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Long-Term Nitrogen Addition Alters the Composition of Soil-Derived Dissolved Organic Matter

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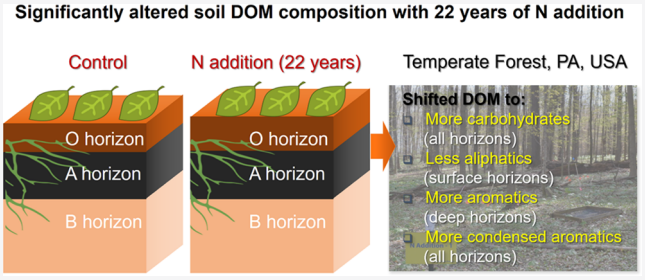
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Supporting Information

ABSTRACT: Forest soil dissolved organic matter (DOM) is a major source of terrigenous dissolved organic carbon (DOC) that is an important component of biogeochemical cycles. While many studies have shown that DOM dynamics are regulated strongly by nitrogen availability, how continued or increasing deposition of reactive nitrogen to forests alters the molecular composition of soil DOM remains unexplored. We studied the storage and molecular-level composition of soil-derived DOM after 22 years of nitrogen addition in a temperate deciduous forest. Soil DOC quantity changes were small (+19.0 g/m^2; ∼10% increase) but the molecular composition changed markedly. Indices based on optical spectroscopy suggested that DOM molecular size and aromaticity were elevated with nitrogen addition. Nuclear magnetic resonance analyses showed that DOM contained more carbohydrates and aromatics but less aliphatic compounds with nitrogen addition. Ultrahigh-resolution mass spectrometric analysis further supports the finding that solid-phase isolated DOM from nitrogen-added soils was larger in molecular size and aromaticity. Condensed aromatic dissolved black carbon, particularly those compounds with high molecular size/carbon number and those stored in deep soils, showed the greatest percent increase with nitrogen addition. These results countered our hypothesis that soluble carbohydrates would be selectively removed from the DOC pool compared to polyphenols and condensed aromatic components under nitrogen enrichment. These changes in DOM molecular composition may also impact microbial communities and downstream metabolism of DOC.



KEYWORDS: *dissolved organic matter, reactive nitrogen deposition, temperate forest soils, Fourier transform ion cyclotron resonance mass spectrometry, nuclear magnetic resonance spectroscopy*

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1. INTRODUCTION

Dissolved organic matter (DOM) can account for as much as 5% of the soil organic matter (SOM), yet it represents the most mobile and reactive carbon (C) fraction in forest soils.[1,2] Its quantity and quality are critical in regulating soil respiration, plant growth, biogeochemical fluxes, and energy fluxes within soils,[3,4] as well as fueling the biological metabolism of receiving aquatic systems.[5] DOM is a highly complex continuum containing components of various molecular sizes (e.g., monomers versus polymers), aromaticity (e.g., fatty acids versus polyphenols versus condensed aromatics), and stability (e.g., biolabile sugars versus more persistent lignin).[2] The compositional heterogeneity of DOM suggests that DOM reactivity is distinct and has varying potential to be mineralized to CO_2. For example, while the labile components such as carbohydrates are a favored substrate that fuels microbial activity in soils and waters,[5] some antimicrobial polyphenols may negatively impact microbial communities.[6] Within the DOM pool, the ubiquitous thermogenic dissolved black carbon (DBC) is of increasing concern[7−9] due to its particularly low biolability,[10] strong photoreactivity,[11,12] high sorption capacity for pollutants,[13] and high reactivity with disinfectants to form nitrogenous toxins during water treatment.[14]

As global nitrogen (N) deposition is expected to continuously increase from 100 Tg/yr in 1995 to 200 Tg/yr in 2050,[15,16] understanding how the soil DOM and DBC storage and flux respond to long-term N addition is critical for

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projecting a future change in their biogeochemical dynamics. C and N cycles often interact, and the soil dissolved organic carbon (DOC) concentrations have been correlated to C/N ratios and NH_4^+−N levels in soils globally.[17] Simulated N addition altered plant community composition and productivity,[18] microbial community composition and activity,[19] and soil properties such as soil pH[20] and soil organic matter (SOM) content.[21] As all of these characteristics are governed by the C and N cycles, the combination of these properties will inevitably alter DOM composition, as well as the storage and export of soil DOM.

The impact of N deposition on soil DOC quantity has been studied for decades across various types of forest ecosystems[22−30] but has yielded variable results. Many studies reported that increased N deposition increased soil DOC storage or concentrations,[22−24] while other studies observed decreased[25] or insignificantly altered[26−30] DOC storage or concentrations. For example, simulated N deposition for 1 year[31] or for 8 years[32] increased the concentrations and fluxes of DOC and dissolved organic nitrogen (DON) in northern temperate broad-leaved forests of the upper Great Lakes Region, although another study[33] observed no significant change in DOC concentration with 7 years of N addition in similar forested ecosystems in Massachusetts. N deposition was also reported to accelerate the decomposition of soil by microbes and thus promoted the DOC export to a nearby river.[34] In contrast, the release of organic acids decreased by 20% months after N addition in a red pine plantation in Maine, the United States.[35] Interestingly, the DOC fluxes from mineral soils were reduced by 17% with 1 year of N addition in beech forest stands on calcareous soils in the Swiss Jura, which could be due to decreasing pH and increasing ionic strength.[36] In N-rich lowland tropical forests of China, 7 years of N addition significantly reduced DOC concentrations in soil solutions but increased the storage of extractable DOC in soil.[37]

Long-term N addition studies show various responses of total SOM and molecular-level SOM components, which could contribute to different net responses of soil organic carbon (SOC) storage across forests.[38−40] Therefore, soil DOM components, which are among the mobile and reactive, but least studied portions of SOM, are also expected to shift with long-term N addition. Indeed, lower DOC/DON ratios have often been reported in soils with N addition[33,41] and in fluvial water from catchments dominated by N fertilized farmlands,[42,43] which suggested that DOM quality is altered by N addition. Lower specific ultraviolet absorbance (SUVA) of soil DOM was found with over 1 year of N addition in beech forest stands, which suggested a shift of DOM to lower aromaticity with N addition.[36] This was attributed to the N-induced suppression of microbially mediated lignin degradation and reduced production of aromatic DOM, and the pH-dependent sorption/desorption responses.[36] In contrast, the SUVA of soil DOM increased after 2[44] and 10 years[45] of N addition in some other forests, which suggested increased aromaticity with N addition. The results from previous studies focused on one or a few aspects of DOM chemistry (mostly from optical properties) and were mostly from short-term studies with the treatment of less than a decade. Currently, a detailed analysis of the molecular biogeochemistry of DOM in soil profiles with long-term N deposition is lacking. Elucidating the response of DOM at the molecular-level to long-term N addition, e.g., whether more labile or more antimicrobial DOM will be yielded, is critical to understanding the DOM reactivity in the environment. Because long-term N addition has been reported to increase SOM storage and stimulate carbohydrate degradation but inhibit lignin degradation,[46] we hypothesize that (1) the soil DOC storage will significantly increase and (2) easily degradable compounds (e.g., soluble carbohydrates) will be selectively removed from the DOC pool compared to more complex DOC components (e.g., polyphenols and condensed aromatics) under long-term N enrichment in all soil horizons. Moreover, as the response of SOM storage to long-term N addition was more significant in the surface soil horizon,[38] our third hypothesis is that the changes in DOC storage and chemistry would be greater in the surface soil horizon than in deeper horizons. To test these hypotheses, we quantified the extractable DOC concentration and molecular composition with soil depth after 22 years of N addition in a temperate forest ecosystem receiving high levels of N deposition. Optical spectroscopy, solution-state nuclear magnetic resonance (NMR) spectroscopy, and high-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) were used to determine the potential shifts in the DOM composition.

