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Article

Chronic Warming and Nitrogen-Addition Alter Soil Organic Matter Molecular Composition Distinctly in Tandem Compared to Individual Stressors

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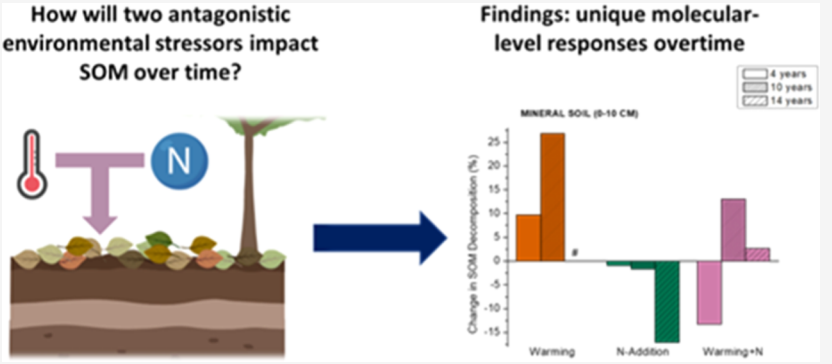

ABSTRACT: Forest soils are major reservoirs of carbon (C), but these stores are threatened by increasing global temperatures and atmospheric nitrogen (N) deposition. These environmental stressors can alter soil microbial communities and soil organic matter (SOM) biogeochemistry through a variety of mechanisms. To investigate the impact of chronic warming, N-addition, and simultaneous warming and N-addition (warming + N) on forest soils, soil samples from the Harvard Forest Soil Warming and Nitrogen Addition (SWaN) experiment were analyzed after 14 years. Elemental analysis, targeted compound analysis by gas chromatography−mass spectrometry, and ^{13}C nuclear magnetic resonance (NMR) spectroscopy were used to analyze changes in SOM in both the organic and mineral (0−10 cm) soil layers. Overall, changes in the molecular composition and microbial biomass were observed, but the extent of differences was unique to warming, N-addition, and warming + N treatments. Specifically, N-addition slowed SOM decomposition as measured via solid-state ^{13}C NMR, while warming and warming + N accelerated SOM decomposition. Continued SOM decomposition after 14 years with warming + N signified a pronounced change to observations made after 4 and 10 years of experimental treatment. This is also demonstrative of how a two-factor approach leads to a unique molecular-level response that cannot be predicted from experiments with individual stressors alone. This study emphasizes the need to observe environmental stressors in tandem using a combination of molecular-level approaches to obtain a comprehensive understanding of how persistent anthropogenic activity will fundamentally alter forest soil systems.

KEYWORDS: *soil carbon, soil warming, soil lipids, cutin-derived, suberin-derived, phospholipid fatty acid profiling, solid-state nuclear magnetic resonance*

INTRODUCTION

Forest soils represent one of Earth's greatest carbon (C) reservoirs[1,2] and are a vital part of the global C cycle.[3] Persistent anthropogenic activity can change both the biotic and abiotic character of these ecosystems,[4,5] hence impacting the manner in which forests participate in global biogeochemical cycles.[6−8] As atmospheric temperatures steadily rise globally,[9,10] continuous warming may limit capacity for soils to sequester C due to enhanced microbial respiration.[11,12] Warming may also enhance both above- and below-ground C inputs to soil and contribute to the sensitive balance of soil C stored versus that respired.[13−16] In addition, increased atmospheric nitrogen (N) deposition to soil is currently occurring at a rate triple than that of 150 years ago[17] and can increase the amount of C sequestered in forests.[18−20] Moreover, warming and N-addition can impact the chemistry

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of soil organic matter (SOM), a complex mixture comprising plant, animal, and microbial matter at differing stages of decay,[21,22] and soil microbial communities,[23,24] which are both intrinsically linked to soil C and the global C cycle.[25] Overall, the link between changing climate conditions and the expectation that persistent human activity will continue to accentuate stressors[26] demonstrates the need for further information on the long-term impacts to SOM biogeochemistry.

Soil warming has contributed to the loss of soil C in forests[11,14,27,28] and increased SOM decomposition.[14,29−32] These impacts have been attributed to changes in microbial biomass and activity[31,33,34] and/or their substrate utilization.[35−37] Enhanced decomposition of preferred microbial substrates, such as sugars and aliphatic lipids, was noted with short-term warming.[30,31,38] Additionally, Feng et al.[30] observed enhanced processing of longer-lived SOM compounds, such as lignin, due to increased fungal biomass with short-term warming. In contrast, long-term warming experiments have demonstrated variability in the magnitude of SOM decomposition, as evidenced by changes in microbial biomass and community structure[39−42] which altered the concentrations of both short- and long-lived SOM compounds.[34] Melillo et al.[15] hypothesized that microbial communities go through multiple "phases" of reorganization with long-term warming, being associated with variation in soil C respired over time. Interestingly, chronic warming was reported to alter the molecular-level composition of SOM even though microbial biomass decreased.[34] Observed changes in the SOM chemical composition reflected a decline in polysaccharides, a preferred microbial substrate, and was likely due to a more active, yet smaller, microbial community.[34,39] Furthermore, Pec et al.[43] reported that short-versus long-term warming altered SOM composition uniquely, in addition to impacts to the soil fungal community composition. Similarly, detailed molecular-level characterization of SOM with warming demonstrated that the extent of changes to specific SOM components intensified with time[31,32] emphasizing the need to better elucidate how warming alters SOM in the long term. Model predictions suggest that warming will continue to enhance soil C losses,[12] which further demonstrates the need to better understand the magnitude with which warming will enhance SOM degradation and alter SOM chemistry over time.[31,32]

In contrast to warming, N-additions to temperate deciduous forest soils tend to increase C sequestration,[18,44−48] lower microbial biomass and diversity,[20,49,50] and decrease SOM decomposition.[31,32,51] Both short- and long-term experiments have demonstrated that the addition of N increases C storage through slowed or suppressed microbial SOM decomposition.[18,31,32,51,52] Primarily, altered biogeochemical processing of plant-derived compounds is observed[51] and generally attributed to changes in microbial biomass, community composition, and enzymatic activity.[18,50,53] For instance, Hasegawa et al.[54] demonstrated that the decomposition of lignin was suppressed after 12 years of N-addition, an observation commonly linked to decreased fungal biomass and activity,[18,48,55,56] a microbial group imperative in the processing of lignin.[57] Molecular-level studies also reported decreased SOM decomposition after 4[31] and 10 years[32] of N-addition, likely due to the accumulation of plant-derived inputs and also suppressed microbial decomposition of SOM. This suggests that while N-addition will continue to suppress SOM decomposition in forest soils, it may do so through a variety of distinct mechanisms, including via degradation by enzymes,[58] suppressed microbial activity,[18] and altered substrate utilization,[59] which together led to a variable response over time.

Warming and N-addition are both environmental stressors that have been extensively studied independently; however, fewer studies have investigated the impact of warming and N-addition when combined (warming + N).[60−64] Crucially, Rillig et al.[65] indicated that the study of singular environmental stressors are not appropriate models for complex systems. This is especially relevant for forests, considering how warming and N-addition antagonistically alter soil C,[28,45] microbial communities,[18,20,34] and specific SOM compound concentrations,[30,44] as well as how these responses are individually modulated by time.[43,51] Previous studies focused on how warming + N will alter soil biogeochemistry noted that while the relationship between warming and N-addition is often in opposition, specific molecular responses vary with time.[31,32,62,66] For example, soil C did not significantly change with warming + N,[31,32,66] and microbial biomass either increased[66] or did not change.[62] Furthermore, studies have demonstrated that in the short term, warming + N alters SOM molecular composition more similar to N-addition alone.[31] However, after 10 years, vandenEnden et al.[32] reported that warming + N combined no longer mirrored the impact to SOM composition as N-addition alone and was more similar to warming alone. A complex web of interactions between microbial communities, C pools, and organic matter chemistry,[21] as well as the difficulty of predicting responses over time, ultimately delineates the need for further research into how warming + N will alter SOM. Moreover, given that warming and N-addition alter soil C biogeochemistry distinctly, predicting how forest soils will respond in the long term requires further study, especially using molecular-level SOM characterization techniques.

