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Atmospheric N Deposition Increases Bacterial Laccase-Like Multicopper Oxidases: Implications for Organic Matter Decay

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Anthropogenic release of biologically available nitrogen (N) has increased dramatically over the last 150 years, which can alter the processes controlling carbon (C) storage in terrestrial ecosystems. In a northern hardwood forest ecosystem located in Michigan in the United States, nearly 20 years of experimentally increased atmospheric N deposition has reduced forest floor decay and increased soil C storage. This change occurred concomitantly with compositional changes in *Basidiomycete* fungi and in *Actinobacteria*, as well as the downregulation of fungal lignocelluloytic genes. Recently, laccase-like multicopper oxidases (LMCOs) have been discovered among bacteria which can oxidize β-O-4 linkages in phenolic compounds (e.g., lignin and humic compounds), resulting in the production of dissolved organic carbon (DOC). Here, we examined how nearly 2 decades of experimental N deposition has affected the abundance and composition of saprotrophic bacteria possessing LMCO genes. In our experiment, LMCO genes were more abundant in the forest floor under experimental N deposition whereas the abundances of bacteria and fungi were unchanged. Experimental N deposition also led to less-diverse, significantly altered bacterial and LMCO gene assemblages, with taxa implicated in organic matter decay (i.e., *Actinobacteria*, *Proteobacteria*) accounting for the majority of compositional changes. These results suggest that experimental N deposition favors bacteria in the forest floor that harbor the LMCO gene and represents a plausible mechanism by which anthropogenic N deposition has reduced decomposition, increased soil C storage, and accelerated phenolic DOC production in our field experiment. Our observations suggest that future rates of atmospheric N deposition could fundamentally alter the physiological potential of soil microbial communities.

Over the past 150 years, atmospheric deposition of biologically available nitrogen (N) has increased 10-fold (e.g., from 0.5 to 1 kg N ha^{-1} $year^{-1}$ to 15 to 20 kg N ha^{-1} $year^{-1}$) across much of the Northern Hemisphere. Although rates of N deposition have recently declined in parts of North America and Europe, global rates of N deposition are expected to increase through the next century (1–3). The extent to which N deposition affects terrestrial C storage has fueled much scientific discourse (4–7), and it is the focus of this work. In N-limited temperate forests, anthropogenic N deposition can increase ecosystem C storage by fostering higher rates of net primary productivity (NPP); it can also slow decomposition of detritus, leading to greater C storage in long-lived soil organic matter (SOM; 4, 6). For nearly 2 decades, we studied the effects of chronic experimental N deposition in replicate forest stands along a 500-km climatic gradient, which spans the north-south geographic range of the sugar maple-dominated northern hardwood forest in the Great Lakes region of North America (see Fig. S1 in the supplemental material and Table 1; 8). We previously demonstrated that experimental N deposition, at a rate expected in some locations by midcentury, has reduced litter decay, increased the level of SOM, and accelerated dissolved organic carbon (DOC) leaching (9, 10). Although N deposition increased NPP (+10%), it has not altered the amount of leaf or root litter entering the forest floor or mineral soil (11, 12). Therefore, we have reason to believe that a microbial mechanism may underlie the accumulation of SOM and accelerated DOC production. Moreover, field observations, coupled with ecosystem simulation models, indicate that chronic N deposition has decreased the extent of microbial decay of the forest floor, leading to an accumulation of SOM (13).

Bacteria and fungi are responsible for ~90% of all organic matter (OM) decomposition (14), a process second to only photosynthesis in controlling C cycling in terrestrial ecosystems. Laccases (EC 1.10.3.2) are enzymes found among bacteria and fungi, and they oxidize β-O-4 linkages in phenolic compounds, such as those in lignin and SOM. Bioinformatic analyses reveal a phylogenetically diverse suite of bacterial laccase-like multicopper oxidases (LMCOs), and to date, their ability to modify phenolic compounds has been demonstrated among members of the *Actinobacteria* (15, 16), *Deinococcus/Thermus* (17), *Firmicutes* (18, 19), and *Alpha-*, *Beta-*, and *Gammaproteobacteria* phyla (15, 20, 21). In addition to laboratory studies, the occurrence, composition, and diversity of LMCOs have been quantified in a forest (22) as well as in bog and fen soils (23); however, we have no understanding of how the bacterial genes may be affected by atmospheric N deposition.

Lignin, a recalcitrant phenol-propane polymer integral to the plant secondary cell wall, acts as a control on litter decomposition rates (24, 25). Lignin decay is mediated by lignin-peroxidases (LiP), manganese-peroxidases (MnP), and laccases thought to be of largely fungal origin and can result in complete metabolism to CO_2 (26–28). The high redox potential of LiP and MnP enables the oxidation of both phenolic and nonphenolic lignin units (~90% of the polymer), whereas the lower redox potential of laccases oxidize phenolic lignin units, often comprising ~10% of

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TABLE 1 Site, climatic, overstory, and ambient nitrogen deposition rates of four study sites receiving experimental NO_3^- additions[a]