2. MATERIALS AND METHODS

2.1. Site Information. Soil samples were collected from the long-term N addition experiment at Bousson Environmental Research Reserve in Meadville, Pennsylvania (41°36′N, 80°2′W), in the spring of 2016 after 22 years of N addition manipulations. The detailed site information and experimental design are described in Chan et al.[47] and Wang et al.[38] Briefly, Bousson Environmental Research Reserve is a temperate forest dominated by black cherry (*Prunus serotina* Ehrh; ∼60% of aboveground biomass) and sugar maple (*Acer saccharum* Marsh; ∼28% of aboveground biomass). The daily temperatures average −4 °C in January and 21 °C in August with an average precipitation of 105 cm/year. The soils are classified as Alfisols with a silty-loam texture, moderately well drained, a pH of 4.0, a cation exchange capacity of 3.73 $cmol_c$/kg, and a bulk density of 0.52 ± 0.01 g/cm^3 in the upper 15 cm of the mineral soil horizon. The experiment consists of three 15 m × 15 m control plots and three 15 m × 15 m N-added plots. Nitrogen was added monthly from May to October in the form of NH_4NO_3, with an annual rate of 100 kg N/ha/yr.[46]

The surface litter was carefully removed before soil sampling. Soil samples from O horizon (depth of approximately 1−3 cm), A horizon (depth of approximately 3−8 cm), and top 10 cm of the B horizon were collected from two 20 cm × 20 cm area soil blocks within each plot. In total, six soil blocks (two blocks in each of the three plots) were collected from both control and N-added plots. The dried mass (105 °C) and bulk density of each soil horizon from each soil block were determined and recorded. All soil samples were sieved (2 mm), freeze-dried, and ground with a ball mill before chemical analyses. The SOC contents in soils were determined with a Thermo Flash 2000 elemental analyzer.

2.2. Soil DOM Extraction and Analyses. The water-extractable dissolved organic matter stored in soil samples was exacted with Milli-Q water using an exhaustive method.[48] Specifically, soil samples (5 g for O horizon, 10 g for A horizon, and 20 g for B horizon) were mixed with 200 mL of Milli-Q water and shaken for 24 h in dark on a reciprocal shaker, and the extract was filtered through a Millipore 0.45 μm membrane. This extraction was repeated five additional

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times with 150 mL of Milli-Q water to exhaustively extract the DOM. All six sequential filtrates were combined, filled up with Milli-Q water to 1000 mL, and well mixed. The exhaustive method was used because we aimed to estimate the maximum storage of DOM, and it should be acknowledged that the exhaustively extracted DOM could be chemically different from the DOM in intact soil pore water. About 20 mL of the extract was acidified to pH = 2 and analyzed for the DOC and total dissolved nitrogen (TDN) concentrations using a total organic carbon analyzer (Shimadzu model TOC-LCPH, Kyoto, Japan). The DOC mass per soil area (noted as "DOC storage"; in g-C/m^2-soil), per soil mass (noted as "DOC concentration"; in mg-C/g-soil), and per SOC (noted as "DOC/SOC"; in %) were calculated.

Ultraviolet−visible absorbance and fluorescence spectroscopies are convenient approaches to study the characteristics of chromophoric and fluorescent DOM, respectively. Each soil extract was analyzed for their ultraviolet−visible absorbance and fluorescence emission excitation matrices (EEMs) using a Horiba Aqualog spectrometer as previously described.[44] Several optical indices including specific ultraviolet absorbance at 254 nm (SUVA), E2/E3 ratio, and freshness index were calculated.[14] SUVA, a widely used indicator for DOM aromaticity, was calculated as ultraviolet absorbance at 254 nm divided by the DOC concentration.[49] E2/E3 ratio, an indicator of DOM molecular size, was calculated as the absorbance at 254 nm divided by that at 365 nm.[50] The freshness index, an index for the contribution of recently produced microbial DOM, was calculated as the signal ratio of emission at 380 nm divided by the maximum emission between 420 and 435 nm for excitation at 310 nm.[51] No parallel factor analysis of EEMs was performed in the present study because the analyses of FT-ICR MS and NMR have provided more detailed and high-resolution molecular-level information of the DOM composition and the sample size was relatively small. Given that the treatment of N addition often interacts with the soil horizon to alter the soil DOM quantity and properties, we used one-way analyses of variances (ANOVA) followed by Tukey's post hoc test to examine the differences in soil DOC storage, soil DOC concentration, DOC/SOC ratio, and the optical indices of DOM between the treatment and control for each soil horizon. SPSS v15.0 (IL) was used for statistical analyses. The difference was considered as significant if $P < 0.0.5$.

