Supporting Information for

Long-term Nitrogen Deposition Disrupts Carbon Cycling and Enhances Sequestration of Plant-derived Matter in a Temperate Forest

Biwei Yang[a], Meiling Man[a], Yuxuan Weng[a], Richard D. Bowden[b], Jun-Jian Wang[c,d,e], and Myrna J. Simpson[a,*]

[a]Environmental NMR Centre and Department of Physical and Environmental Sciences, University of Toronto, Toronto, Ontario M1C 1A4 Canada

[b]Department of Environmental Science, Allegheny College, Meadville, PA 16335, USA

[c]State Key Laboratory of Soil Pollution Control and Safety, Southern University of Science and Technology, Shenzhen, 518055, China

[d]State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Southern University of Science and Technology, Shenzhen, Guangdong 518055, China

[e]Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control, Southern University of Science and Technology, Shenzhen, Guangdong 518055, China

* Myrna J. Simpson

Email: myrna.simpson@utoronto.ca

Method details

To isolate soil organic matter (SOM) compounds, sequential solvent extractions were performed on forest floor (1 g freeze-dried soil) and mineral layer (5 g freeze-dried soil) samples. The extraction protocol employed progressively polar solvents—beginning with dichloromethane (DCM, Optima grade) to isolate non-polar compounds, followed by a 1:1 (v/v) DCM:methanol mixture (Optima grade) to extract intermediate polarity compounds, and concluding with pure methanol (Optima grade) to recover more polar components.[1,2] Each 20-minute ultrasonication step (VWR ultrasonic bath, 35 kHz) was conducted in 30 mL solvent aliquots at room temperature (22 ± 1°C) in 50 mL Teflon tubes, with phase separation achieved by centrifugation (2700 rpm, 10 min). After each extraction, supernatants were filtered through glass fiber filters (Whatman GF/A 1.6 μm and GF/F 0.7 μm) to remove particulate matter. Combined extracts were concentrated by rotary evaporation (40°C water bath) and transferred to 2 mL amber vials for final drying under gentle nitrogen flow at 50°C. Processed extracts were stored at −20°C until gas chromatography-mass spectrometry (GC-MS) analysis, with soil residues preserved for base hydrolysis.

To target cutin-, suberin-, and microbial-derived hydrolysable compounds, half of the solvent-extracted soil residues underwent alkaline cleavage in 15 mL of 1 M methanolic potassium hydroxide (ACS grade) at 100°C for 3 hours using Teflon-lined digestion bombs.[3] After cooling, supernatants were decanted into clean Teflon tubes, and residual solids were extracted twice with 10 mL of 1:1 DCM:methanol (v/v) via ultrasonication (15 min) and centrifugation (2700 rpm, 10 min). Combined extracts were acidified to pH 1 with 6 M HCl (ACS grade) and concentrated by rotary evaporation (40°C water bath). Prior to liquid-liquid extraction, 40 mL of deionized water (<18.2 MΩ·cm at 25°C, Millipore) was added to redissolve salts. The aqueous phase was then extracted three times with 30 mL anhydrous diethyl ether (ACS grade) per aliquot. Ether fractions were pooled, dried over anhydrous sodium sulfate, filtered through pre-rinsed glass wool, and concentrated under reduced pressure (room temperature water bath). Final extracts were transferred to 2 mL amber vials, dried under a gentle nitrogen stream at room temperature, and stored at −20°C until GC-MS analysis. The hydrolyzed soil residues were retained for subsequent lignin-derived SOM compounds analysis.