Characteristic	Value for:			
	Site A	Site B	Site C	Site D
Location				
Latitude (N)	46°52″	45°33″	44°23″	43°40″
Longitude (W)	88°53″	84°52″	85°50″	86°9″
Climate				
Mean annual temp (°C)	4.8	6.1	6.5	7.7
Mean annual precipitation (cm)	91.9	93.3	92.8	86.6
Ambient N deposition (kg N ha^{-1} yr^{-1})	5.9	6.1	7.4	7.4
Vegetation				
Overstory biomass (Mg ha^{-1})	261	261	274	234
Acer saccharum biomass (Mg ha^{-1})	237	224	216	201
Environment				
Leaf litter (Oe/Oa horizons)				
Litter C:N	63.7	57.1	52.9	43.4
Litter mass (g)	412	396	591	550
Soil (0–10 cm)				
Sand (%)	85	89	89	87
pH (1:1 soil/H_2O)	4.8	5.0	4.5	4.7
Base saturation, %	71	96	73	80

[a] Data represent site, climatic, overstory, and ambient nitrogen deposition rates of four study sites receiving experimental NO_3^- additions. (Adapted from reference 7 with permission from ASA, CSSA, SSSA.)

the polymer (29). Bacteria also can depolymerize lignin (15, 30), which results in the production of soluble polyphenols (~60% of products) with minimal amounts of CO_2 (<4%) (23, 31). In our long-term field experiment, experimental N deposition has decreased the activity of phenol oxidase and peroxidase expression (~−30%) and fungal laccase (*lcc*) expression (−80%) (32) as well as the diversity of genes mediating detritus decay (−35%) (33).

It is plausible that LMCOs of bacterial origin, which may be more efficient than fungal laccases because bacterial LMCOs contain no introns or posttranslational modification (23), will play a greater role in organic matter decay under future rates of atmospheric N deposition. Here, we sought to determine if experimental N deposition increased the relative abundance and altered the composition of saprotrophic bacteria and LMCOs, which would be consistent with the previously observed slowing of decay, the accumulation of SOM, and the greater production and leaching of phenolic DOC (6). To address our objectives, we amplified bacterial 16S rRNA and LMCO genes using quantitative PCR (qPCR) and utilized "third-generation" high-throughput Pacific Biosciences DNA sequencing technology to determine if the abundance and composition of bacterial and LMCO gene assemblages were altered by experimental N deposition.

MATERIALS AND METHODS

Site description and sample collection. The influence of experimental N deposition on soil bacterial communities was investigated in four sugar maple (*Acer saccharum* Marsh.)-dominated northern hardwood forest stands in lower and upper Michigan in the United States (see Fig. S1 in the supplemental material). The sites are floristically and edaphically similar and span the north-south geographic range of the northern hardwood forests in the Great Lakes region (8); they lie along a climatic and atmospheric N deposition gradient (Table 1). The thin Oi horizon is composed of sugar maple leaf litter, whereas the thicker Oe horizon is interpenetrated by a dense root mat. The soils are sandy (85% to 90%), well-drained, isotic, frigid Typic Haplothods of the Kalkaska series. Six 30-m-by-30-m plots were established at each stand in 1994; three of the plots receive ambient N deposition, and three receive experimental N deposition. During the growing season, the experimental N deposition plots receive six equal applications of $NaNO_3$ pellets broadcast over the forest floor (30 kg N ha^{-1} $year^{-1}$). In our study sites, NO_3^- comprises ~60% of wet plus dry atmospheric N deposition (6).

Forest floor and mineral soil sampling occurred in May 2012, a time in which ample soil moisture favors high rates of microbial activity. Previously, it has been documented that microbial decay has been reduced in the Oe, Oa, and upper A horizons (6), horizons that were sampled for this study. In each 30-m-by-30-m plot, 10 random 0.1-m-by-0.1-m forest floor samples (Oe/Oa horizons) were collected after removing the Oi horizon. Mineral soil (A horizon) samples were collected from the center of each 0.1-m-by-0.1-m area using a 2.5-cm-diameter soil corer (10 cm depth). All samples were composited within each plot and homogenized by hand in the field. The samples were transported on ice to the University of Michigan, where they were held at 4°C for enzyme assays or at −80°C for DNA extraction.

Enzyme analysis. Enzyme assays were initiated 48 h after sampling. Laccase activity potential was determined by extracting extracellular enzymes from 5 g forest floor or 10 g mineral soil (32). Samples were placed in 150 ml acetate buffer (pH 5.0), mixed for 30 min on a shaker, and then centrifuged for 30 min at 2,500 × *g*. A total of 50 μl of the supernatant was combined with 2,6-DMP substrate (ε_{470} = 27.5 mM^{-1} cm^{-1}; 3 mM final concentration) in 96-well microplates and measured spectrophotometrically at 3-min intervals using a Synergy HT microplate reader (Gen5, version 2.00.18; BioTek, Winooski, VT). Laccase activity potential was expressed relative to the dry weight of forest floor or mineral soil and quantified in units, where 1 unit = 1 μm min^{-1} of the oxidized reaction product. The effects of location, experimental atmospheric N deposition, and their combined interaction on laccase activity potential were determined by analysis of variance (ANOVA); means were compared by the use of a protected Fisher's least significant difference (LSD) test (SPSS Statistics, Version 20, IBM Corp., Armonk, NY).

DNA extraction. Genomic DNA was extracted from 2.5 g (fresh weight) of forest floor and from 5 g of mineral soil samples using a PowerMax Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instructions. To remove possible coextracted PCR inhibitors (e.g., humic substances), extracted DNA was purified using a PowerClean DNA Cleanup kit (MoBio). Extracted DNA quality was determined using an ND 8000 NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and quantified by Quant-iT PicoGreen (Invitrogen, Carlsbad, CA) using a Synergy HT fluorometer according to the manufacturer's instructions. All DNA was stored at −80°C.