Consequently, our aim in this study was to identify how warming, N-addition, and warming + N alter SOM chemistry and cycling following 14 years of experimental treatment. Soil samples were collected from the Soil Warming and Nitrogen Addition (SWaN) Study at the Harvard Forest Long-term Ecological Research (LTER) site and assessed using a combination of targeted and non-targeted molecular-level techniques. We used two complementary analytical approaches including gas chromatography−mass spectrometry and solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy to characterize changes in SOM chemistry.[31,32,67] When used in tandem, these methods quantify distinct classes of plant- and microbial-derived compounds that is combined with an overview of SOM chemistry from solid-state ^{13}C NMR spectroscopy. We also performed C and N analyses, which are indicative of general pool sizes and offer insights into substrate availability. We hypothesized that warming alone would continue to accelerate SOM decomposition via preferential use of specific SOM compounds, while N-addition would continue to suppress SOM degradation and result in the preservation of SOM compounds. We also hypothesized that warming + N would mirror warming and continue to accelerate SOM decomposition, but not to the same extent as warming alone[31,32] given that warming impacts can be variable over time.[42] Overall, this study investigates how SOM chemistry is altered after 14 years of warming, N-addition, and warming + N, with the goal of obtaining molecular-level insights into how different anthropogenic stressors, alone and combined, uniquely impact forest soils.

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METHODS

Site Experimental Design and Sample Collection. Soil samples were collected from the SWaN plots at Harvard Forest located in Petersham, Massachusetts, USA, a forest characterized by a closed canopy made up primarily of hardwoods including red oak, red maple, and American beech.[66] This forest has an average mean temperature of 7 °C and a mean annual precipitation of 110 cm.[68] Harvard forest soils are characterized as Gloucester-series with a fine loam texture.[66,69] The SWaN plots (3 m × 3 m) include the following treatments: ambient (control), warming, nitrogen addition (N-addition), or simultaneous warming + N addition (warming + N), and each treatment is replicated six times. Warming and warming + N plots are heated with buried cables running 10 cm below the soil surface, continuously elevating the temperature to 5 °C above ambient soil temperature. Nitrogen is supplied to N-addition and warming + N plots monthly from May through October via addition of aqueous ammonium nitrate at a rate equivalent to 5 g N m^{-2} y^{-1}. Further information about the SWaN experimental design may be found in previously published studies.[18,66,69] All plots have similar above-ground inputs as there are no canopy trees within the plots. Soil samples were collected in July of 2020 (after 14 years of treatment). For each plot, two individual samples were taken and included a 10 cm × 10 cm forest floor (organic horizon) sample followed by 0−10 cm core (5 cm diameter) of mineral soil. Soils were stored at 4 °C and then passed through a 2 mm sieve to remove rocks, roots, and organic debris >2 mm. Replicate cores were pooled and homogenized, and then, soil moisture was determined gravimetrically by drying organic horizon and mineral soil subsamples at 60 and 105 °C, respectively. Additional fresh subsamples were immediately frozen at −20 °C and then freeze dried and finely ground for subsequent molecular analyses.[70]

Soil Carbon and Nitrogen Measurements. Total organic C and N were measured using a Thermo Flash 2000 Elemental Analyzer which treated samples with O_2 gas at a temperature of 950 °C. Each soil sample was measured in duplicate.

Targeted Analysis of SOM Compounds. A sequential extraction method which targets various plant- and microbial-derived compounds was used.[71] Each soil sample was extracted in duplicate. First, all solvent-extractable sugars and lipids were isolated from samples.[72] Approximately 1 g of soil was used for organic horizon samples, while 2 g of soil was used for mineral (0−10 cm) layer samples. Samples were extracted via sonification with 30 mL of dichloromethane (Optima grade, Fisher Scientific), 1:1 dichloromethane/methanol and methanol (Optima grade, Fisher Scientific), respectively, and then centrifuged at 2500 rpm for 5 min. Samples were next filtered using two glass microfiber filter papers (GF/A and GF/F, Whatman), both with a diameter of 55 mm and a pore size of 0.7 and 1.6 μm, respectively. Following filtration, extracts were concentrated using rotary evaporation. Samples were placed into 2 mL glass vials and evaporated to dryness using N_2 gas prior to quantification using gas chromatography−mass spectrometry.

Base hydrolysis was used to cleave ester-bound lipids in cutin-, suberin-, and microbial-derived compounds.[73] Soil samples that were previously solvent-extracted were placed within Teflon-lined bombs with 15 mL of 1 M methanolic (Optima grade, Fisher Scientific) potassium hydroxide (ACS grade, Fisher Scientific) and heated for 3 h at a constant temperature of 100 °C. Following heating, samples were cooled and extracted with 1:1 dichloromethane/methanol (Optima grade, Fisher Scientific) and centrifuged twice (10 min at 2500 rpm). The supernatant was isolated and acidified using 6 M hydrochloric acid (ACS grade, Sigma-Aldrich) to a pH of 1. Following concentration via rotary evaporation, liquid−liquid extraction was performed using anhydrous ethyl ether (ACS grade, Fisher Scientific) and deionized water (18.2 MΩ cm). Samples were then dried over anhydrous sodium sulfate (ACS grade, Fisher Scientific), concentrated using rotary evaporation, and dried using N_2 gas prior to quantification using gas chromatography−mass spectrometry.

Copper(II) oxide (>99%, Fisher Scientific) was used to isolate vanillyl, syringyl, and cinnamyl phenols from lignin.[74] Previously extracted soil samples were placed within Teflon-lined bombs with 1 g of copper(II) oxide (>99%, Fisher Scientific), 100 mg of ammonium iron(II) sulfate hexahydrate (ACS grade, Sigma-Aldrich), and 15 mL of 2 M sodium hydroxide (ACS grade, Fisher Scientific) in deionized water (18.2 MΩ cm). Samples were purged using N_2 gas prior to being heated at 170 °C for 2.5 h. Samples were cooled, deionized water (18.2 MΩ cm) was added, and the mixture was centrifuged (8 min at 2500 rpm). Samples were maintained in the dark at room temperature for 1 h to prevent polymerization of cinnamic acids.[75] Lignin-derived compounds were isolated using solid-phase extraction[76] with 60 mg of Oasis HLB cartridges (Waters Corporation). Solid-phase extraction cartridges were conditioned using methanol (Optima grade, Fisher Scientific) and deionized water (18.2 MΩ cm) prior to the sample loading and washing using 30% methanol (Optima grade, Fisher Scientific) in deionized water (18.2 MΩ cm). Samples were eluted using a mix of 70:25:5 dichloromethane (Optima grade, Fisher Scientific): methyl acetate (>99.8%, Sigma-Aldrich): pyridine (ACS grade, Fisher Scientific) and methanol (Optima grade, Fisher Scientific), dried over anhydrous sodium sulfate (ACS grade, Fisher Scientific), and concentrated in 2 mL glass vials using N_2 gas.