To investigate the lignin-derived SOM compounds, we employed alkaline copper oxide oxidation to cleave ether bonds in polymeric lignin to isolate lignin phenol monomers and dimers.[4,5] Half of the air-dried soil residues from base hydrolysis were reacted with 1 g cupric oxide (97%) and 100 mg ferrous ammonium sulfate (ReagentPlus) in 15 mL of 2 M sodium hydroxide (reagent grade) under oxygen-free conditions (nitrogen-purged Teflon bombs) at 170°C for 2.5 hours. This controlled oxidative degradation effectively releases vanillyl, syringyl, and cinnamyl phenol monomers. After cooling, supernatants were transferred to clean Teflon tubes, and residual solids were extracted twice with 10 mL deionized water (sonication for 10 min, centrifugation at 2700 rpm for 10 min). Combined extracts were acidified to pH 1 with 6 M HCl

(ACS grade) and stored in darkness at room temperature for 1 h to prevent cinnamic acid polymerization. Target compounds were then isolated using reversed-phase solid phase extraction (Oasis HLB cartridges, 3 mL, 60 mg sorbent, Waters; preconditioned with 4 mL methanol and 4 mL deionized water). After loading, cartridges were washed with 1.5 mL of 30% methanol (v/v in water) and dried under air. Lignin phenols were eluted sequentially with optimized elution employing a 70:25:5 (v/v/v, freshly prepared) dichloromethane:methyl acetate:pyridine mixture and 1 mL methanol to recover the lignin phenols while excluding interfering compounds. Eluates were dried over anhydrous sodium sulfate, concentrated under nitrogen stream at 55°C, and stored at −20°C until GC-MS analysis.

Soil microbial biomass was assessed through phospholipid fatty acid (PLFA) analysis following an optimized Bligh-Dyer extraction protocol.[6] Soil samples (1 g forest floor; 5 g mineral layer) were subjected to a 24-hour solvent extraction in the dark using a methanol:chloroform:citrate buffer system (16:8:6 v/v/v, 0.15 M citrate buffer at pH 4) with continuous agitation. Phase separation was achieved through sequential centrifugation and addition of chloroform:buffer (7:7 v/v), with the lipid-containing chloroform phase carefully collected and evaporated under nitrogen stream. The total lipid extract was fractionated via silicic acid column chromatography, sequentially eluting neutral lipids (10 mL chloroform, Optima grade), glycolipids (20 mL acetone, Optima grade), and polar lipids (10 mL methanol, Optima grade) to isolate phospholipids. The polar lipid fraction was converted to fatty acid methyl esters (FAMEs) through mild alkaline methanolysis (0.2 M methanolic potassium hydroxide, ACS grade, 37°C for 15 min) in the presence of toluene:methanol (1:1 v/v, Optima grade). Following acidification (1 M acetic acid, ACS grade) and aqueous washing, FAMEs were extracted with hexane:chloroform (4:1 v/v) and concentrated under nitrogen stream. Final extracts were stored at −20°C in amber vials until GC-MS analysis.

Soil extracts were derivatized and analyzed by GC–MS within two weeks after extraction. Samples (1 μL) were injected at an inlet temperature of 280°C onto a HP-5MS fused silica capillary column (30 m 9 0.25 mm i.d. 9 0.25 μm film thickness). The oven temperature program was 65°C for 2 min, followed by an increase of 6°C min^{-1} to 300°C, followed by a 20 min isothermal hold. The carrier gas (helium) flow rate was 1 $mL \cdot min^{-1}$. All compounds were analyzed by GC–MS with electron impact (70 eV) ionization. Data acquisition was performed using Agilent Mass Hunter GC–MS Acquisition (version B.07.03.2129) and data were processed using Agilent Enhanced ChemStation (version F.01.03.2357) and MassHunter (version B.07.03.2129) software. External standards (from Sigma-Aldrich) were used for quantification: tetracosane, 1-docosanol, methyl tricosanoate and ergosterol for solvent extracts; methyl tricosanoate for base hydrolysis products; syringic acid and syringaldehyde for copper oxidation products; and methyl oleate for PLFAs. Compounds were identified using the Wiley Registry (9th edition), National Institute of Standards and Technology (2008 edition) mass spectral databases, and lab internal library.