Bacterial, LMCO, and fungal Abundance. To determine the effect of experimental N deposition on the abundance of bacteria and LMCO genes, quantitative PCR (qPCR) was performed. The procedure was conducted in triplicate on a Stratagene MX3000P real-time PCR system with MaxPro software (version 3.0; Agilent Technologies). All qPCRs were performed using Brilliant III Ultra-Fast SYBR green qPCR master mix (Agilent Technologies) following the manufacturer's instructions. Bacterial abundance was determined using primers 338f and 518r (34, 35), which amplify 180 bp of the 16S rRNA gene (V3 region; see Table S1 in the supplemental material). In addition to SYBR green master mix, the reaction mixtures contained 200 nM primers and 2.5 μl bovine serum albumin (BSA; New England BioLabs, Ipswich, MA) (10 mg ml-1), and the mixtures were brought to a 25-ml final volume with diethyl pyrocarbonate (DEPC)-treated water. PCR cycles consisted of 10 min at 95°C followed by 35 amplification cycles of 30 s at 95°C, 57°C, and 72°C.

Primer sets Cu1Af and Cu2R were used to amplify a 140-bp region of the bacterial LMCO gene (22). The reaction mixture contained 1 μM primers and 3 μl BSA in addition to SYBR green master mix. PCR cycles

consisted of 10 min at 95°C, followed by 45 amplification cycles of 30 s at 95°C and 48°C and then 20 s at 72°C.

Fungal abundance was estimated using primers ITS1f (36) and 5.8S (37), which amplify an ~300-bp region of the fungal internal transcribed spacer (ITS) region. The reaction mixture contained 500 μM primers and 2.5 μl BSA in addition to master mix. PCR cycles consisted of 10 min at 95°C, followed by 40 amplification cycles of 40 s at 95°C, 20 s at 59°C, and 20 s at 72°C. SYBR green fluorescence was measured at the conclusion of each elongation step and was normalized to the fluorescence of 6-carboxyl-X-rhodamine (ROX); excitation and emission wavelengths were 584 and 612 nm, respectively. In all reactions, a melt curve was performed at completion to verify the reaction products.

Standard curves for 16S rRNA and LMCO qPCRs were generated from *Sinorhizobium fredii* genomic DNA using an UltraClean Microbial DNA isolation kit (MoBio, Carlsbad, CA). Fungal standard curves were generated from plasmid standards containing the ITS region using DNA extracted from *Mycena epipterygia*. PCR products were purified using a MinElute PCR Purification kit (Qiagen, Valencia, CA) and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), from which plasmids were isolated using a Wizard plus SV kit (Promega, Madison, WI). To confirm the specificity of the amplification, the amplified products were run on a 1.5% agarose gel and cloned using a TOPO TA cloning kit (Invitrogen). Ten positive clones were picked, and plasmids were isolated from the 10 positive clones using a Wizard Plus SV kit (Promega) and sequenced on an Applied Biosystems 3730 automated sequencer using a BigDye Terminator kit (v3.1) at the University of Michigan DNA sequencing core. Sequences were screened for vector contamination using VecScreen (NCBI; https://www.ncbi.nlm.nih.gov/tools/vecscreen/), and the taxonomic affiliation of the resulting sequence was determined using the BLASTn algorithm on the National Center for Biotechnology Information server (NCBI; http://blast.ncbi.nlm.nih.gov). One hundred percent of bacterial and fungal clones were identified as the correct target group, whereas 90% of the cloned bacterial LMCO amplicons were identified as such (see Table S1 in the supplemental material). Nontarget LMCO clones were identified as fungal laccase.

Mass-based estimates of bacterial 16S rRNA and LMCO genes as well as fungal ITS regions were converted to copy number per μg DNA by assuming a molecular mass of 660 g mol^{-1} per base pair for the average length of the target region. All copy numbers were transformed (log_2) before statistical analysis to meet the assumption of normality. The effects of location, experimental N deposition, and their combined interaction on bacterial, fungal, and LMCO assemblage relative abundances were determined by ANOVA; means were compared by the use of a protected Fisher's LSD test.

PCR amplification and high-throughput sequencing. All PCR amplifications were performed in triplicate using an Expand High Fidelity PCR system (Roche, Indianapolis, IN) on a Mastercycler ProS thermocycler (Eppendorf, Hauppauge, NY), and the reaction mixtures were pooled prior to purification. The V1-to-V3 region of the 16S rRNA gene was amplified from each plot ($n = 48$) using universal bacterial primers 27F and 519R (34) (see Table S1 in the supplemental material). PCR conditions included an initial denaturation stage of 95°C for 10 min and then 25 cycles of 95°C for 30 s followed by 1 min each at 55°C and 72°C. Amplicon purifications were performed using a MinElute PCR purification kit (Qiagen, Valencia, CA). Bacterial LMCO genes were similarly amplified using primer sets Cu1Af and Cu2r under previously described conditions (22); amplicons were purified using a QiaQuick gel extraction kit (Qiagen). Libraries were created from PCR products pooled in equimolar concentrations by plot and were sequenced on a PacBio-RS II system (Pacific Biosciences, Menlo Park, CA) at the University of Michigan DNA Sequencing Core, using standard protocols (38) and C_2 chemistry. PCR amplicons from two and four experimental plots were pooled per SMRT cell for 16S rRNA and LMCO gene assemblages, respectively. For this study, we utilized PacBio circular consensus technology, which can generate at 99.5% to 99.9% sequence accuracy for DNA fragments ranging in size from 150 to 500 bp (39). For more detailed information regarding the use of PacBio circular consensus technology in high-throughput amplicon sequencing, please refer to Fichot and Norman (40).