Phospholipid fatty acids (PLFAs) were extracted to measure microbial biomass and community composition.[70] Approximately 1 and 5 g of soil were used for organic and mineral samples, respectively. Samples were mixed with 6.3 mL of a 0.15 M citrate buffer, 15.8 mL of methanol (Optima grade, Fisher Scientific), and 7.9 mL of chloroform (Optima grade, Fisher Scientific) and shaken for 24 h. Afterward, samples were centrifuged at 2500 rpm for 10 min and separated into fractions following the addition of chloroform (Optima grade, Fisher Scientific) and citrate buffer. The chloroform (Optima grade, Fisher Scientific) phase was dried under N_2 gas. Extracts were redissolved in 1 mL of chloroform (Optima grade, Fisher Scientific), and compounds were separated through silicic acid columns. Following fractionation of the extract, the fraction containing polar lipids was treated with methanolic (Optima grade, Fisher Scientific) potassium hydroxide (ACS grade, Fisher Scientific) and heated, converting these compounds to fatty acid methyl esters. These compounds were further extracted using a 4:1 mixture of hexanes (Optima grade, Fisher Scientific) and chloroform (Optima grade, Fisher Scientific)[77] and transferred into 2 mL glass vials prior to analysis via gas chromatography−mass spectrometry.

Targeted SOM Compound Quantification and Data Analysis. Dried isolated extracts were stored at −15 °C prior

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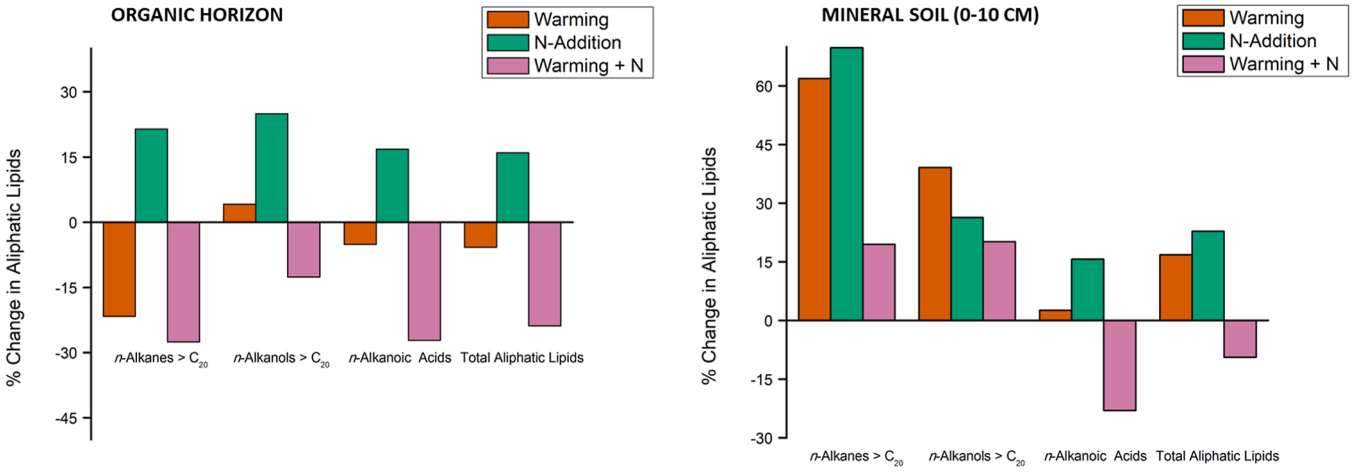

Figure 1. Concentration of aliphatic lipids reported as percent change relative to control as measured in organic and mineral (0−10 cm) soil samples. *n*-Alkanes and *n*-alkanols with chain length $<C_{20}$ were not detected (Tables S1 and S2).

to quantification using gas chromatography−mass spectrometry. Prior to analysis, samples were derivatized using *N,O*-bistrifluoroacetamide (>98.5%, Sigma-Aldrich) and anhydrous pyridine (>99.8%, Sigma-Aldrich). Base hydrolysis extracts were further derivatized using *N,N*-dimethylformamide dimethyl acetal (ACS grade, Fisher Scientific) to methylate compounds.[78] External standards were prepared and derivatized using the same technique/materials. The external standards included 1-docosanol (>98%, Sigma-Aldrich), methyl tricosanoate (>99%, Sigma-Aldrich), cholesterol (>99%, Sigma-Aldrich), and tetracosane (>99%, Sigma-Aldrich) and were used for solvent extraction samples. Methyl tricosonoate (>99%, Sigma-Aldrich) was used for base hydrolysis, and syringaldehyde (>98%, Sigma-Aldrich) and syringic acid (>98%, Sigma-Aldrich) were used for copper oxidation compounds. Oleic acid (>99%, Sigma-Aldrich) was used as an external standard for PLFA quantification.

Samples and standards were analyzed using an Agilent 7890B gas chromatograph hyphenated to an extractor ion source operated in the electron impact (70 eV) mode and a 5977B mass spectrometer. Compounds were identified and quantified through use of Agilent ChemStation Data Analysis software (version F.01.03) and a National Institute of Standards and Technology (NIST) and Wiley 275 mass spectral library and mass spectra from standards. Targeted SOM compounds and PLFAs were quantified via area integration and normalized to the mass of soil used in the extraction. The yield of SOM compounds is typically proportional to the soil C content;[31,32] and as such, concentrations for targeted SOM compounds were further normalized to the mass of soil C.

SOM Composition Using Non-Targeted Solid-State ^{13}C NMR Spectroscopy. Composite samples were prepared for both organic and mineral layer samples for each treatment. This approach was used for consistency to previous studies that collected NMR data on these plots after 4 and 10 years of the experiment.[31,32] For each treatment, 2 g of each mineral layer replicate was measured, ground, and combined to create a composite sample, whereas 0.1 g was used for each organic layer replicate. Mineral layer composites were repeatedly treated with 10% hydrofluoric acid (ACS grade, BDH Chemicals) to remove any paramagnetic materials, hence enhancing signal and improving the observed signal-to-noise ratio.[79] Following repeated extraction with hydrofluoric acid, samples were washed with deionized water (18.2 MΩ cm) until the electrical conductivity of the supernatant indicated low salt contents. Samples were then frozen and freeze-dried prior to analysis.

Samples were packed into 4 mm zirconium rotors, sealed with a Kel-F cap, and then placed within a 500 MHz Bruker BioSpin Avance III spectrometer with a 4 mm H-X MAS probe. Both organic and mineral composite samples were acquired using 21,000 scans under magic angle spinning (MAS) at a rate of 11 kHz. NMR data were acquired using a 1 ms ramp-cross-polarization contact time and with a 1 s recycle delay.[80] NMR spectra were analyzed using Bruker TopSpin 3.6.2., and spectra were divided into four chemical shift regions: 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).[81] The integration of these four regions of the NMR spectra provides information regarding the amount of each compound class contained in each sample. The alkyl/*O*-alkyl C ratio was calculated by dividing the area of alkyl C by that of *O*-alkyl C, and this ratio is used to provide a relative indicator of SOM degradation.[67,81] Studies have observed increasing alkyl/*O*-alkyl C ratios with progressive decomposition of SOM.[31,32,51] We also compared the results from this study (14 years of experimental treatment) to results obtained after 4 and 10 years of the experiment.[31,32]

Statistical Analyses. Soil C and N contents were analyzed for statistically significant differences ($p \leq 0.05$) using a one-way analysis of variance (ANOVA) and post-hoc Bonferroni analysis [SPSS Statistics (Software; version 28)], using field replication ($n = 5$) as a fixed factor. Differences in SOM-targeted compounds between treatment and control plots were tested for statistical significance ($p \leq 0.05$) using ANOVA) with post-hoc Bonferroni analysis (SPSS Statistics Software; version 28) with field replication constrained as a fixed factor ($n = 5$) and analytical replication set as a random factor ($n = 2$). We also compiled select SOM compound concentrations from years 4, 10, and 14 that represent a gradient of relative stability in soil for a broader analysis using non-metric multidimensional scaling (NMDS). Concentrations for aliphatic compounds, simple sugars, and cutin-, suberin-, and lignin-derived compounds were compiled and compared for both organic and mineral soil horizons. We transformed the