Preliminary data from the absorbance and fluorescence spectroscopy showed that at least one of the optical properties was significantly different between the control and treatment for a soil horizon based on ANOVA. That is, there was great between-treatment variability over the with-treatment variability although there must be spatial variations caused by soil heterogeneity. To examine discrete molecular-level DOM compositional changes with N addition, six replicates (3 plots × 2 blocks) belonging to the same soil horizon of either control or N-added plots were well mixed as one composite sample for the analyses of FT-ICR MS and NMR. Forty milliliters of the composite water extract was acidified (pH = 2) and solid-phase-extracted using PPL SPE cartridges following the method by Dittmar et al.[52] The solid-phase extracted DOM was eluted by MeOH, dried by N_2 gas in a 2 mL glass vial, and kept frozen before the analysis by FT-ICR MS. The solid-phase extracted DOM was reconstituted in a 1:1 methanol/water mixture to achieve a concentration of 50 μg of the sample DOC per milliliter before analysis by a 12 Tesla Bruker SolariX FT-ICR MS (Bruker Daltonics Inc., Billerica, MA) with electrospray ionization source using negative ion mode and 200 scans. All samples were analyzed at a resolving power of 400 000 ($m/\Delta m$ 50% at m/z 400) with a mass range of 114−1200 m/z. Bruker Daltonik (version 4.2) software was used to convert raw spectra to peak lists by applying peak picker with a signal-to-noise threshold of 7 and an absolute intensity threshold of 100. Elemental formula is assigned to each monoisotopic mass using the Formularity software,[53] which was based on the compound identification algorithm developed by Kujawinski and Behn,[54] with the following requirement $C_{0-100}H_{0-200}O_{0-50}N_{0-10}S_{0-2}P_{0-2}$, H/C < 2.5, O/C < 1.2, N/C < 0.5, S/C < 0.2, P/C < 0.1, and (S + P)/P < 0.2.[55,56] The aromaticity and unsaturation degree of each compound were evaluated using the modified aromaticity index (AI_{mod}) and double bond equivalents (DBEs), respectively.[55,57] The nominal oxidation state of carbon (NOSC), an indicator of DOM polarity, was calculated as 4-[(4C + H − 3N − 2O − 2S)/C].[58] The average molecular weight, AI_{mod}, DBE, NOSC, H/C, O/C, N/C, S/C, and P/C ratios were calculated using the intensity weighted methods.[59] The molecular lability boundary for more labile contributions (MLBL %), which is a proxy for DOM lability, was calculated as described in D'Andrilli et al.[60] Molecular formulae were assigned to different groups as previously described:[61] (1) condensed aromatics ($AI_{mod} \geq 0.67$), including dissolved black carbon if $C \geq 15$,[62] (2) aromatic compounds including polyphenols and condensed aromatics with aliphatic side chains[55] ($0.67 > AI_{mod} \geq 0.50$), (3) highly unsaturated compounds, including lignin degradation products[63] ($AI_{mod} < 0.50$ and H/C < 1.5), subdivided into oxygen-rich (group 3a, O/C > 0.5) and oxygen-poor (group 3b, $O/C \leq 0.5$) compounds, (4) unsaturated aliphatic compounds ($2.0 \geq H/C > 1.5$, N = 0), (5) unsaturated aliphatic compounds containing N, including peptide molecular formulae ($2.0 > H/C \geq 1.5$, N > 0), (6) saturated compounds, including lipids (H/C > 2.0 and O/C < 0.9), and (7) saturated compounds (H/C > 2.0) with O/C > 0.9, including carbohydrate molecular formulae. However, because the signals for the saturated lipids and carbohydrates were consistently less than 0.2% of the total signal, the results of groups (6) and (7) were not shown in the present study. The relative abundance of different compound classes was intensity weighted and calculated as the ratio of the summed intensity of formulae in each class to the summed intensity of all detected formulae.[64] Furthermore, the unique formulae in control and N-added soils (noted as control-specific and N-added-specific formulae) were identified and analyzed based on Kendrick mass defect (KMD) plots to display the reaction trends of (1) hydrogenation/dehydrogenation (H_2 series), (2) methylation/demethylation (CH_2 series), (3) ring-opening reactions (O_2 series), and (4) carboxylation/decarboxylation (CO_2 series).[65,66]

For NMR analysis, ∼900 mL of composited water extract was frozen, freeze-dried, and further dried under a vacuum over P_2O_5. Approximately 5 mg of dried sample was reconstituted in 60 μL of NaOD/D_2O solution (pH = 12), centrifuged to remove any insoluble materials, and transferred into a Bruker BioSpin 1.7 mm NMR tube.[67] All samples were characterized using a Bruker BioSpin Avance III 500 MHz NMR spectrometer (Karlsruhe, Germany) equipped with a $^1H-^{15}N-^{13}C$ TXI 1.7 mm microprobe fitted with an actively shielded Z gradient.[67] First, one-dimensional ^1H NMR spectra were collected using presaturation utilizing relaxation gradients

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and echoes (PURGE) with a scan number of 256, a recycle delay of 2 s, and time domain points of 32 K.[68] Spectra were then processed using a zero-filling factor of 2 and were apodized by multiplication with an exponential decay corresponding to a 0.3 Hz line broadening. Second, diffusion-edited (DE) ^{1}H NMR spectra were collected using a bipolar pulse longitudinal encode−decode sequence.[69] A total of 2048 scans were collected using a 2.5 ms, 53.5 gauss cm^{-1}, sine-shaped gradient pulse, a diffusion time of 200 ms, 16 K time domain points, and a 10 Hz line broadening. The one-dimensional ^{1}H NMR spectra were integrated into four regions: (1) materials derived from linear terpenoids (MDLTs), 0.6−1.6 ppm; (2) carboxyl-rich alicyclic molecules (CRAMs), 1.6−3.2 ppm; (3) carbohydrates and peptides, 3.2−4.5 ppm; and (4) aromatics and phenolics, 6.5−8.4 ppm.[70,71] Furthermore, two-dimensional ^{1}H−^{13}C heteronuclear single quantum coherence (HSQC) spectra were obtained using digital quadrature detection with an echo−antiecho gradient selection. A total of 1200 scans were collected with 2 K and 96 time domain increments in the F2 and F1 dimensions, respectively. The two-dimensional spectra were processed using a function corresponding to a 25 Hz line broadening in F2 and an unshifted sine-squared function in F1 with a zero-filling factor of 2 in both dimensions.

For NMR and FT-ICR MS analyses, we did not perform statistical analyses on the difference of molecular-level DOM composition between the treatment and control because we analyzed the composite sample instead of all replicates. However, the percent change in NMR constituent or solid-phase extracted DOM component i relative to the control (% ΔMass(i)) was roughly estimated as follows by assuming that (1) the quantity of NMR-detected dissolved organic hydrogen was approximately in proportion to the quantity of dissolved organic carbon in both the control and N-added plots, and (2) the differences in solid-phase extraction recovery and the ionization efficiency for a specific solid-phase extracted DOM were negligible between the control and N-added plots

$$\%\ \Delta\mathrm{Mass}(i) \approx \left(\frac{M(\mathrm{DOC,\ N\text{-}added}) \times P(i,\ \mathrm{N\text{-}added})}{M(\mathrm{DOC,\ control}) \times P(i,\ \mathrm{control})} - 1\right) \times 100$$

where M(DOC, control) and M(DOC, N-added) are the DOC mass per soil area (in g-C/m^2-soil) in a specific soil horizon for the control and the N-added plots, respectively, and P(i, control) and P(i, N-added) are the relative abundances of the DOM component i in a specific soil horizon for the control and the N-added plots, respectively.