DNA sequence processing. Sequences were initially processed using the pbh5tools package (Pacific Biosciences), and downstream processing was performed in mothur (Version 1.31.1; 41). Initial quality control (QC) measures removed any sequence with a consensus fold coverage < 5, average quality score < 25 (50-bp rolling window), ambiguous length (<450 or >550 bp), an ambiguous base, >8 homopolymers, or a >1-bp mismatch to either the barcode or primer (42).

High-quality reads were dereplicated and aligned with the SILVA ribosomal database (43) for 16S rRNA gene assemblages; a local LMCO database was used to align LMCO assemblages using kmer searching (8mers) with Needleman-Wunsch global, pairwise alignment methods (44). Both were checked for chimeras using UCHIME (45). The local LMCO database consisted of 641 known LMCO gene sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) in July 2012 and aligned using ClustalO (46).

Analysis of bacterial community composition. Operational taxonomic units (OTUs) were selected at 99%, 97%, 95%, and 90% sequence similarity to explore how experimental N deposition has altered the composition of bacterial and LMCO gene assemblages. Bacterial 16S rRNA gene sequences were taxonomically assigned via a Bayesian classifier (47) with a bootstrap cutoff of 80 using the SILVA database; LMCO sequences were assigned using the BLASTn algorithm (NCBI). Taxonomy was classified as "unknown" if the nearest BLAST hit was <70% similar in coverage or identity to the query sequence. Assemblage diversity was estimated using the Shannon index (H′; 48) and richness using the Chao1 estimator (49); significance was determined by two-way ANOVA, and means were compared using the protected Fisher's LSD test.

Pairwise distances between 16S rRNA and LMCO gene assemblage compositions were determined by (i) taxonomic composition, i.e., Bray-Curtis dissimilarity based on OTU abundance (50), and (ii) phylogenetic membership, i.e., the unweighted UniFrac distance (51). Calculations of Bray-Curtis dissimilarity, UniFrac distance, and all downstream analysis were performed using mothur and Primer (version 6; Primer-E Ltd., Plymouth, United Kingdom). The significance of the taxonomic dissimilarity between ambient and experimental N deposition gene assemblages was confirmed by analysis of similarity (ANOSIM; 52). Maximum-likelihood trees for phylogenetic analysis were generated using representative sequences from each OTU and FastTree (53). The phylogenetic similarity between ambient and experimental N depositions was determined by the unweighted UniFrac algorithm, which measures the sum of unique branch lengths attributable to one treatment or the other but not to both (e.g., ambient N deposition versus experimental N deposition; 51).

Ordinations were obtained from principal coordinate analysis (PCoA) to further test the hypothesis that experimental N deposition altered the taxonomic and phylogenetic composition of bacterial and LMCO gene assemblages. Spearman correlations were calculated from PCoAs to ascertain which specific OTUs contributed to the observed compositional shift in bacterial and LMCO gene assemblages in response to experimental N deposition. Taxonomic and phylogenetic compositional differences were statistically determined by permutational multivariate analysis of variance (PerMANOVA) (54). PerMANOVA allows multivariate information to be partitioned according to the experimental design (with interaction terms) and determines significance by random permutation but cannot determine whether observed shifts are due to location and/or dispersion. Therefore, a distance-based test for the homogeneity of multivariate dispersions (permutational analysis of multivariate dispersions [PERMDISP]) was used to discern the directional nature of observed assemblage shifts.

Defining ecological significance. The four experimental forest stands in our long-term ecological study span a 500-km north-south range that comprises the north-south geographic range of northern hardwood forests in the Great Lakes region of North America. Two-way ANOVA and

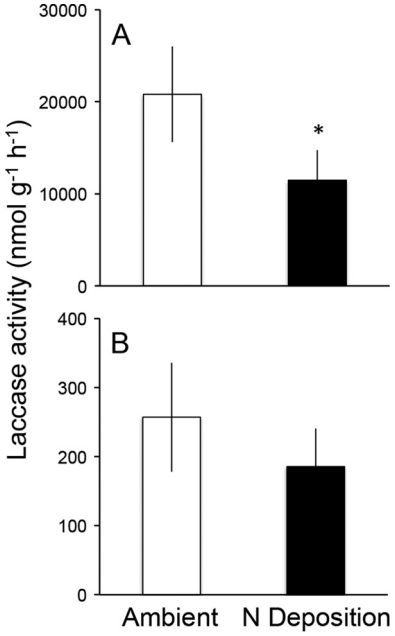

FIG 1 The effect of experimental N deposition on laccase activity potential in forest floor (A) and mineral soil (B). Mean ± standard error ($n = 12$) values are presented; eight analytical replicated were included. *, $P < 0.10$.