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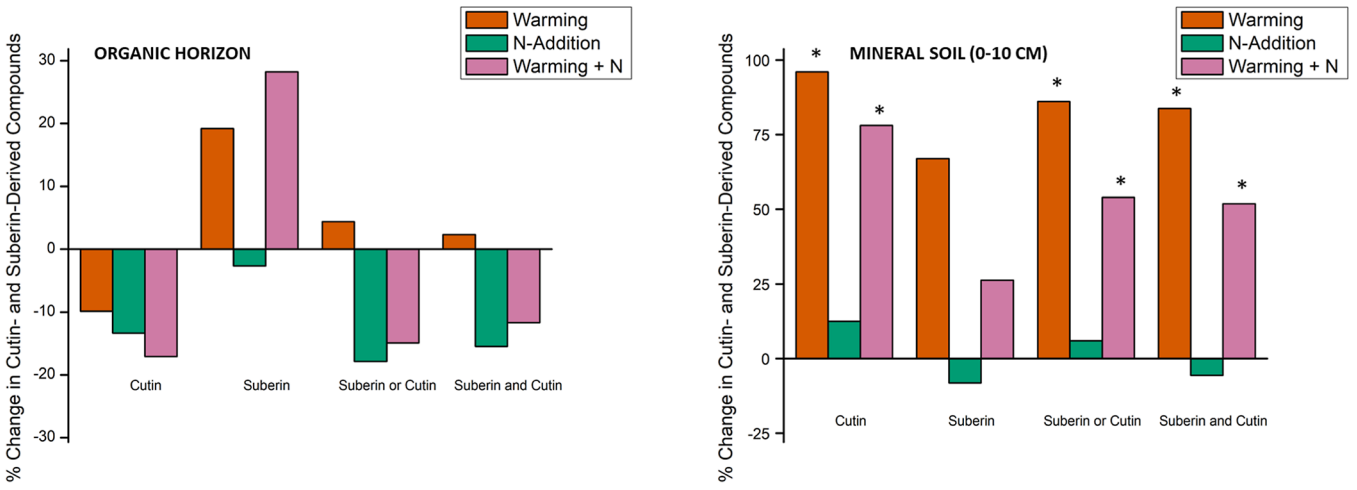

Figure 2. Total cutin- and suberin-derived compound concentrations reported as percent change relative to control as measured in organic and mineral (0−10 cm) soil samples. Asterisks (*) indicate a difference from control treatment which is statistically significant ($p < 0.05$; Table S3).

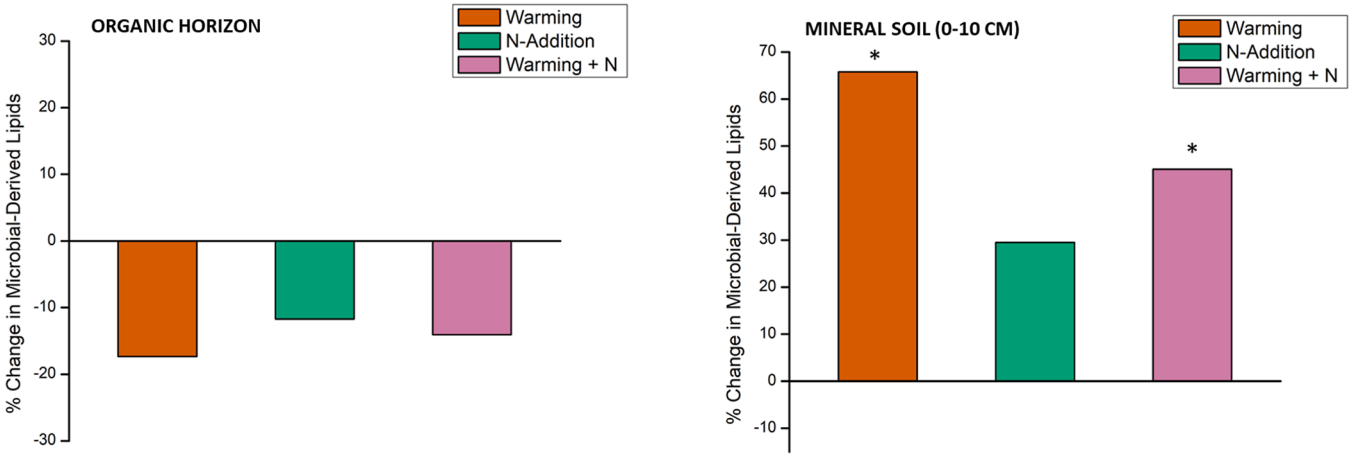

Figure 3. Microbial-derived compound concentrations reported as percent change relative to control as measured in organic and mineral (0−10 cm) soil samples. Asterisks (*) indicate a difference from control treatment which is statistically significant ($p < 0.05$; Table S4).

data by taking the square root of the relative abundance of each compound within a plot to correct the skewness in the data set across the three sampling years and to place less importance on SOM compounds found in high concentrations. We tested for significance between groups using a PERManova ($p \leq 0.05$). We used the vegan package[82] of R[83] to perform all multivariate analyses.

RESULTS

Soil Carbon and Nitrogen Contents. In the organic horizon, warming, N-addition, and warming + N plots, all had lower total soil C relative to the control plots (Figure S1), but these differences were not statistically significant. Similarly, in the mineral layer, no significant differences in soil C were observed (Figure S1), but decreases relative to the control plots were noted. No significant changes were observed with total soil N content in both layers (Figure S1).

Targeted Analysis of SOM Compounds. *Solvent-Extractable Compounds.* Solvent-extractable compounds from both organic and mineral samples included a variety of lipids arising from both plant and microbial sources (Tables S1 and S2). Solvent-extractable compounds included aliphatic lipids (*n*-alkanes, *n*-alkanols, and *n*-alkanoic acids), cyclic lipids (terpenoids and plant-derived steroids), and sugars. The concentrations of both long-chain (>C_{20}) aliphatic lipids (*n*-alkanes, *n*-alkanols, and *n*-alkanoic acids) and short-chain (<C_{20}) aliphatic lipids (*n*-alkanoic acids) provide information about the microbial- or plant-derived sources of SOM,[71] and differences in the distribution of these compounds (Table S1) indicated variable warming impacts to the SOM composition. *n*-Alkanes and *n*-alkanols with chain lengths less than C_{20} were not detected (Tables S1 and S2). Overall, we did not observe any significant differences in solvent extractable compound concentrations with warming, N-addition, and warming + N in the organic or mineral soil layers (Figure 1; Tables S1 and S2). Warming decreased the total concentration of aliphatic lipids within the organic horizon and increased the aliphatic lipids in the mineral soil (Figure 1) but not significantly. In both the organic and mineral layer, N-addition increased the overall concentration of aliphatic lipids (both long-chain and short-chain compounds) but not significantly (Figure 1; Table S1). Warming + N decreased the concentration of aliphatic lipids (both long-chain and short-chain lipids in both soil layers) but not significantly (Figure 1; Table S1). Cyclic lipids were not significantly changed with any treatment or in any soil layer (Table S2). Similarly, sugar concentrations were not significantly different with warming, N-addition, or warming + N in the organic and mineral layers (Table S2). A