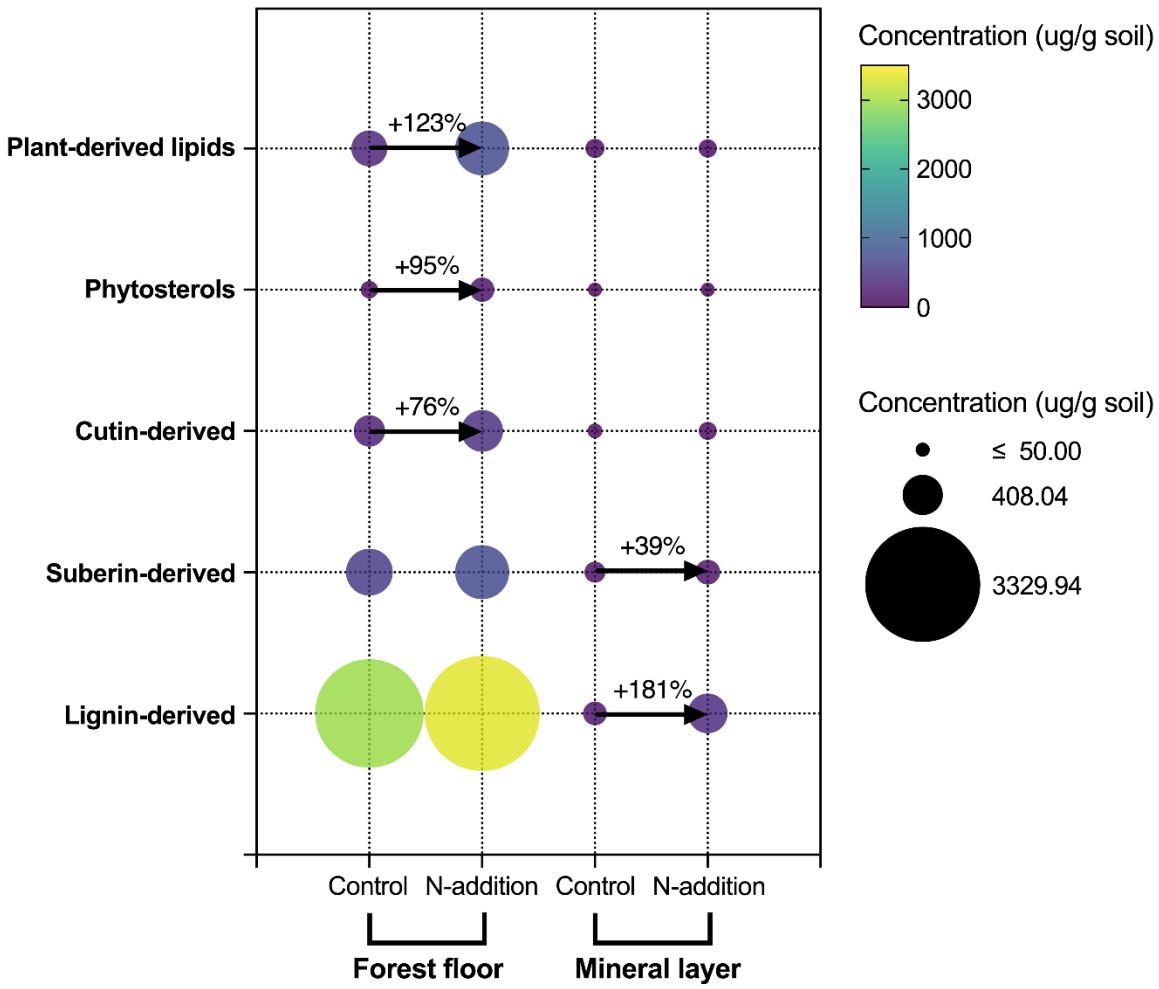

Fig. S1. Bubble plot showing the accumulation of plant-derived organic matter in the forest floor and mineral soil layers after long-term nitrogen addition, compared with the control. Concentrations of targeted soil organic matter components are represented by both color and bubble size. Significant changes ($p < 0.05$) are labeled as percentages.