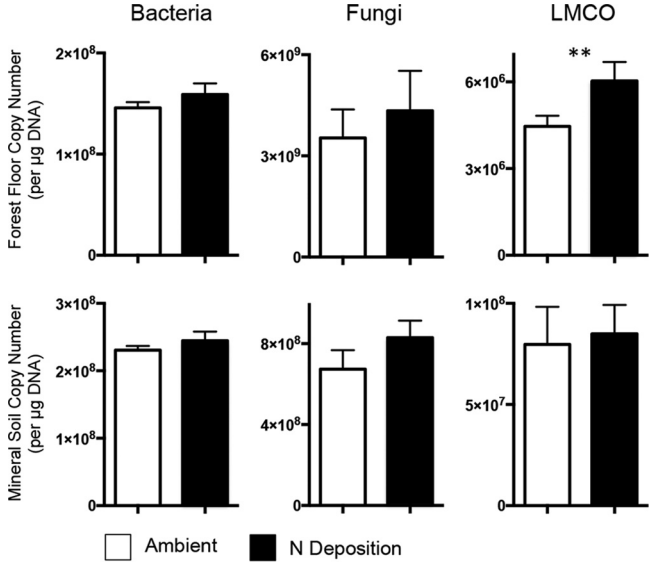

FIG 2 Microbial abundance in mineral soil or forest floor exposed to ambient or experimental N deposition. Values represent treatment means for forest floor (Oe/Oa horizon) and surface soil (upper 10 cm of A horizon) in four replicate northern hardwood stands in Michigan. Error bars represent standard errors ($n = 12$). **, $P < 0.05$.

PerMANOVA are central to our statistical analyses performed with site and experimental N deposition treatment as independent variables. If we were able to reject the site by treatment interaction, it would imply a uniform response to future rates of atmospheric N deposition across an expansive ecosystem in eastern North America. Given the broad geographic scope encompassed by our four experimental sites and the inherent variability associated with a study of this scale, we chose to reject H_0 at $\alpha = 0.10$.

Nucleotide sequence accession numbers. Fastq files of the data set used in this analysis have been deposited in the NCBI SRA database under project accession numbers SRR1011531 and SRR1012312.

RESULTS

Extracellular enzyme activity. In the forest floor, experimental N deposition reduced mean laccase activity by 81% (Fig. 1; $P = 0.09$). In mineral soil, laccase activity decreased under experimental N deposition conditions (−38%), albeit this response was not significant. In both forest floor and mineral soil, levels of laccase activity differed considerably among the four sites; however, we observed no significant site-by-treatment interactions, indicating that experimental N deposition affected laccase activity similarly across sites.

Bacterial and fungal abundance. In mineral soil and the forest floor, bacterial 16S rRNA gene abundance and fungal ITS abundance were unaffected by experimental N deposition (Fig. 2; see Table S2 in the supplemental material). However, bacterial LMCO genes were 26% more abundant in the forest floor under experimental N deposition conditions relative to ambient N deposition ($P = 0.04$); there was no response in mineral soil. No site-by-treatment interaction was observed, indicating that the effect of experimental N deposition on bacterial, fungal, and bacterial LMCO abundance was consistent among all four sites in both forest floor and mineral soil.

Diversity and composition of bacteria and bacterial LMCO under ambient and experimental N deposition conditions. We utilized "third-generation" high-throughput DNA sequencing to test the hypothesis that experimental N deposition altered the composition of saprotrophic bacterial and LMCO gene assemblages. Prior to quality control (QC), we obtained 878,717 16S rRNA sequences and 434,025 LMCO sequences. The numbers of sequences remaining after each step of our pipeline are summarized in Table S3 in the supplemental material; in total, 411,321 16S rRNA (53% loss) and 270,655 LMCO (38% loss) gene sequences passed QC. For downstream analyses, all sites were rarified to 6,000 and 1,000 sequences per site for 16S rRNA and LMCO genes, respectively. The observed responses were phylogenetically robust, as the directionality and significance results were largely similar regardless of the OTU cutoff value. Therefore, results from OTUs generated at 97% sequence similarity are presented for conciseness; relevant statistics at all OTU levels included can be found in the supplemental material.

Bacterial assemblages were dominated by OTUs attributable to *Proteobacteria* (~41%), *Bacteroidetes* (~20%), *Actinobacteria* (~16%), and *Acidobacteria* (~10%) in the forest floor (see Fig. S2 in the supplemental material). However, in mineral soil, *Acidobacteria* (~29%), *Proteobacteria* (28%), and "unclassified" (~16%) accounted for the majority of the OTUs. Mainly, bacterial phyla were similarly abundant under conditions of ambient deposition and experimental N deposition; however, *Bacteroidetes* were relatively less abundant under experimental N deposition conditions in the forest floor (20.9% ± 0.7% and 19.0% ± 0.7% of the community in ambient and experimental N deposition plots, respectively; $P = 0.03$).

Taxonomic affiliations of LMCO OTUs were individually determined for those present in at least half of the experimental plots ($n = 6$ plots). In all, 444 LMCO OTUs (46% of the community) were taxonomically assigned in the forest floor and 507 LMCO OTUs (51% of the community) were assigned in mineral soil. LMCO core OTUs were dominated by *Alpha-*, *Beta-*, and *Gam-*