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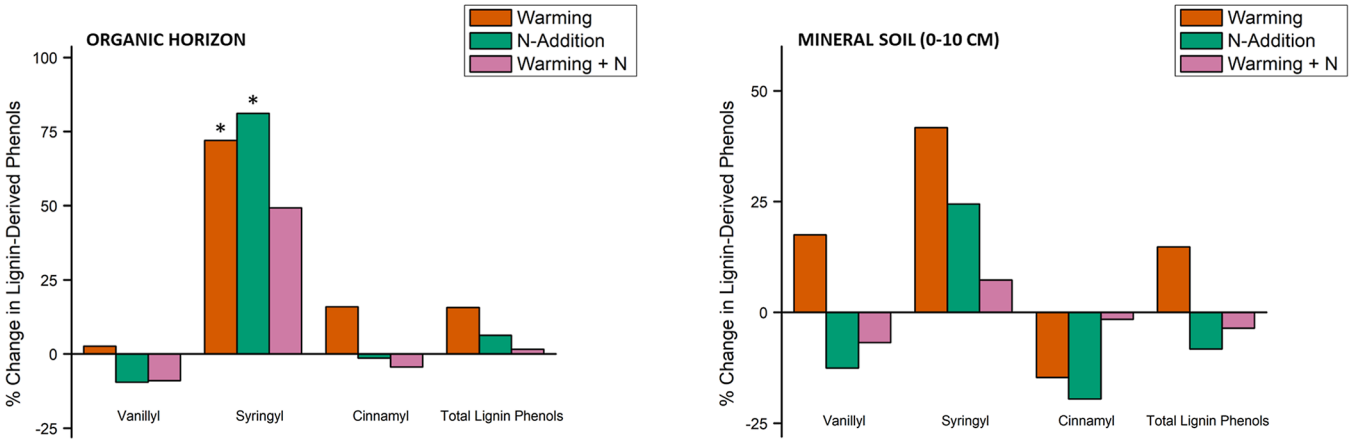

Figure 4. Lignin-derived compound concentrations, including vanillyl, syringyl, and cinnamyl phenols, reported as percent change relative to control as measured in organic and mineral (0−10 cm) soil samples. Asterisks (*) indicate a difference from control treatment which is statistically significant ($p < 0.05$; Table S5).

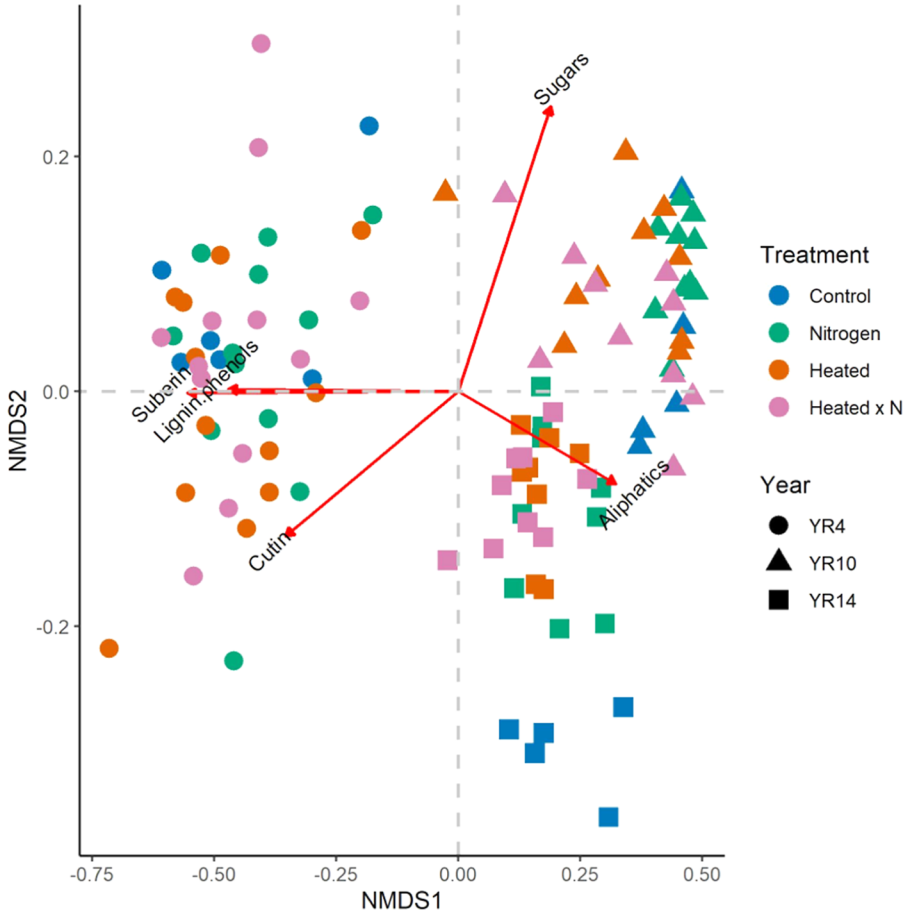

Figure 5. Comparison of different SOM compound classes with varying persistence in soil with treatments over time using NMDS. Data from 14 years of the experiment were obtained from this study. Data from years 4 and 10 were published in detail in studies by Pisani et al.[31] and vandenEnden et al.,[32] respectively.

comparison of aliphatic lipid and sugar concentrations over time using NMDS (Figure 5) shows more similarities between treatments at years 10 and 14 ($p = 0.008$) in comparison to year 4.

Cutin- and Suberin-Derived Compounds. Cutin- and suberin-derived compounds, which are derived from above- and below-ground plant sources, respectively,[84] were detected in both organic and mineral layer samples (Figure 2; Table S3) but to varying extents. In the organic horizon, there were no significant differences in cutin- or suberin-derived compounds across treatments. In the mineral soil, we observed significantly higher concentrations of compounds found in cutin and suberin with warming and warming + N. The ratio of ω-hydroxyalkanoic acids to total hydrolyzable acids of corresponding chain length (ω-$C_{16}/\Sigma C_{16}$; ω-$C_{18}/\Sigma C_{18}$)[71,72] has been used to assess the degree of degradation of cutin-derived

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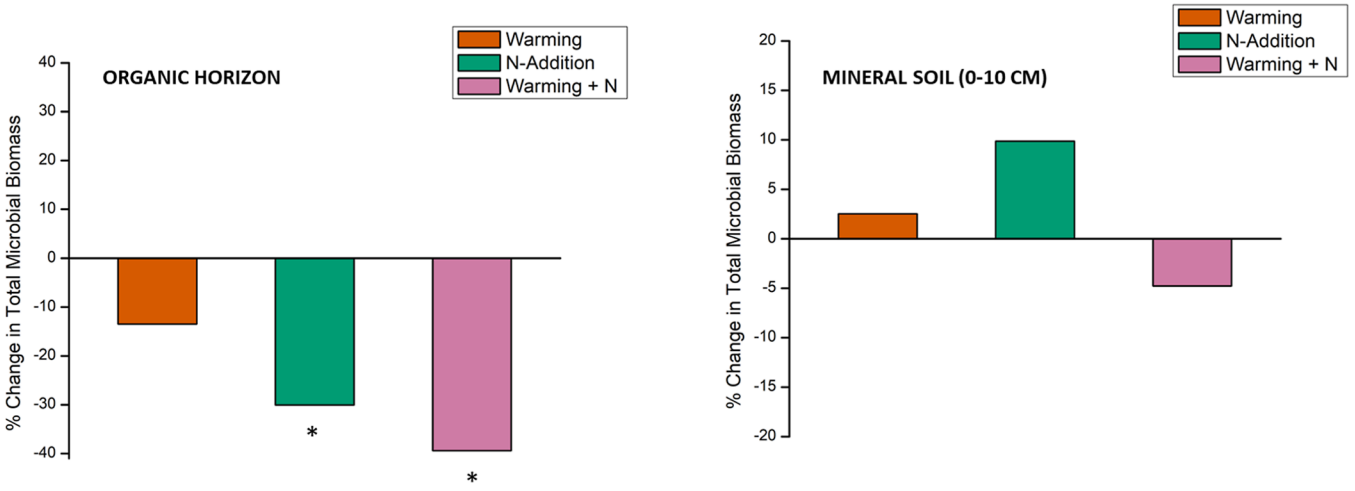

Figure 6. Total microbial biomass (PLFA concentrations) reported as percent change relative to control as measured in organic and mineral (0−10 cm) soil samples. Asterisks (*) indicate a difference from control treatment which is statistically significant ($p < 0.05$).

compounds. There was no significant difference in this ratio with warming, N-addition, or warming + N in both the organic and mineral soils relative to the control (Table S3), indicating that cutin decomposition was not enhanced with treatment. Cutin- and suberin-derived compounds were included in the NMDS comparison and further demonstrate a change in biogeochemical trajectory over time (Figure 5).