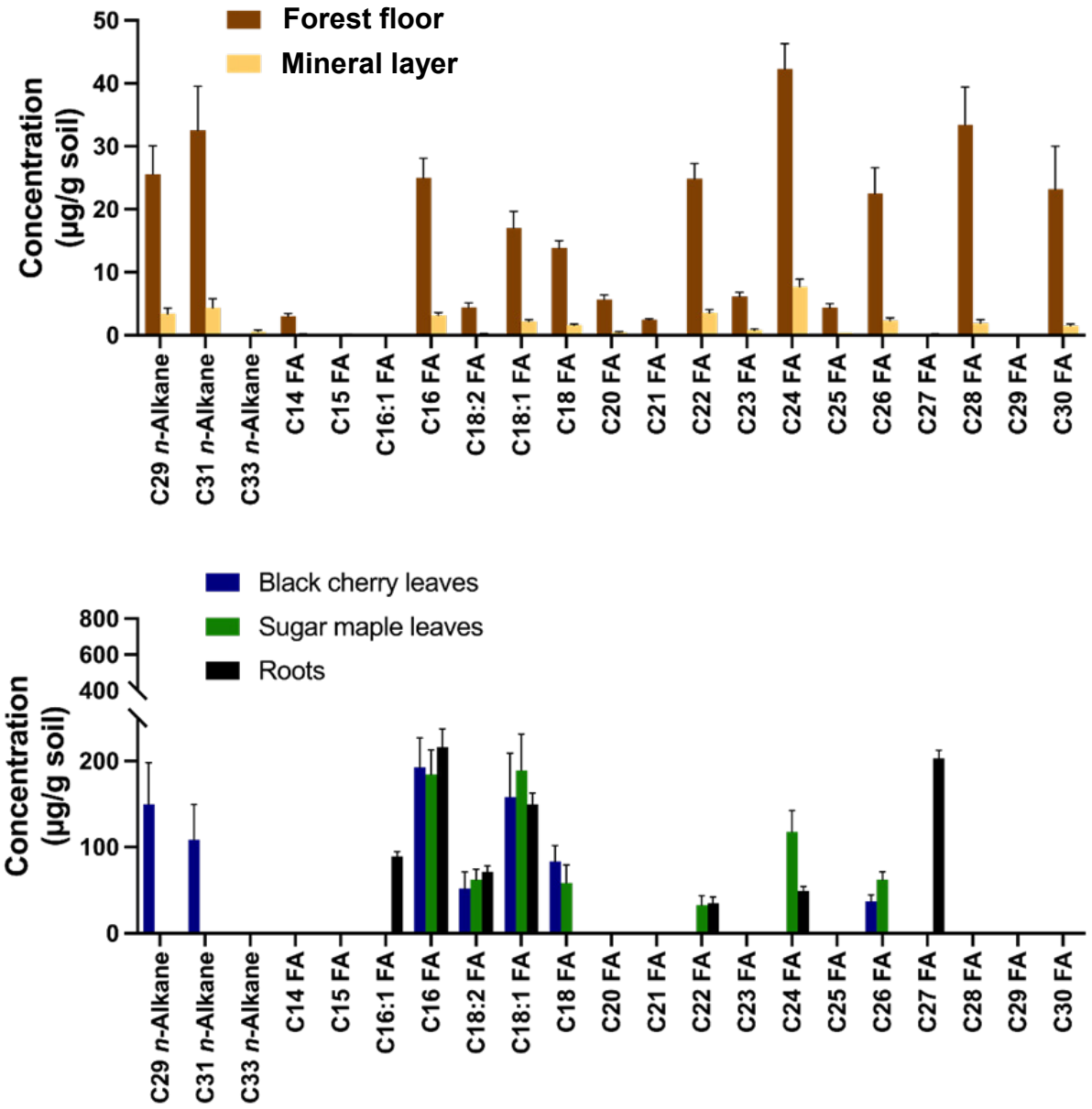

Fig. S2. Distribution patterns of solvent-extractable lipids, including n-alkanes and fatty acids (FA), in the forest floor and mineral layer from previous analysis of Bousson Forest vegetation and soils are shown in the top plot,[7] patterns in black cherry litter, sugar maple litter, and roots collected from Bousson Forest are shown in the bottom plot.

