decreased with warming + N. These results suggests that even though the SOM biogeochemistry with warming + N is more like warming alone; the added N overall plays a potentially antagonistic role with a few clear exceptions in which particular compounds accumulated exclusively under warming + N (i.e. cutin-derived compounds in the mineral soil). This suggests that the response of SOM to future environmental change will be dynamic, complex, and a complete understanding can only be elucidated by studying a variety of different forest ecosystems over time and by including paired treatments that may reflect global environmental changes more accurately.

Conclusions

After 10 years, SOM decomposition increased with warming and warming + N, while N-addition suppressed SOM degradation. Overall, the observations indicate that simultaneous warming and N-addition resulted in SOM composition and degradation patterns more like warming and represent a marked shift from the observations made after 4 years. These findings emphasize that the response of SOM to simultaneous warming and N-addition vary with time, and as future anthropogenic changes are anticipated to result in increased temperatures and environmental N-deposition, long-term monitoring is essential to make

accurate predictions about resulting changes in soil C cycling and storage. Our study has shown unequivocally that studying multiple stressors comprehensively is critical for assessing future changes in soil C storage and biogeochemistry and such changes cannot be predicted from studying ecosystem impacts (warming and N-addition) individually. Furthermore, using sensitive molecular-level methods has revealed that biogeochemical processes can evolve and change trajectory over time. This highlights the need for a combination of integrative molecular biogeochemical approaches for monitoring SOM composition and soil C biogeochemistry with multiple stressors over time.

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Data availability All compound concentrations and solid-state ^{13}C NMR spectra are listed in detail within the Supplementary Materials. Raw data files from instrumental analysis, soil bulk density and edaphic data are available from the corresponding author.

Declarations

Conflict of interest The authors do not have any conflicts of interest or competing interests to declare.

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