Microbial-Derived Lipids. Microbial-derived lipids, which included C_{14}−C_{19} branched alkanoic acids, long-chain *n*-alkanols (C_{18}), and long-chain *n*-alkanoic acids (C_{16} + C_{18}),[85] were observed in both the organic and mineral layers (Figure 3; Table S4). These lipids encompass both cellular and extracellular lipids but differ from PLFAs as they are not membrane lipids.[86] In the organic horizon, there was no significant variation with warming, N-addition, or warming + N. However, in the mineral soil, warming and warming + N significantly increased microbial-derived lipid concentrations.

Lignin-Derived Compounds. Lignin-derived compounds, which included vanillyl, syringyl, and cinnamyl phenols, were detected in both organic and mineral soil samples (Figure 4; Table S5). With warming, syringyl phenols were significantly higher in the organic horizon, but warming did not change the total lignin-derived compound concentrations significantly relative to the control in either soil layer (Figure 4). Acid-to-aldehyde ratios for vanillyl and syringyl compounds (Ad_v/Al_v; Ad_s/Al_s) are used to assess lignin oxidation.[74] However, no significant changes were observed in either the organic or mineral soil with any treatment (Table S5). In comparison to other classes of SOM compounds by NMDS (Figure 5), lignin phenol concentrations were more like year 10 than year 4.

Phospholipid Fatty Acids. PLFA concentrations were altered with warming, N-addition, and warming + N treatments (Figure 6; Table S6). N-addition and warming + N exhibited significantly lower microbial biomass (total PLFAs) in the organic horizon. There were no significant changes to total microbial biomass in the mineral soil. Differences in the soil microbial community composition were also observed. With warming, the ratio of Gram-negative to Gram-positive bacteria was significantly lower in the organic horizon. However, the microbial community composition did not change significantly in mineral soil samples (Table S6). N-addition did not lead to any significant change with regard to community composition in either the organic or mineral layers (Table S6). Warming + N significantly lowered the ratio of Gram-negative to Gram-positive bacteria but did not significantly change the community composition in the mineral layer (Table S6). Specific ratios of PLFAs (monoenoic: saturated PLFAs, cy17:0/16:1ω7c, cy19:0/18:1ω7c) have been reported to increase when microbial biomass is faced with substrate limitations or other stress, such as changes in the size of the biomass and shifts in the community composition.[87] In the organic horizon, warming and warming + N decreased the ratio of monoenoic to saturated PLFAs significantly relative to the control. Furthermore, the ratio of cy17:0/16:1ω7c increased significantly with warming, and the ratio of cy19:0/18:1ω7c increased significantly with warming + N (Table S6). No significant changes in any stress ratios were noted with any treatment in the mineral soil (Table S6).

Solid-State ^{13}C NMR Spectroscopy. Using ^{13}C NMR spectroscopy, changes in the integration values of chemical shift regions representing different forms of SOM relative to the control were observed. The integrated regions (Table S7) included alkyl C region (0−50 ppm), which represents resonances from aliphatic lipids and side chains from cutin and suberin; *O*-alkyl C region (50−110 ppm), which arises from peptides and carbohydrates, such as cellulose and methoxy C from lignin; aromatic and phenolic C region (110−165 ppm), which includes lignin and aromatic amino acids, and the carboxyl and carbonyl C region (165−215 ppm), which stems from a range of side-chain functionalities from lipids, peptides, and other oxidation products.[88] Warming increased alkyl C and decreased aromatic and phenolic C relative to the control in the organic horizon but did not impact *O*-alkyl C or aromatic and phenolic C (Table S7). In the mineral soil, aromatic and phenolic C, as well as carboxyl and carbonyl C, differed from the control (Table S7). N-addition altered alkyl, *O*-alkyl, and carboxyl and carbonyl C in both organic and mineral layers, respectively, increasing (*O*-alkyl C, carboxyl, and carbonyl C) and decreasing (alkyl C) these integration values relative to the control (Table S7). Warming + N increased alkyl C and decreased carboxyl and carbonyl in both organic and mineral layers relative to the control (Table S7). Furthermore, warming + N decreased *O*-alkyl C and aromatic and phenolic C in the organic layer only (Table S7). The alkyl/*O*-alkyl C ratio is used to assess the degree of SOM degradation relative to the control.[81] Warming

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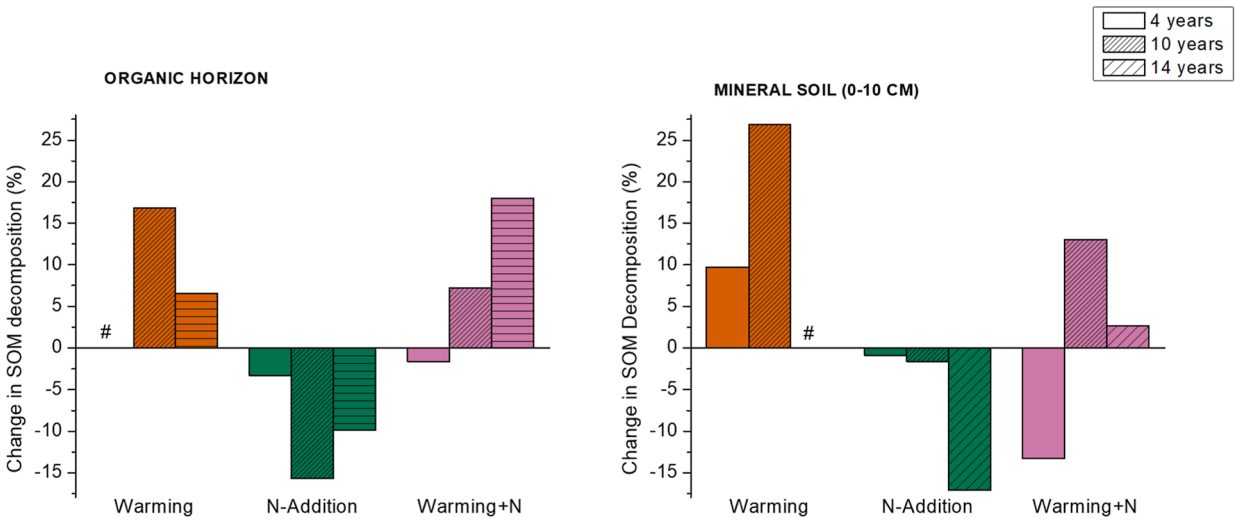

Figure 7. Percent change in SOM decomposition relative to the control treatment, as indicated by the alkyl/*O*-alkyl C ratio from solid-state ^{13}C NMR spectroscopy. # indicates no measured difference (Table S7).

enhanced SOM degradation in the organic horizon but did not change the relative state of SOM decomposition in the mineral layer. With N-addition, suppressed SOM degradation was observed in both the organic and mineral layers. Warming + N accelerated SOM degradation in both the organic and mineral soils relative to the control (Figure 7). The relative change in SOM decomposition over time shows that some responses have intensified depending on the horizon, whereas others have weakened (Figure 7).