Table S1. Carbon (C) and nitrogen (N) concentrations, and storage in forest floor and mineral layers under control and N-addition. Means and standard errors (n = 3) are listed. Significant differences ($p < 0.05$) between treatments (paired two-sample t-test) are highlighted in bold font.

	Forest floor		Mineral soil	
	Control	N-addition	Control	N-addition
Total C (%)	**12.4 ± 0.8**	**22.9 ± 0.8**	4.3 ± 0.4	4.3 ± 0.4
Total N (%)	**0.9 ± 0.0**	**1.3 ± 0.0**	0.4 ± 0.0	0.3 ± 0.0
Organic C (%)	**12.3 ± 0.8**	**22.8 ± 0.8**	4.2 ± 0.4	4.2 ± 0.4
Carbon-to-nitrogen ratio	**14.0 ± 0.2**	**17.1 ± 0.8**	**12.2 ± 0.2**	**14.3 ± 0.4**
C storage (kg/m^2)	**1.1 ± 0.3**	**2.0 ± 0.3**	3.7 ± 0.4	3.9 ± 0.3
N storage (kg/m^2)	**0.1 ± 0.0**	**0.1 ± 0.0**	0.3 ± 0.0	0.3 ± 0.0

Table S2. Solid-state ^{13}C nuclear magnetic resonance characteristics of control and nitrogen-addition forest floor and mineral layer soil samples.

	Forest floor		Mineral layer	
	Control	N-addition	Control	N-addition
Alkyl carbon (%) 0–50 ppm	38	33	39	43
O-alkyl carbon (%) 50–110 ppm	41	44	42	37
Aromatic + phenolic carbon (%) 110–165 ppm	12	15	9	11
Carboxyl + carbonyl carbon (%) 165–215 ppm	9	8	10	9

Table S3. Concentration and ratios of phospholipid fatty acids (PLFAs) detected by gas chromatography-mass spectrometry in soil samples. Means and standard errors were calculated based on six replicates (i.e., three field replicates with two analytical replicates). Significant differences ($p < 0.05$) between treatments were determined by repeated measures analysis of variance (ANOVA) and are highlighted in bold.

	Forest floor		Mineral layer	
	Control	N-addition	Control	N-addition
Total microbial biomass	179.3 ± 9.6	151.6 ± 12.4	11.4 ± 1.3	7.6 ± 0.5
Microbial composition				
Total bacteria[a]	151.2 ± 8.1	125.9 ± 10	9.5 ± 1.1	6.3 ± 0.5
Total fungi[b]	7.8 ± 0.8	7.8 ± 0.7	0.3 ± 0	0.2 ± 0
Gram-positive bacteria[c]	21.4 ± 0.9	19.1 ± 1.6	0.9 ± 0.2	0.6 ± 0.1
Gram-negative bacteria[d]	**95.9 ± 5.9**	**75.3 ± 6.1**	6.4 ± 0.8	3.9 ± 0.3
Actinobacteria[e]	12.6 ± 0.7	10.2 ± 0.9	1.1 ± 0.1	0.8 ± 0
Gram-negative/gram-positive	4.5 ± 0.1	4 ± 0.1	7.8 ± 0.6	6.5 ± 0.5
Gram-negative/(gram-positive & Actinobacteria)	2.8 ± 0.1	2.6 ± 0.1	**3.2 ± 0.1**	**2.8 ± 0.1**
Fungi/bacteria	0.1 ± 0	0.1 ± 0	0 ± 0	0 ± 0
Monounsaturated PLFAs	**66.6 ± 5**	**48.8 ± 4.1**	**3.3 ± 0.5**	**1.6 ± 0.2**
Saturated PLFAs	33 ± 1.5	31.1 ± 2.4	2.2 ± 0.2	1.8 ± 0.1
Microbial stress ratios				
Monounsaturated/saturated	**0.5 ± 0.0**	**0.6 ± 0.0**	**0.7 ± 0.1**	**1.1 ± 0.1**
Cy17:0/16:1ω7c	**0.7 ± 0**	**1.0 ± 0**	0.3 ± 0	0.3 ± 0