3. RESULTS AND DISCUSSION

3.1. Soil DOM Storage and Concentration. After 22 years of N additions, the total water-extractable soil DOC storage was 184.0 ± 27.4 g/m^2 (mean ± standard error) in control plots and 203.0 ± 12.5 g/m^2 in N-added plots in all three horizons studied, with no significant difference between the control and treatment (Figure 1). The average horizon-specific soil DOC storage in g/m^2-soil and soil DOC concentration in mg/g-soil were also not significantly different between N-added plots and control plots for all three horizons (Figure 1). However, the soil DOC/SOC for the O horizon was significantly lower in N-added soils (2.4 ± 0.2%) compared to that in the control (3.5 ± 0.3%; Figure 1). Percentagewise, the slight, insignificant increase of ∼10% in

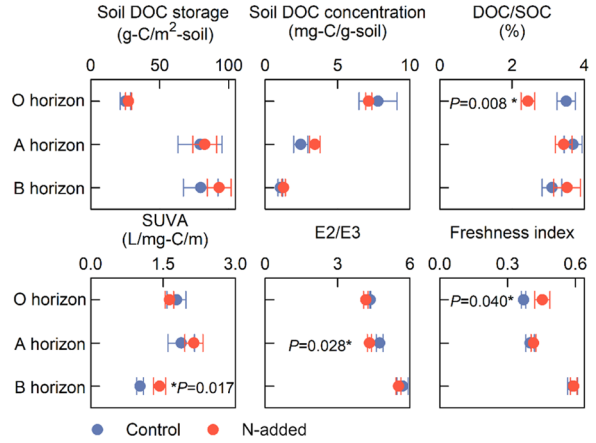


Figure 1. Dissolved organic carbon (DOC) storage (per m^2 soil area) and concentration in soil (per gram soil) and soil organic carbon (SOC) in control and nitrogen-added plots. Error bars present the standard errors of six replicates. Asterisks and P-values show significant differences (P < 0.05) between the treatment and control in a specific horizon.

average soil DOC storage here is similar to the increase of ∼17% in SOC storage in these three horizons as previously reported.[38,46] This percentage was slightly lower than the range of 12.03−25.29%, the 95% confidence interval of a percent increase in DOC concentration of N-added soils from 58 tropical, temporal, and boreal forest sites in a meta-analysis.[22]

Many mechanisms involving DOM production, leaching, and mineralization have been proposed to explain the increased soil DOM storage or concentration.[36,72] N addition may (1) increase DOM storage by increasing forest productivity, which produces more organic materials that act as a source of DOM production;[72] (2) alter microbial production of DOM from the litter or soil by promoting or inhibiting the activities of certain microbes;[34,73] and (3) reduce the export of DOM by altering the solute chemistry such as increasing ion strength and acidity.[36] At this study site, the first mechanism is unlikely to be an important process because the plant inputs such as litterfall did not significantly increase with N addition.[38,46] The second and third mechanisms are more likely to be dominant. Particularly, the second mechanism has been supported by the reduced microbial biomass (measured via phospholipid fatty acids) and lignin-degrading enzyme activity with N addition at this site.[38,46] Therefore, the reduced soil DOC/SOC ratio in the O horizon of the N-added plots compared to that of the control may be partially explained by the suppressed microbial production of DOM from SOM in the O horizon with N addition.[38,46] In addition, increased soil acidity can greatly reduce DOC leaching[36,37] and DOC levels in water bodies.[74] Therefore, the acidification (pH decrease of approximately 0.5 unit) resulting from nitrification found at this site[46] may have reduced DOC leaching and likely caused more DOC accumulation with long-term N addition. Interestingly, deeper horizons have a greater percent increase in the DOC storage and DOC/SOC with treatment, which contradicts our third hypothesis and differs from our earlier finding that deeper horizons have the least percent increase in the total SOC storage with N addition.[38] It is possible that because N addition decreased the microbial and enzyme activities to a greater extent in the O horizon than in A and B horizons,[38,46] lower proportions of DOC were yielded from

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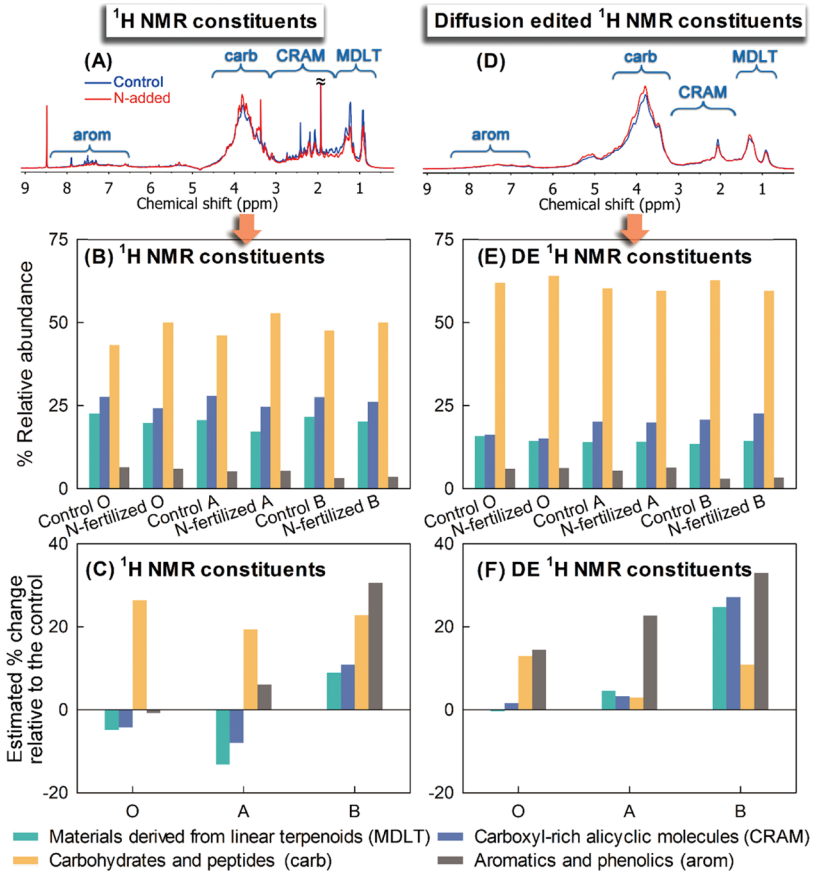


Figure 2. Integration results (B, E) of dissolved organic matter constituents in ^{1}H and diffusion-edited (DE) ^{1}H nuclear magnetic resonance (NMR) spectra (A and D showing examples for O horizon soil extracts) and the estimated percent change with long-term N addition (C, F).

microbial depolymerization of SOC in the surface horizon than in the deeper horizons of N-added plots.