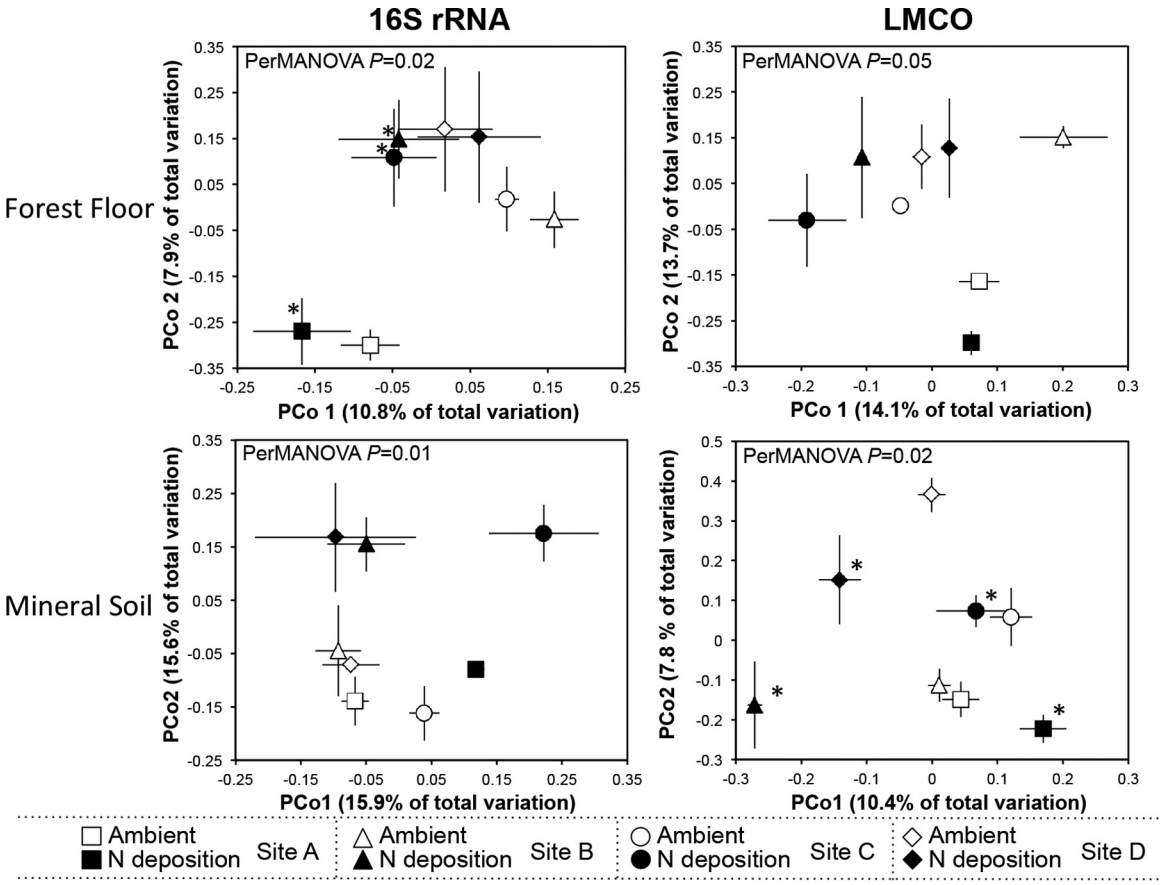

FIG 3 Ordinations obtained from principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity of OTUs generated at 97% sequence similarity. Pairwise significance is presented if there is a site whose results were statistically significantly different with respect to treatment interactions ($P < 0.10$). **, $P < 0.05$; *, $P < 0.10$ (by pairwise PerMANOVA).

maproteobacteria, as well as by *Actinobacteria*, together accounting for 85% of the OTUs in the forest floor and 88% of the OTUs in mineral soil (see Fig. S3 in the supplemental material). LMCOs of most phyla did not respond to the experimental N deposition treatment. However, in the forest floor, *Betaproteobacteria* were more abundant under experimental N deposition conditions than under ambient conditions (7.1% ± 0.6% and 9.8% ± 0.9% of the communities in ambient N deposition and experimental N deposition, respectively; $P = 0.09$). In mineral soil, *Alphaproteobacteria* were less abundant under experimental N deposition conditions (33.7% ± 2.0% and 25.8% ± 2.2% of the communities in ambient N deposition and experimental N deposition, respectively; $P = 0.07$), whereas *Gammaproteobacteria* were more abundant under experimental N deposition conditions (24.3% ± 1.3% and 30.3% ± 2.7% in ambient N deposition and experimental N deposition, respectively; $P = 0.06$).

In the forest floor, bacterial diversity, as indicated by Shannon index (H′) values, decreased under experimental N deposition conditions (−3.0%, $P = 0.07$; see Table S4 in the supplemental material), whereas Chao1 richness was unaffected. In mineral soil, both H′ and Chao1 were unaffected by experimental N deposition. LMCO gene assemblages were less diverse under experimental N deposition conditions in both forest floor and mineral soil (forest floor, −4.0%; mineral soil, −3.5%; $P < 0.10$). LMCO assemblage richness declined in mineral soil (−21.8%; $P < 0.01$) and was unaffected in the forest floor.

16S rRNA and LMCO gene composition was considered to represent either (i) taxonomic composition, i.e., Bray-Curtis dissimilarity calculated from OTU abundance, or (ii) phylogenetic membership, i.e., unweighted UniFrac distance calculated from maximum-likelihood phylogenetic reconstructions. Ordinations obtained by principal coordinate analysis (PCoA) reveal clear taxonomic and phylogenetic compositional shifts due to experimental N deposition among both 16S rRNA and LMCO gene assemblages (Fig. 3; see also Fig. S4 in the supplemental material). These compositional shifts were supported by ANOSIM (see Table S5; $P < 0.10$) for taxonomic composition and by the UniFrac metric for phylogenetic composition ($P < 0.05$) for both 16S rRNA and LMCO gene assemblages. Observed treatment effects on both the taxonomic and the phylogenetic compositions of 16S rRNA and LMCO gene assemblages were further supported by PerMANOVA (see Table S5; $P < 0.10$). However, bacterial assemblages in the forest floor of site D did not respond to the experimental N deposition (site by treatment, $P < 0.10$; pairwise PerMANOVA, $P > 0.10$). Bacterial and LMCO assemblages of ambient and N deposition treatments were similarly heterogeneous in all assemblages (PERMDISP, $P > 0.10$; data not shown).