[a] Total bacterial PLFAs: Gram-positive bacterial PLFAs + Gram-negative bacterial PLFAs + (11:0 + 13:0 + 14:0 + i15:1ω7 + i15:1ω5 + 15:0 + 16:0 + 17:0 + 18:0 + 19:0 + 20:0 + 21:0 + 22:0).

[b] Total fungal PLFAs: 18:2ω6,13 + 18:2ω6,9 + 18:1ω9c.

[c] Total Gram-positive PLFAs: i12:0 + i14:0 + i15:0 + i16:0 + i17:0 + a17:0 + i18:0

[d] Total Gram-negative PLFAs: 16:1ω7 + 16:1ω9 + 16:1ω7t + 16:1ω7c + 16:1ω5t + 16:1ω5c + 17:1ω9 + 17:1ω7 + cy17:0 + 18:1ω3t + 18:1ω9t + 18:1ω12 + 18:1ω10 + 19:1ω9 + 20:1ω7 + cy19:0 + 20:1ω9t + 20:1ω9c + 20:1ω5c + 22:1ω9.

[e] Actinobacterial PLFAs: 10Me16:0 + 10Me17:0 + 10Me18:0

Table S4. Summary of changes in concentration and ratios of specific phospholipid fatty acids (PLFAs) detected by gas chromatography-mass spectrometry in soil samples. Means and standard errors were calculated based on six replicates (i.e., three field replicates with two analytical replicates multiplied). Significant differences ($p < 0.05$) between treatments were determined by repeated measures analysis of variance (ANOVA) and are highlighted in bold.

	Forest floor		Mineral layer	
	Changes	Significance	Changes	Significance
Total microbial biomass	−15%	−	−33%	−
Microbial composition				
Total bacteria	−17%	−	−34%	−
Total fungi	0%	~	−33%	−
Gram-positive bacteria	−11%	−	−33%	−
Gram-negative bacteria	**−21%**	**−***	−39%	−
Actinobacteria	−19%	−	−27%	−
Gram-negative/gram-positive	−11%	−	−17%	−
Gram-negative/(gram-positive & Actinobacteria)	−8%	~	−12%	**−***
Fungi/bacteria	0%	~	bld	bld
Monounsaturated PLFAs	**−27%**	**−***	**−52%**	**− −***
Saturated PLFAs	−6%	−	−18%	− −
Microbial stress ratios				
Monounsaturated/saturated PLFAs	**+27%**	**+****	**+63%**	**++***
Cy17:0/16:1ω7c	**+43%**	**+*****	0%	~

‘‘~’’ indicates minor alteration within ± 10%; ‘‘−’’ a n d ‘‘− −’’ show > 10% and > 50% decreases from control, respectively; ‘‘+’’ and ‘‘++’’ show > 10% and > 50% increases from control, respectively; *, **, and *** indicate statistically significant differences at p value < 0.001, < 0.01 and < 0.05, respectively.

Table S5. Concentration and ratios of organic compounds from solvent extraction, base hydrolysis, and CuO oxidation detected by gas chromatography-mass spectrometry in soil samples. Means and standard errors were calculated based on six replicates (i.e., three field replicates with two analytical replicates). Significant differences ($p < 0.05$) between treatments were determined by repeated measures analysis of variance (ANOVA) and are highlighted in bold.