3.2. Optical Properties of Soil DOM. Despite that the total soil DOM quantity was not significantly (only ~10%; $P > 0.05$) altered by long-term N addition, soil DOM quality was more sensitive to the long-term addition as indicated by the absorbance and fluorescence spectroscopy (Figure 1). SUVA of soil DOM was significantly higher in the N-added (1.43 ± 0.12 L/(mg-C m)) than control soils (1.02 ± 0.07 L/(mg-C m)) in the deep B horizon (Figure 1). As SUVA is a common indicator of DOM aromaticity,[49] this result suggests that the long-term N addition shifted soil DOM to a higher relative abundance of aromatics in the deeper soil horizons. Short-term N additions in different forests have shown distinct responses of SUVA to N addition.[36,44] However, the present results coincide with an earlier finding that 4 decades of N addition increased the SUVA of soil-derived DOM in B horizons but not in O horizons in a boreal forest[75] and another finding that SUVA and soluble phenols in soil DOM at 75 cm were all increased at four different forests with 10 years of N addition.[45] Furthermore, the average E2/E3 ratios were consistently lower in the N-added soils than those in control soils in all horizons (Figure 1). Because a lower E2/E3 ratio usually associates with higher molecular size,[50] our results indicate that long-term N addition may increase the overall molecular size of the soil-derived DOM in all horizons. The increased molecular size may be due to suppressed microbial processing[38] or less leaching[36] of the DOM of relatively larger molecular weight. However, the freshness index was significantly higher in N-added soils than that in control soils in the O horizon (Figure 1), which suggests a higher relative abundance of fresh microbial DOM inputs in the O horizon.[51]

3.3. DOM Composition by NMR. Based on the two-dimensional ^{1}H−^{13}C HSQC analyses, soil-derived DOM was dominated by materials derived from linear terpenoids (MDLTs), carboxyl-rich alicyclic molecules (CRAMs), carbohydrates (with signal contribution from peptides), and aromatics and phenolics (Supporting Information Figure S1). In addition to the general compound class regions, a series of small molecules that exhibit sharp and distinct resonances were also identified[48] and included formic acid, lactic acid, methanol, succinic acid, and acetic acid (SI Figure S1).

^{1}H NMR analyses were used to quantify the DOM composition in soil samples of different horizons and treatments. In all samples, carbohydrates were dominant, which is consistent with an earlier study that applied ^{1}H NMR spectroscopy to soil DOM characterization.[48] Compared to the control, the N-added soil DOM consistently had a higher percentage of carbohydrates in all horizons (Figure 2). This result is different from the first part of our second hypothesis that N addition likely reduces the concentration of soluble carbohydrates. N addition can stimulate cellulase enzyme activity and accelerate cellulose and *O*-alkyl-C decomposition,[76,77] which was also observed at this site.[38,46] As such, the greater net production (production minus mineralization/leaching loss) of soluble carbohydrates from *O*-alkyl-rich polymeric carbohydrates with the long-term N addition compared to that in the control is likely responsible for the increased carbohydrate fraction in DOM. In addition, compared to the control, the N-added soil DOM had

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Table 1. Molecular Characterization of the Solid-Phase Extracted DOM As Determined by FT-ICR MS[a]

	Control O horizon	N-added O horizon	Control A horizon	N-added A horizon	Control B horizon	N-added B horizon
total no. of assigned molecules	6561	6408	6206	6001	6101	6112
average mass (Da)	492	500	495	508	499	515
average formula	$C_{22.0}H_{22.4}O_{12.4}N_{0.3}S_{0.1}$	$C_{22.3}H_{21.3}O_{12.7}N_{0.3}S_{0.1}$	$C_{22.1}H_{23.2}O_{12.4}N_{0.4}S_{0.1}$	$C_{22.3}H_{20.7}O_{13.2}N_{0.4}S_{0.1}$	$C_{22.4}H_{24.5}O_{12.4}N_{0.3}S_{0.1}$	$C_{22.8}H_{23.4}O_{13.1}N_{0.3}S_{0.1}$
average H/C atom ratio	1.02	0.95	1.05	0.93	1.09	1.03
average O/C atom ratio	0.56	0.57	0.56	0.59	0.55	0.58
average DBE	12.1	13.0	11.8	13.2	11.4	12.4
average AI_{mod}	0.36	0.40	0.34	0.41	0.31	0.34
average NOSC	0.16	0.24	0.12	0.31	0.05	0.17
molecular lability boundary for more labile contributions (MLBL %)	9.1	6.0	6.2	3.9	3.7	2.5
% condensed aromatics ($AI_{mod} \geq 0.67$)	4.7	6.4	2.4	5.0	0.7	1.3
-% high molecular weight condensed aromatics $\geq C_{15}$	4.2	5.8	2.1	4.5	0.6	1.2
-% low molecular weight condensed aromatics $<C_{15}$	0.6	0.7	0.3	0.5	0.1	0.2
-% O-rich condensed aromatics (O/C > 0.5)	3.8	5.1	1.3	3.5	0.3	0.7
-% O-poor condensed aromatics (O/C ≤ 0.5)	1.0	1.3	1.0	1.4	0.4	0.6
% aromatics ($0.67 > AI_{mod} \geq 0.5$)	19.3	24.0	13.9	23.5	8.6	13.0
-% high molecular weight aromatics $\geq C_{15}$	18.1	22.8	13.2	22.6	8.2	12.6
-% low molecular weight aromatics $<C_{15}$	1.2	1.2	0.7	0.9	0.4	0.4
-% O-rich aromatics (O/C > 0.5)	15.7	19.9	10.7	19.7	6.3	10.4
-% O-poor aromatics (O/C ≤ 0.5)	3.6	4.1	3.3	3.7	2.3	2.6
% percentage of highly unsaturated molecules (HU)	55.2	49.8	63.5	52.1	74.3	68.5
-% O-rich HU (O/C > 0.5)	34.9	31.3	40.2	37.8	46.2	47.8
-% O-poor HU (O/C ≤ 0.5)	20.3	18.5	23.3	14.3	28.0	20.7
% unsaturated aliphatic compounds ($2.0 \geq H/C > 1.5$, N = 0)	5.9	3.7	3.1	1.5	1.9	1.0
% unsaturated aliphatic compounds containing N, including peptide molecular formulae ($2.0 > H/C \geq 1.5$, N > 0)	2.1	1.5	2.1	1.7	1.3	1.1

[a]Relative abundance of the total signal of all detected formulae.

consistently lower percentages of CRAM and MDLT in all horizons (Figure 2). This can be explained by the reduced microbial production of soluble aliphatics because the microbial processing of aliphatic SOM (alkyl-C) has been suppressed in these N-added plots.[38] Previous studies have hypothesized that a significant portion of CRAM is microbial-derived.[78] Thus, the reduced percentage of CRAM in soil-derived DOM with N addition is also consistent with a reduction in microbial biomass (phospholipid fatty acid) as previously reported.[38] With N addition, the relative abundance of aromatic and phenolic constituents in soil DOM declined to a lesser extent (<5%) in the O horizon but increased in the A and B horizons (Figure 2), consistent with the SUVA results. We have recently reported increased lignin-derived compounds with long-term N addition at this site.[38] The increased soluble aromatic and phenolic constituents with treatment in A and B horizons suggest that the N addition increased the solubility of aromatics and phenolics. The slightly decreased abundance of soluble aromatic and phenolic constituents with N addition in the O horizon is likely tied to the reduced peroxidase activity[46] and thus reduced the microbial production of dissolved aromatics from biopolymers in the O horizon, or due to dilution induced by the increased soluble nonaromatic carbohydrate fraction. Taking the amount of DOM storage into consideration, the percent change of each DOM constituent was estimated (Figure 2c). In terms of the overall DOM composition, the carbohydrate storage increased by ~20% in all horizons. Both the CRAM and MDLT decreased their storage in O and A horizons but increased their storage in the B horizon. The aromatic and phenolic constituents had increased storage in deeper A and B horizons but had a minor change in storage (<5%) in the surface O horizon.