DISCUSSION

Overall, we observed differences in the SOM chemical composition after 14 years of warming, N-addition, and warming + N. However, these changes were unique to each treatment and reflect different patterns and perturbations to biogeochemical cycling of soil C. Using several molecular proxies, we observed that warming and warming + N accelerated SOM degradation, while N-addition suppressed the decomposition of SOM. Furthermore, the magnitude and direction of these differences varied, especially when comparing warming alone to warming + N. Warming + N demonstrated the greatest enhancement in SOM decomposition, indicating that the combination of two antagonistic stressors (warming vs N-addition) results in unique changes to SOM biogeochemistry. Below, we outline in detail how after 14 years, distinct shifts in SOM chemistry were observed with warming, N-addition, and warming + N. We also compare these results to our earlier studies at this same site, which includes the same characterization approach after 4 years, published in a study by Pisani et al.[31] and 10 years, published in a study by vandenEnden et al.[32] of the experiment (Figures 5 and 7).

Soil Warming. Several studies showed that continued warming accelerates SOM decomposition.[14,29,30] Some studies have indicated that the flux of soil CO_2 from microbial respiration with prolonged warming is variable and dependent on the use of substrates by soil microbes.[89,90] Furthermore, the magnitude of warming impacts is also dependent on time, and observed changes in the SOM composition, soil respiration, and overall microbial activity have been reported to respond non-linearly with continued warming.[31,32,39,40] Other studies have indicated that above- and below-ground inputs can increase with soil warming.[13,30] In this study, the organic horizon exhibited decreased soil C concentrations (Figure S1) from 28 to 22%, although not significantly, which suggests that prolonged warming likely continued to enhance microbial decomposition of soil C.[91] Similarly, solid-state ^{13}C NMR spectroscopy indicated an overall increase in SOM degradation based on the alkyl/*O*-alkyl C ratio.[81] Microbial biomass (PLFA concentrations) did not change significantly but differences in microbial community composition observed were consistent with possible changes in substrate use (Table S6). After 10 years of warming, vandenEnden et al.[32] also reported no change in total soil microbial biomass indicating that the observed enhanced SOM decomposition was likely due to shifts in microbial substrate utilization. Frey et al.[41] reported that long-term warming altered substrate utilization patterns in an adjacent soil warming experiment at Harvard Forest (Figure S1 and Table S6). Anthony et al.[92] reported that fungal community evenness declined, and the community composition was altered with 10 years of soil warming which could also impact substrate utilization patterns. We observed that the ratio of Gram-negative to Gram-positive bacteria shifted, suggesting that bacteria may be responding to changes in substrate availability.[93] Furthermore, we observed increased production of cyclic PLFAs (cy17:0/16:1ω7c and monoenoic/saturated ratios; Table S6) which signify increased stress with warming, likely related to changes in substrate availability or continuous soil warming, which has been shown to increase microbial stress independent of substrate availability.[94] Interestingly, these shifts in microbial community structure did not reflect extensive changes in the use of specific SOM compounds, as previously observed in the organic horizon after 10 years of the experiment.[32] After 14 years of the experiment, specific SOM compounds (Tables S1−S5) did not exhibit the same extent of significant differences relative to the control organic horizon. However, enhanced SOM decomposition observed via solid-state ^{13}C NMR spectroscopy (Figure 7) indicated that changes to the microbial community composition continued to subsequently alter the SOM composition but to a lesser extent.[32] Collectively, these results indicate that the extent to which warming has impacted SOM degradation has

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weakened over time in the forest floor and is consistent with the patterns observed for long-term warming.[43]

In contrast, the mineral (0−10 cm) soil did not exhibit any differences in total soil organic C concentrations or microbial biomass (PLFAs) and community composition (Figures S1 & 6; Table S6). Furthermore, short-chain lipids, cyclic lipids, and simple sugars (Tables S1 and S2), which are typically categorized as preferred microbial substrates, were not statistically different after 14 years of warming. Higher concentrations of suberin-derived compounds relative to the control were observed, signifying continued preservation of these root-derived compounds with warming and/or increased below-ground inputs.[32] There were also higher concentrations of C_{16}, C_{18}, and mid-chain lipids relative to the control in the mineral soil (Table S3). Increased root-derived compounds are consistent with the results of Kwatcho Kengdo et al.[13] who reported increased fine root turnover with 15 years of warming in a European mountain forest. Microbial-derived lipids, which are thought to result from plant-derived SOM processing and associated SOM turnover,[85] increased in the mineral soil further suggesting enhanced decomposition of plant-derived compounds. Although some changes in specific SOM compounds were detected, there was no overall difference in the SOM composition observed by solid-state ^{13}C NMR in the mineral layer (Figure S1) and no difference in SOM decomposition relative to the control (Figure 7; Table S7). These observations are distinct from previous studies where enhanced SOM decomposition was observed with continued warming[31,32] but likely indicate that the overall composition of SOM reflects an apparent equilibrium between increased plant-derived inputs and microbial decomposition.

After 10 years of warming, vandenEnden et al.[32] reported a significant decrease in plant-derived aliphatic and cyclic lipids (steroids and terpenoids). In our study, no significant decrease was observed in either the organic or mineral soils after 14 years (Figure 1). Furthermore, vandenEnden et al.[32] reported that warming had elevated SOM degradation by 16 and 26% relative to the control in organic and mineral soils, respectively. After 14 years, we noted that warming had only elevated degradation by 6.5% in the organic horizon and not at all in mineral soil (0%) (Figure 7). Our results are consistent with our hypothesis, which stipulates that warming will continue to accelerate SOM degradation. Furthermore, NMDS analysis shows similarities between concentrations of aliphatic compounds between years 10 and 14 but not year 4 (Figure 5). However, the magnitude of accelerated SOM decomposition is very different from that observed at 4 and 10 years,[31,32] demonstrating that sustained warming is dynamic and selectively impacts SOM compounds uniquely over time.

N-Addition. Previous studies have indicated that N-addition slows SOM decomposition in temperate deciduous forests[45,54] via the suppression of microbial processing of SOM.[51] In our study, N-addition did not significantly alter soil C or N concentrations in both organic and mineral layers (Figure S1), which may be consistent with the time required to increase the soil C content with long-term N-addition.[18,95] Furthermore, we observed a decrease in the alkyl/*O*-alkyl C ratio in both organic and mineral layers, suggesting that SOM decomposition was suppressed[81] relative to the control (Figure 7; Table S7) and was consistent with observations made at years 4 and 10. In addition, microbial biomass was significantly lower with N-addition in the organic horizon (Figure 6; Table S6). Previous studies reported decreased microbial biomass with continued N-addition, related to the inhibition of oxidative enzyme activity, especially peroxidases which are responsible for the degradation of lignin.[18,59] In the mineral layer, no significant changes were noted with regard to microbial biomass or community composition; however, the response of microbial communities and substrate utilization patterns may differ with soil depth.[46,96] Furthermore, higher concentrations of long-chain aliphatic and cyclic (steroids and terpenoids) lipids were found in both soil layers (Figure 1; Table S1 and S2), which is indicative of suppressed processing of plant-derived inputs and SOM.[51] An increase in sugars and *O*-alkyl C relative to the control further corroborates the slowing of SOM decomposition, given these compounds are preferred microbial substrates (Table S7).[97] No significant change was observed in the concentrations of microbial-derived lipids (Table S4) relative to the control in either soil layer which is consistent with previous studies that reported decreases in microbial biomass with N-addition.[51] As such, the reduced microbial biomass and accumulation of plant-derived compounds with N-addition have subsequently reduced the production of cellular and extracellular microbial-derived lipids, suggesting that this group of compounds may be useful indicators to assess how anthropogenic change is altering soil C biogeochemical processes. Overall, the continued suppression of SOM decomposition in both horizons is consistent with previous observations as well as our hypothesis which stipulates that 14 years of continuous N-addition would suppress microbial biomass and SOM degradation in these temperate forest soils.