	Forest floor		Mineral layer	
	Control	N-fertilized	Control	N-fertilized
Solvent-extracted products (µg/g soil) and ratios				
Short-chain lipids ($< C_{20}$)	**233.9 ± 3.3**	**350.1 ± 7.5**	27 ± 4.9	41.9 ± 3.5
Long-chain lipids ($\geq C_{20}$)	**326.1 ± 7.6**	**728.4 ± 25**	92 ± 6.1	83.7 ± 5.1
Short-chain *n*-alkanes ($< C_{20}$)	Bdl	Bdl	Bdl	Bdl
Long-chain *n*-alkanes ($\geq C_{20}$)	**31.7 ± 1**	**180.3 ± 10.6**	15.6 ± 1.8	10.3 ± 2.4
Short-chain *n*-alkanoic acids ($< C_{20}$)	**198.6 ± 5.9**	**286.2 ± 5.1**	23.6 ± 4.4	35.5 ± 2.9
Long-chain *n*-alkanoic acids ($\geq C_{20}$)	**201.4 ± 5.4**	**381.4 ± 14.4**	44 ± 3.9	43.7 ± 1.4
Short-chain *n*-alkanols ($< C_{20}$)	3.5 ± 0.3	6.5 ± 0.2	0.4 ± 0	0.5 ± 0
Long-chain *n*-alkanols ($\geq C_{20}$)	55.8 ± 3.3	83 ± 12.1	21.1 ± 1.9	15.8 ± 3.2
Phytosterols	**76.6 ± 5.7**	**149.4 ± 11.2**	6.6 ± 0.3	8.9 ± 1.1
Triterpenoids	61 ± 13.5	103.6 ± 14.3	4.3 ± 0.5	6.9 ± 1.3
Base hydrolyzed products (µg/g soil) and ratios				
Cutin-derived lipids	**248.9 ± 13.2**	**438.7 ± 45.6**	56.2 ± 4.9	81.7 ± 11.6
Suberin-derived lipids	552.1 ± 61.1	734.4 ± 62.7	**111.4 ± 13.5**	**154.4 ± 15.3**
Cutin- or suberin-derived lipids	512.3 ± 50.3	764.2 ± 92.4	191.8 ± 12.3	158.6 ± 15.3
Cutin- and suberin-derived lipids	**1313.3 ± 91.3**	**1937.4 ± 138.8**	**359.4 ± 25.6**	**394.6 ± 22.7**
Cutin/suberin ratio	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Microbial-derived lipids	42.1 ± 4.6	46.6 ± 5.5	27.8 ± 3.4	21.4 ± 2.5
CuO oxidized products (µg/g soil) and ratios				
Vanillyls	1869.8 ± 177.8	2098.8 ± 284.4	**94.1 ± 7.2**	**249.5 ± 22.5**
Syringyls	1000.9 ± 77.4	1140.1 ± 157.3	**29.8 ± 3.7**	**95.4 ± 11.0**
Cinnamyls	85.8 ± 15.0	91.0 ± 21.0	17.3 ± 4.5	51.1 ± 9.7
Lignin-derived phenols	2956.6 ± 231.5	3329.9 ± 434.9	**141.2 ± 11.6**	**396.0 ± 30.8**
Syringyls/vanillyls	0.6 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
Cinnamyls/vannilyls	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
$(Ad/Al)_V$[a]	0.4 ± 0.0	0.5 ± 0.1	1.1 ± 0.3	0.9 ± 0.1
$(Ad/Al)_S$[a]	0.3 ± 0.0	0.3 ± 0.1	1.0 ± 0.3	0.8 ± 0.1

[a](Ad/Al)$_V$ and (Ad/Al)$_S$ are the acid to aldehyde ratios for vanillyls and syringyls, respectively.
Bdl = below detection limits.

Table S6. Summary of changes in concentration and ratios of organic compounds from solvent extraction, base hydrolysis, and CuO oxidation detected by gas chromatography-mass spectrometry in soil samples. Means and standard errors were calculated based on six replicates (i.e., three field replicates with two analytical replicates). Significant differences ($p < 0.05$) between treatments were determined by repeated measures analysis of variance (ANOVA) and are highlighted in bold.