The DE ^{1}H NMR experiment displays signals from molecules that do not move more than ~1 μm within the NMR tube over the diffusion time of 200 ms.[44] Therefore, the DE ^{1}H NMR spectra reflect relatively large, macromolecular, and aggregated compounds and differs from ^{1}H NMR spectra that display signals from all DOM compounds.[71] Compared to the ^{1}H NMR results, the percentage of carbohydrates in DE ^{1}H NMR exhibited smaller differences between the treatment and control (Figure 2). This suggests that the increased storage of carbohydrates with N addition is mainly contributed by small carbohydrates rather than macromolecule/aggregated carbohydrates. Opposite to the results of ^{1}H NMR, DE ^{1}H NMR showed that the percentages of the MDLT and CRAM were higher in N-added plots than in control plots in the A and B horizons. Therefore, although the overall percentages of soluble aliphatic materials decreased, the relative abundance of soluble macromolecular/aggregated lipids increased with N addition. Similarly, the relative abundance of macromolecular aromatic and phenolic constituents was consistently higher with N amendment for all horizons. This suggests that the soluble macromolecular/aggregated aromatics accumulated with N addition in all horizons, possibly due to increased production or reduced solubilization of macromolecular/aggregated aromatics. Taking the amount of DOM storage into consideration, the percent change of each macromolecular DOM constituent was estimated (Figure 2f). All macromolecular constituents increased in storage, and the deepest horizon displayed the greatest macromolecular enrichment. Overall, the differences between ^{1}H and DE ^{1}H NMR spectra

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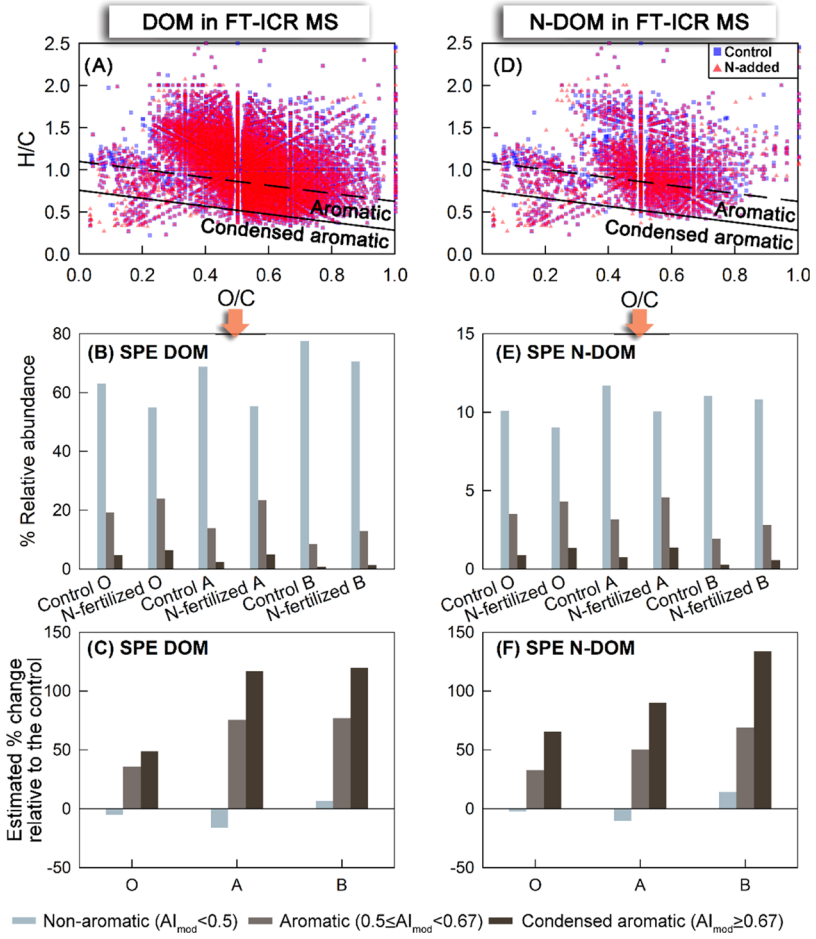


Figure 3. Relative abundances (B, E) of solid-phase extracted dissolved organic matter (DOM) components and nitrogen-containing DOM (N-DOM) components based on formulae assignment results of Fourier transform ion cyclotron resonance mass spectrometric analyses (A and D showing van Krevelen plots for each assigned formula for O horizon soil extracts) and the estimated percent change with long-term nitrogen addition (C, F).

indicated that all four major DOM constituents except the carbohydrates shifted to higher molecular size, which is consistent with the results of the E2/E3 ratio measurements. Therefore, N addition increased the storage of most of the macromolecular constituents, and the macromolecular aromatic and phenolic constituents consistently showed the greatest accumulation among the four DOM categories shown in the DE ^{1}H NMR. This result agrees with the latter part of our second hypothesis that N addition may increase the aromatics.

3.4. DOM Composition Based on FT-ICR MS. To gain increased molecular insight into the alteration of DOM chemistry by long-term N addition, FT-ICR MS was also used to characterize the solid-phase extractable and electrospray ionizable DOM constituents. About 6000 molecular formulae can be identified for each sample, and 4860 of them were found in all six samples with various abundances (Table 1). Despite both N-added and control soils always sharing large numbers of DOM formulae in common, their specific DOM formulae in each horizon have distinct distributions in van Krevelen plots, DBE vs carbon number plots, and molecular weight vs carbon number plots (SI Figures S2–S4). This clearly demonstrates that the DOM composition is altered by long-term N addition. The average molecular weight of DOM was consistently higher with N addition in all horizons (Table 1). This observation agrees with the decreased E2/E3 ratio results and preferentially enriched NMR resonances from macromolecule (except carbohydrates) with N addition. Specifically, the increase in average molecular weight is greatest in the deeper horizon (+16 Da in B horizon vs +8 Da in O horizon; Table 1), which is consistent with the NMR results that the deepest horizon displayed the greatest macromolecule enrichment. With N addition, the average H/C ratio of the DOM consistently decreased and both average AI$_{mod}$ and DBE of DOM were elevated in all horizons (Table 1). These results indicate that DOM contained more double bonds and became more aromatic with long-term N addition, which is highly consistent with the increased DOM aromaticity as reflected by the increased SUVA and aromatic signals in NMR. The average O/C ratio and NOSC were consistently higher in N-added soils than those in control soils (Table 1). As higher O/C ratio and NOSC usually indicate more O-containing functional groups and higher polarity,[58] the increased NOSC with N addition may suggest that the bulk DOM has stronger mobility to translocate from O and A horizons to the B horizon and contribute to the relatively larger difference in DOM storage in B horizon. The MLBL % was consistently lower in N-added soils than that in control soils (Table 1). As lower MLBL % is usually associated with

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lower DOM biolability,[79,80] it suggests that the DOM biolability may be decreased with long-term N addition.