Soil Warming + N-Addition. When applied separately, warming and N-addition impact SOM differently.[51,61] Therefore, it is important to study combined anthropogenic stressors to better understand how different components of global environmental change will alter soil C biogeochemistry in forests. Our previous investigations after 4 and 10 years documented[31,32] that warming + N impacted SOM decomposition differently over time. With 4 years of experimental treatment, warming + N-addition behaved more similar to N-addition[31] but after 10 years, warming + N accelerated SOM decomposition and was more similar to warming alone[32] (Figure 7). After 14 years, and consistent with our hypothesis, warming + N has continued to mimic warming alone but to a different extent than observed previously. The soil C content was not significantly different to the control, but this does not reflect changes that have occurred with regard to specific SOM compounds or the overall rate of degradation.[98] For example, alkyl/*O*-alkyl C ratios demonstrated that warming + N continued to accelerate degradation in both the organic and mineral layers through the microbial processing of preferred substrates (Figure 7; Table S7), despite a lack of increase in the microbial biomass. Notably, after 10 years, the rate of SOM degradation was accelerated with warming + N but not to the same extent as with warming alone (Figure 7).[32] Analysis of select SOM compounds by NMDS further confirms that warming + N results are more similar between years 10 and 14 as compared to year 4 (Figure 5). Our results here demonstrate a shift in the biogeochemical trajectory with warming + N over time, however, exemplifying that the overall differences in the SOM composition are indeed distinct from warming alone. After 14 years, warming alone is no longer accelerating degradation, but warming + N is continuing to accelerate SOM decomposition in both soil layers. With warming + N, we also observed significantly lower microbial

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biomass (PLFA concentrations) in the organic horizon but no difference in microbial biomass in the mineral layer, indicating unique responses that are horizon-dependent (Figure 6; Table S6). In the organic horizon, increased microbial stress (cy19:0/18:1ω7c ratio) was observed with warming + N as compared to the other treatments which may be related to many factors including substrate stress and changes in the microbial environment.[99] Interestingly, microbial stress (as measured via different PLFA ratios) has also been linked to temperature variance alone and has been shown to increase with higher temperatures independent of changes in substrate availability.[94] This may explain why increased microbial stress was observed with both warming and warming + N but not with N-addition. Furthermore, Melillo et al.[15] suggested that microbial community reorganization is indicative of long-term soil warming and respiration patterns, suggesting that these processes are dynamic over time. Our results indicate that as with warming, warming + N is also dynamic, and the changes in SOM decomposition are offset by increased plant inputs. As with warming, the concentrations of several specific SOM compounds did not change significantly (Figure 1; Tables S1 and S2), suggesting that the impact of warming + N has also weakened over time but not to the same magnitude as warming alone. More long-lived compounds, such as cutin- and suberin-derived lipids, increased significantly in the mineral layer with both warming and warming + N (Figure 2; Table S3) and likely reflect the use of other preferred substrates as well as preservation of below-ground inputs that may also have shifted after 14 years of the experiment.[13] Furthermore, there were higher concentrations of C_{16}, C_{18} and mid-chain lipids relative to the control in the mineral soil. As with warming, this may be due to a shift in microbial processing. This significant increase in microbial-derived lipids was observed with warming + N in the mineral soil (Figure 3; Table S4), indicating that despite no change in the size of the microbial biomass, the sustained metabolic activity continued to alter SOM biogeochemical trajectories and enhanced the overall SOM decomposition uniquely. Moreover, warming + N appears to have a more pronounced and long-lived impact on the SOM composition than warming alone after 14 years of the experiment, which is consistent with our third hypothesis.

As warming and N-addition alter SOM chemistry in opposite manners,[69] it is difficult to predict how this combined treatment will continue to impact SOM decomposition. However, our hypothesis that warming + N will continue to mirror warming in both organic and mineral layers is supported by both NMR and molecular biogeochemistry data. Compared to previous studies, there is a smaller difference with warming + N relative to the control in both soil layers, consistent with CO_2 flux and expected warming behavior. Furthermore, we did not observe additive impacts with regard to warming and N-addition as other studies have noted.[100] This may demonstrate that N-addition acts to offset certain warming-induced SOM responses and that these two environmental stressors do not work through the same mechanisms, as has been reported in other studies studying the response of SOM to warming + N.[101,102] For instance, Song et al.[103] proposed that N-addition may create a negative feedback loop and lessen the impact warming would otherwise have on SOM. However, most experiments are short term in nature and may not be sufficient in predicting how SOM will react after 14 years of continued treatment. In some regards, warming + N leads to a more pronounced change in SOM than warming alone, especially in the mineral layer, highlighting the need for further long-term studies of combined ecosystem stressors.

CONCLUSIONS

Following 14 years of treatment and using a combination of targeted and non-targeted techniques, we observed several changes to SOM degradation patterns with warming, N-addition, and warming + N in both organic and mineral (0−10 cm) soils. This approach offers insight into both specific compound concentrations associated with plant- and microbial-processing, as well as a holistic overview of the SOM molecular composition via solid-state ^{13}C NMR spectroscopy. Warming and warming + N accelerated SOM degradation, whereas N-addition suppressed the decomposition of SOM, consistent with our initial hypotheses, as well as SOM behavior following 10 years of treatment. However, changes in the magnitude of SOM acceleration and suppression were noted, especially in the case of warming, wherein fewer molecular compound changes occurred in both organic and mineral soils. Likewise, while suppression of SOM degradation due to N-addition has been reported previously, 14 years of addition led to a stronger impact, especially in the mineral horizon. This is distinct from our previous observations after 4 and 10 years of experimental manipulations, wherein N-addition had a greater impact on the organic horizon. While warming + N continues to mirror warming alone, some aspects are markedly different, demonstrating the dynamic responses of SOM over time in forests. Overall, our study shows how two individually antagonistic, but very prevalent, environmental stressors interact with one another to change the character of SOM uniquely and over time. As persistent anthropogenic activity continues to heighten the concern surrounding these stressors, our results highlight the need for additional research studying the long-term and combined impact that warming + N, and ultimately, climate change will have upon forest soils. Our study further emphasizes that when combined, environmental stressors behave uniquely at the molecular-level, and these responses cannot be predicted from studies that consider individual stressors alone.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.2c00380.

Carbon and nitrogen content, solid-state ^{13}C NMR spectra, all measurements conducted using solvent extraction, base hydrolysis, copper oxidation, PLFA analysis, and solid-state ^{13}C NMR spectroscopy (PDF)

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Author Contributions

The manuscript writing was led by I.S. and edited by all authors. All authors have given approval to the final version of the manuscript. S.D.F., M.K., and T.M. carried out fieldwork and sampling. T.M. processed soils and carried out soil carbon and nitrogen measurements and NMDS and PERManova analyses. I.S. carried out solvent extraction, base hydrolysis, copper(II) oxide extractions, and GC-MS analysis. I.S. also carried out sample preparation and analysis by solid-state ^{13}C NMR. M.T.A. carried out PLFA extraction and data collection. The SWaN experiment was conceptualized and designed by S.D.F. S.D.F. and M.J.S. supervised the work and conceived the initial idea for the project. Measurement techniques were conceived by M.J.S. and S.D.F.

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Notes

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