Soil layers	Forest floor (Oh)		Mineral layer (Ah)	
	Changes	Significance	Changes	Significance
Solvent-extracted products (µg/g soil) and ratios				
Short-chain lipids ($< C_{20}$)	**50%**	**++*** **	55%	++
Long-chain lipids ($\geq C_{20}$)	**123%**	**++*** **	**−9%**	**−***
Short-chain *n*-alkanes ($< C_{20}$)	Bdl	Bdl	Bdl	Bdl
Long-chain *n*-alkanes ($\geq C_{20}$)	**469%**	**++*** **	−34%	−
Short-chain *n*-alkanoic acids ($< C_{20}$)	**44%**	**+*** **	50%	++
Long-chain *n*-alkanoic acids ($\geq C_{20}$)	**89%**	**++*** **	−1%	~
Short-chain *n*-alkanols ($< C_{20}$)	86%	++	25%	+
Long-chain *n*-alkanols ($\geq C_{20}$)	49%	+	−25%	−
Phytosterols	**95%**	**++***	35%	+
Triterpenoids	70%	++	60%	++
Base hydrolyzed products (µg/g soil) and ratios				
Cutin-derived lipids	**76%**	**++****	45%	+
Suberin-derived lipids	33%	+	**39%**	**+****
Cutin- or suberin-derived lipids	49%	+	-17%	−
Cutin- and suberin-derived lipids	**48%**	**+***	10%	+
Cutin/suberin ratio	25%	+	0%	~
Microbial-derived lipids	11%	+	-23%	−
CuO oxidized products (µg/g soil) and ratios				
Vanillyls	−40%	−	**161%**	**++****
Syringyls	−39%	−	**229%**	**++***
Cinnamyls	−43%	−	200%	++
Lignin-derived phenols	−40%	−	**176%**	**++****
Syringyls/vanillyls	−17%	−	33%	+
Cinnamyls/vannilyls	0%	~	0%	~
$(Ad/Al)_V$[a]	25%	−	−18%	−
$(Ad/Al)_S$[a]	0%	~	−20%	−

[a](Ad/Al)$_V$ and (Ad/Al)$_S$ are the acid to aldehyde ratios for vanillyl and syringyl compounds, respectively.
‘‘~’’ indicates minor alteration within ± 10%; ‘‘−’’ a n d ‘‘− −’’ show > 10% and > 50% decreases from control, respectively; ‘‘+’’ and ‘‘++’’ show > 10% and > 50% increases from control, respectively; *, **, and *** indicate statistically significant differences at p value < 0.001, < 0.01 and < 0.05, respectively.
Bdl =below detection limits.

References

1 A. Otto and M. J. Simpson, Degradation and Preservation of Vascular Plant-derived Biomarkers in Grassland and Forest Soils from Western Canada, *Biogeochemistry*, 2005, **74**, 377–409.

2 A. Otto, C. Shunthirasingham and M. J. Simpson, A comparison of plant and microbial biomarkers in grassland soils from the Prairie Ecozone of Canada, *Org. Geochem.*, 2005, **36**, 425–448.

3 A. Otto and M. J. Simpson, Sources and composition of hydrolysable aliphatic lipids and phenols in soils from western Canada, *Org. Geochem.*, 2006, **37**, 385–407.

4 J. R. Ertel and J. I. Hedges, The lignin component of humic substances: Distribution among soil and sedimentary humic, fulvic, and base-insoluble fractions, *Geochim. Cosmochim. Acta*, 1984, **48**, 2065–2074.

5 A. Otto and M. J. Simpson, Evaluation of CuO oxidation parameters for determining the source and stage of lignin degradation in soil, *Biogeochemistry*, 2006, **80**, 121–142.

6 E. G. Bligh and W. J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.*, 1959, **37**, 911–917.

7 J.-J. Wang, O. Pisani, L. H. Lin, O. O. Y. Lun, R. D. Bowden, K. Lajtha, A. J. Simpson and M. J. Simpson, Long-term litter manipulation alters soil organic matter turnover in a temperate deciduous forest, *Sci. Total Environ.*, 2017, **607–608**, 865–875.