The relative abundances of both condensed aromatic ($AI_{mod} \geq 0.67$) and aromatic ($0.67 > AI_{mod} \geq 0.5$) DOM increased greatly with N addition, whereas the relative abundance of the nonaromatic DOM ($AI_{mod} < 0.5$) decreased with N addition in all horizons (Figure 3). The percent changes in condensed aromatic DOM in the O, A, and B horizons were estimated to be 49, 117, and 120%, respectively (Figure 3). The corresponding values for aromatic DOM were 36, 76, and 77%, respectively, with weaker responses compared to those of the condensed aromatic DOM in all horizons. These results also support the latter part of our second hypothesis that N addition increases the polyphenols and condensed aromatics. The increase in condensed aromatics was greater for the larger molecules (carbon number ≥ 15) than for the smaller molecules (carbon number < 15) and were greater for oxygen-rich (O/C ratio >0.5) than for oxygen-poor molecules (O/C ratio ≤0.5; Table 1). The increased condensed aromatic and aromatic composition in the mass spectra strongly agreed with the results of SUVA and NMR. Based on the classification in Seidel et al.,[81] the highly unsaturated molecules ($AI_{mod} <$ 0.50 and H/C < 1.5), unsaturated aliphatic compounds (2.0 ≥ H/C > 1.5, N = 0), and unsaturated aliphatic compounds containing N (2.0 > H/C ≥1.5, N > 0; containing peptides) all decreased with N addition in O and A horizons (Table 1). These decreases are consistent with the NMR results, which showed less relative abundances of CRAM and MDLT constituents in O and A horizons with long-term N addition. Considering all studied DOM components, the condensed aromatics had the greatest estimated percent increase in storage.

Similar to the overall solid-phase extracted DOM, the solid-phase extracted nitrogen-containing DOM (N-DOM) showed increased average molecular weight, AI_{mod}, and DBE, as well as decreased average H/C ratio with the long-term N addition treatment. The N-DOM also shifted to higher relative abundances and storage of aromatic and condensed aromatic N-DOM. The condensed aromatic N-DOM in O, A, and B horizons was estimated to increase by 66, 90, and 134%, respectively. The corresponding values for aromatic N-DOM were 33, 50, and 69%, respectively (Figure 3). This suggests that long-term N addition favors the accumulation of condensed aromatic and aromatic DON over the nonaromatic DON, and this impact was also stronger in deeper soil horizon compared to that of the surface horizon.

These results indicate that condensed aromatics were the category with the greatest response to the long-term N addition. High molecular weight condensed aromatics ($AI_{mod} \geq 0.67$; carbon number ≥15) have commonly been referred to the operationally defined "DBC".[81] This DBC was estimated to increase by 52, 123, and 139% in O, A, and B horizons respectively, higher than all studied DOM components, as well as the carbohydrates identified in the ^{1}H NMR (26, 19, and 23% in O, A, and B horizons, respectively). To further examine which DBC fraction has the highest response to N addition, the percent change in dissolved-black-carbon-like DOM was plotted against the carbon number (Figure 4). With increasing carbon number, the percent change in DBC-like compounds increased, and the increase was greatest in deep soil horizon (Figure 4). Specifically, for carbon number 15 (C15), the percentage increases of DBC relative to the control were estimated to be 46% in B horizon and 48% in O horizon, which

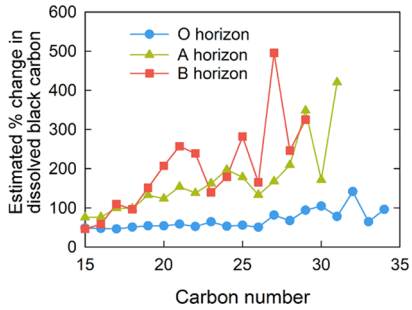


Figure 4. Estimated percent change in dissolved black carbon in different soil horizons with long-term nitrogen addition (relative to the control).

were very similar (Figure 4). However, when the carbon number increased to 27, the corresponding values were estimated to be 495% in B horizon, much higher than the 82% in O horizon (Figure 4). This result is consistent with the NMR analysis, which showed that the aromatics of larger molecular size had much higher accrual in soil than small-sized aromatics, particularly for the deep soil horizon.

The increased DBC in soils with N addition could be attributed to the increased production and decreased loss of DBC. The DBC was believed to be mainly derived from a fire event and anthropogenic pollution sources, which did not occur at this site and thus could not explain the difference in DBC storage between the control and N-added soils. Instead, recent studies have revealed that DBC can be produced via the reactive oxygen species (ROS)-involved reactions.[66,82] Indeed, KMD plots support that this pathway is a possible mechanism for the observed increase in DBC storage with N addition. Specifically, the KMD plots showed that the DOM formulae specifically in N-added soils shift toward lower molecular masses for the KMD H_2 and CH_2 series and shift toward higher molecular mass for the KMD O_2 and KMD CO_2 series compared to the control-specific formulae (Figure 5). These patterns are indicative of higher degrees of demethylation and carboxylation induced by ring-opening and polymerization with N addition, which is consistent with the ROS-induced DBC formation from lignin as reported previously.[66,82] The N addition may possibly strengthen such processes via both abiotic and biotic mechanisms. First, the soil acidification caused by N addition[46] will favor hydroxyl radical production via Fenton-like reactions that occur under acidic conditions. Second, the increased ratio of fungal to bacterial biomass by N addition[38] may also increase the degree of fungal transformation of DOM via hydroxyl radical formation[66] compared to the bacterial transformation of DOM. In addition to the possibly increased production of DBC, the reduced loss of DBC may also contribute to the increased DBC storage. For example, the overall microbial activity has been suppressed by N addition,[38] and thus the microbial community is less likely to degrade DBC. Also, the reduced soil pH may increase the DBC retention[36] via sorptive interaction with the clay minerals or other organic matter.

3.5. Environmental Implications. Although optical spectroscopy, NMR spectroscopy, and FT-ICR MS have been previously applied to understand the soil DOM characteristics,[48,83,84] the combination of these approaches to study soil-derived DOM is rare and hence novel.[85] Each of the analytical methods has its own advantages and disadvantages in characterizing soil-derived DOM. In the present study, the