Spearman correlations were calculated from principal coordi-

nate ordinations to determine which OTUs may have accounted for the observed compositional differences under ambient and experimental N deposition conditions. In all bacterial and LMCO principal coordinate ordinations, the majority of variation could be attributed to PCo1 (Fig. 3; see also Fig. S4 in the supplemental material). For bacterial assemblages in mineral soil, PCo1 and PCo2 accounted for similar amounts of variation (Fig. 3). Using the taxonomic composition of OTUs generated at 97% sequence similarity, experimental N deposition affected bacterial assemblage composition in the negative direction on PCo1 in the forest floor and in the positive direction on both PCo1 and PCo2 in mineral soil. Further analysis of bacterial assemblages in the forest floor revealed that 728 OTUs (44% of the community) were significantly ($P < 0.05$) negatively correlated to axis 1 (see Table S6). Taxonomic identities of significantly correlated OTUs were dominated by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, together accounting for 76% of correlated OTUs. In mineral soil, 397 (19% of the community) and 329 (34% of the community) OTUs were positively correlated to PCo1 and PCo2, respectively. *Proteobacteria*, *Bacteriodetes*, *Acidobacteria*, and *Actinobacteria* account for 78% of the correlated OTUs on PCo1, whereas *Proteobacteria*, *Acidobacteria*, unclassified taxa, and *Bacteroidetes* together account for 77% of the correlated OTUs on PCo2. Among LMCO assemblages, 126 OTUs (7.6% of the community) and 184 OTUs (5.1% of the community) were significantly negatively correlated to PCo1 in forest floor and mineral soil, respectively. OTUs attributable to *Actinobacteria* and *Proteobacteria* drove compositional differences between gene assemblages under ambient and experimental N deposition conditions, accounting for 81% of correlated OTUs in the forest floor and 87% of those in mineral soil.

DISCUSSION

A sustained biogeochemical response to experimental N deposition. Litter decomposition is an enzymatically complex process that is sensitive to N availability in the environment (6, 55). This process is mediated by extracellular enzymes, which are secreted by a species-rich group of saprotrophic bacteria (15, 56) and fungi (32, 57). In our long-term field study, experimental N deposition led to a decline in activity of peroxidase, phenol oxidases, and laccase in the forest floor (32, 58–60), and this trend continues to the present (Fig. 1; peroxidase and phenol oxidase data not shown). These enzymatic changes underlie a biogeochemical response, as 20 years of experimental N deposition has reduced litter decay in our long-term experiment (6), indicative of a larger phenomenon observed among terrestrial ecosystems exposed to simulated N deposition (4, 61). Here, we present evidence that an increase in the abundance of bacterial LMCO genes under experimental N deposition is consistent with these biogeochemical responses, which may indicate the increased bacterial metabolism of plant litter and soil organic matter.

Selection for bacterial LMCO under elevated N deposition conditions. Our hypothesis that chronic N deposition increased the relative abundance and diversity of putative bacterial OM-modifying assemblages was partially supported, as the relative abundance of LMCOs increased and diversity decreased in the forest floor (Fig. 2; see also Tables S2 and S4 in the supplemental material). The relative abundances of bacteria and fungi were unchanged in the forest floor; thus, it appears that bacteria harboring the LMCO gene are favored under experimental N deposition conditions. To draw a stronger causal relationship between LMCO abundance and increased phenolic DOC production, further studies examining effects of experimental N deposition on LMCO expression are needed. For example, fungal laccase expression significantly decreased under N deposition conditions (32) despite previous studies indicating no change in fungal laccase abundance (62). Nevertheless, an increase in the bacterial LMCO relative abundance is consistent with the slowing of decay, accumulation of SOM, and greater production of phenolic DOC that we previously documented under experimental N deposition conditions, thereby identifying a plausible mechanism for these biogeochemical responses.

Experimental N deposition led to significant and consistent compositional shifts in LMCO gene assemblages (Fig. 3; see also Fig. S4 in the supplemental material). Although previous studies investigated bacterial LMCO composition and diversity (22, 23), our study utilized bacterial LMCO assemblages in a hypothesis-based, replicated long-term field manipulation experiment. Shannon diversity estimates of LMCO levels in our study ($H' = 7.3$ to 8.5) were notably higher than those reported from fen and bog soil ($H' = 4.04$ and 4.53, respectively) as well as those reported from a European beech-oak forest ($H' = 4.67$; 22). This difference may be solely due to sequence coverage, because diversity estimates from previous studies were calculated from ≤200 sequences whereas ~50,000 were used here. Observed changes in LMCO composition occurred concomitantly with a shift in bacterial community composition (Fig. 3). Such responses have been previously noted; N enrichment elicited a shift in bacterial community composition in agricultural, alpine, tundra, and grassland soils (63–66) as well as in laboratory assays in soil from 28 sites across the United States (67). Similarly, ectomycorrhizal and saprotrophic fungal compositions are sensitive to N enrichment (32, 61, 68).

Results presented here suggest both specific and broad microbial responses to experimental N deposition, because compositional shifts among 16S rRNA and LMCO gene assemblages remained significant and consistent regardless of how we defined OTUs (see Table S5 in the supplemental material). This differs from the results of a previous study of N enrichment on soil fungi, which revealed a response in mineral soil at fine scales of taxonomic resolution whereas the response in the forest floor was driven by broad groups of fungi (69). Furthermore, in our study, microbial communities were defined by OTU abundance and unique phylogenetic distance, which responded similarly to experimental N deposition. Together, these results provide evidence that chronic N deposition has fundamentally altered the saprotrophic bacterial community at both broad and fine taxonomic resolutions. Further, our results suggest that studies focused on broad taxonomic groups may be sufficient to detect bacterial responses to environmental changes, although potential responses at a finer taxonomic resolution may be overlooked.