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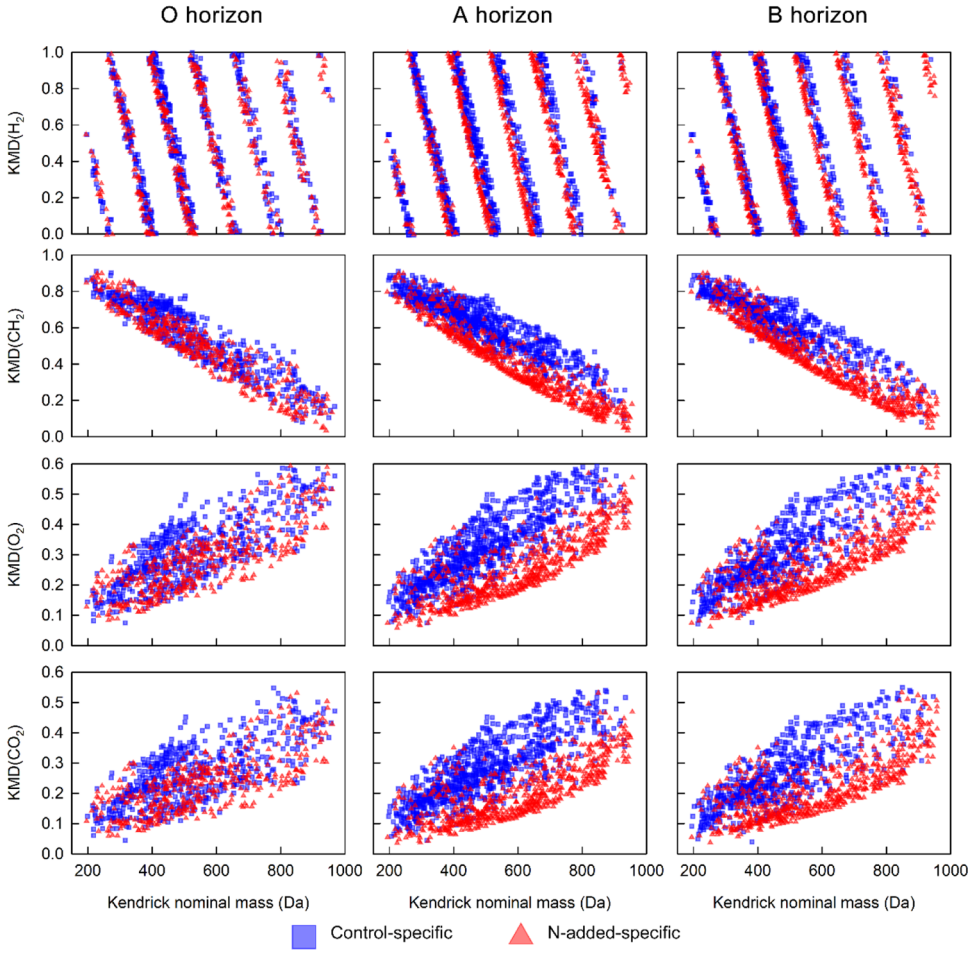


Figure 5. Kendrick mass defect (KMD) plots for dissolved organic matter formulae specifically in control or N-added soils belonging to the O (left), A (middle), and B (right) horizons. The four rows display KMD for H_2, CH_2, O_2, and CO_2 series.

absorbance and fluorescence spectroscopies are rapid, nondestructive, and economical but these methods do not provide information about the nonchromophoric DOM components, such as carbohydrates and lipids, which are abundant in soils. FT-ICR MS is one of the most advanced MS techniques and yields thousands of molecular ions that aid in the identification of DOM structure. However, it is semiquantitative and also restricted by the ionization method used. Additionally, while FT-ICR MS provides useful information on the elemental composition of DOM, it does not yield functional group information unless it is used as MS/MS. NMR spectroscopy is nonselective and thus can provide complete information about DOM chemistry (including functional groups), but it is not as sensitive as MS methods and does not provide the same level of resolution as FT-ICR MS due to the complex nature of DOM. Therefore, the combination of these complementary analytical methods is advantageous and provides a more holistic identification of soil-derived DOM characteristics and their responses to environmental change.

While several studies have hypothesized that DOM may respond differentially to the long-term addition,[36,44,45,75] this study identified previously unreported shifts in DOM composition with long-term N addition. After 22 years of N addition, soil-derived DOM exhibited a minor increase in its storage (an increase of ~10%) but had significant alterations in its optical properties and molecular-level composition. Long-term N addition increased the soluble carbohydrates in all soil horizons, decreased the aliphatic components (MDLT and CRAM) in the O and A horizons, and increased the aromatic and phenolic components in the deeper B horizon. The observed higher carbohydrate storage should have fueled the microbial activity because it is a preferred microbial substrate in soils. However, the suppressed microbial activity was often found with long-term N addition and was usually attributed to the increased acidity caused by nitrification.[86,87] Here, we identified that the antimicrobial condensed aromatics (e.g., DBC) and highly unsaturated polyphenols (e.g., tannins) increased greatly with long-term N addition, which may also have contributed to the suppressed microbial activity. These antimicrobial compounds may have played an important role in the widely found reduced soil respiration and increased SOC pools with N deposition.[86] Importantly, DBC, particularly those with high carbon numbers, was identified as the category having the greatest percent increase with N addition, especially in the deeper soil horizons. Despite that DBC usually accounts for <10% of the bulk DOM,[7] it is known as a critical component that affects carbon, nutrient, and pollutant cycles. For example, the organic carbon-normalized sorption coefficient of phenanthrene to DBC was over 10-fold higher than dissolved soil humic acid.[13] If so, then long-term N addition may significantly increase the accumulation of hydrophobic organic contaminants in deep soil horizons due to the increased DBC storage in deep soils. Although the decreased microbial processing of black carbon and altered soil pH and

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ion strength may be possible mechanisms that contribute to the DBC accumulation in soils with long-term N addition, it is still not fully understood which process is more critical or whether other factors also drive the accumulation of DBC. Future studies are needed to examine the detailed mechanisms and whether the phenomenon observed here also manifests in other types of soils and ecosystems. Nevertheless, we demonstrate that long-term N deposition can greatly alter DOM composition in soils, inevitably altering numerous critical forest soil biogeochemical processes, and which may alter the quality and quantity of DOC inputs and metabolism downstream in aquatic ecosystems.

4. CONCLUSIONS

With 22 years of N addition, the quantity of soil-derived DOM in the studied temperate deciduous forest increased slightly but the molecular composition changed markedly. Optical spectroscopy, NMR spectroscopy, and FT-ICR MS collectively indicated that the molecular size of DOM increased in all horizons and the aromaticity of DOM increased in the A and B horizons. FT-ICR MS showed that the average molecular weight increased by 8, 13, and 16 Da for the solid-phase extracted DOM in O, A, and B horizons, respectively. For the nonaromatic DOM components, NMR analysis showed that more carbohydrates but fewer aliphatic compounds were observed with N addition. For the aromatic DOM components, the macromolecular/aggregated ones (indicated by NMR analysis) and condensed aromatic ones (indicated by FT-ICR MS analysis) exhibited marked increases with N addition. Condensed aromatic dissolved black carbon, specifically compounds with high molecular size/carbon number and those stored deeper in the soil profile, showed the greatest percent increase with N addition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.9b00262.

Elemental composition of the solid-phase extracted dissolved organic matter as determined by Fourier transform ion cyclotron mass spectrometry; molecular characterization of the solid-phase extracted nitrogen-containing dissolved organic matter; example $^{1}H-^{13}C$ heteronuclear single quantum coherence; van Krevelen plots; and double bond equivalent versus carbon number plots (PDF)

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Notes

The authors declare no competing financial interest.

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