Atmospheric N deposition and the importance of bacterial organic matter metabolism. Differences in microbial composition may influence rates of ecosystem-level processes (70, 71). Our observations add to an existing body of literature detailing ecosystem responses to long-term experimental N deposition in a northern hardwood ecosystem (Fig. 4), wherein 20 years of experimental N deposition has led to increased N in soil solution and plant litter, which together have repressed the activities of fungal lignocellulolytic exoenzymes and fungal laccase expression, leading to incomplete lignin decay and increased SOM accumulation and

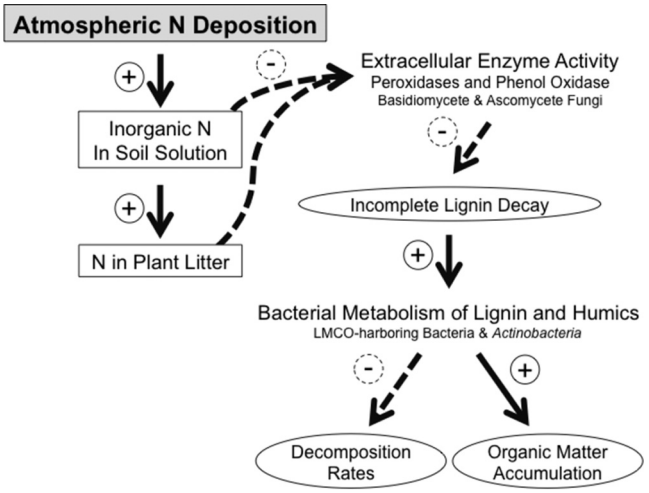

FIG 4 Conceptual diagram of positive (solid arrows) and negative (dashed arrows) fluxes in N pools (squares) and C pools (ovals) and the biological processes (no border) that are affected by experimental N deposition.

phenolic DOC leaching (6, 32, 68, 72). In this report, we present evidence that a more abundant, less diverse, and compositionally different bacterial LMCO assemblage occurred concomitantly with a decreased extent of litter decay and increased DOC production in our long-term field experiment, supporting the hypothesis that experimental N deposition favors bacteria with the physiological ability to metabolize both plant litter and humidified soil organic matter. Experimental N deposition can elicit compositional changes in functional assemblages, including genes mediating C and N cycling (33, 73, 74), as well as numerous metagenomic functional gene categories (64), whereas this study directly examined the abundance and composition of bacterial assemblages, in which observed microbial responses appear to be a mechanism(s) plausibly mediating reduced decay and increased DOC leaching under conditions of experimental N deposition.

There is a growing body of evidence indicating that bacteria can metabolize lignin (15, 30, 75, 76), and LMCOs may mediate this process. Laccase activity, facilitated by LMCO, has been demonstrated in pure cultures of 12 bacterial species encompassing the phyla *Actinobacteria*, *Deinococcus/Thermus*, *Firmicutes*, and *Alpha-*, *Beta-*, and *Gammaproteobacteria* (77). Furthermore, bioinformatic analyses revealed 1,240 LMCO homologs found in 807 published bacterial genomes, encompassing 20 phyla (77), indicating that the vast majority of LMCO diversity remains uncharacterized. However, the causal link between bacterial LMCO and the metabolism of lignin or humic compounds in soil organic matter is not understood (30, 78). Laccases are able to oxidize various phenols, including mono-, di-, and polyphenols; thus, the phenolic subunits of lignin are possible substrates for laccases (79, 80). However, direct biochemical studies are necessary in order to further develop our understanding of the relationship between laccase and lignin decay. In this study, we observed that OTUs representing phyla that have demonstrated lignin-modifying capabilities (i.e., *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Alpha-*, *Beta-*, and *Gammaproteobacteria*) were most abundant and accounted for a shift in bacterial community composition under experimental N deposition conditions (see Fig. S2 and S3 and Table S6 in the supplemental material). This was especially true in the forest floor, in which OTUs attributable to *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* account for 76% of those contributing to the composition shift we observed. In combination, the observations presented here, taken together with previous work (6, 32, 33), suggest that experimental N deposition has favored bacteria with lignin-modifying potential and has disfavored the activity of lignolytic fungi (32).

In conclusion, experimental N deposition consistently and significantly altered bacterial and LMCO community composition in mineral soil and the forest floor. Observed changes were robust, as evidenced by complementary metrics of community composition at multiple levels of taxonomic resolution. Furthermore, the relative abundance of bacterial LMCOs in the forest floor increased under conditions of experimental N deposition, which occurred despite no change in the relative abundance of the bacterial or fungal communities. From this, we suggest that experimental N deposition over nearly 2 decades has favored bacteria with the physiological potential to modify lignin and other polyphenols (i.e., humic compounds) and may be one factor underlying reduced decay, the accumulation of soil organic matter, and greater phenolic DOC production (10). Overall, chronic experimental N deposition has fundamentally altered the composition and functional potential of the saprotrophic bacterial community. Our observations support the idea that the extent of microbial litter decay, in addition to the rate of decay, may be an important mechanism by which anthropogenic N deposition increases soil C storage.

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We declare that we have no conflict of interest.

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