Received Date : 31-Mar-2016
Revised Date : 13-Jul-2016
Accepted Date : 25-Jul-2016
Article type : Primary Research Articles

Anthropogenic N Deposition Increases Soil Organic Matter Accumulation Without Altering Its Biochemical Composition

Revision II for *Global Change Biology*

Running Title: *Biochemical Changes in SOM Under Chronic N Deposition*

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Abstract

Accumulating evidence indicates that future rates of atmospheric N deposition have the potential to increase soil C storage by reducing the decay of plant litter and soil organic matter (SOM). Although the microbial mechanism underlying this response is not well understood, a decline in decay could alter the amount, as well as biochemical composition of SOM. Here, we used size-density fractionation and solid state ^{13}C-NMR spectroscopy to explore the extent to which declines in microbial decay in a long-term (*ca.* 20 yrs.) N deposition experiment have altered the biochemical composition of forest floor, bulk mineral soil, as well as free- and occluded particulate organic matter. Significant amount of organic matter have accumulated in occluded particulate organic matter (~20%; oPOM); however, experimental N deposition had not altered the abundance of carboxyl, aryl, alkyl, or O/N-alkyl C in forest floor, bulk mineral soil, or any soil fraction. These observations suggest that biochemically equivalent organic matter has accumulated in oPOM at a greater rate under experimental N deposition, relative to the ambient treatment. Although we do not understand the process by which experimental N deposition has fostered the occlusion of organic matter by mineral soil particles, our result highlight the importance of interactions among the products of microbial decay and the chemical and physical properties of silt and clay particles that occlude organic matter from microbial attack. Because oPOM can reside in soils for decades to centuries, organic matter accumulating under future rates of anthropogenic N deposition could remain in soil for long periods of time. If temperate forest soils in the Northern Hemisphere respond like those in our experiment, then unabated deposition of anthropogenic N from the atmosphere has the potential to foster greater soil C storage, especially in fine-textures forest soils.

Key Words: *Soil organic matter; anthropogenic N deposition; ^{13}C-NMR; particulate organic matter; soil C storage*

Introduction

By the end of this century, terrestrial ecosystems in eastern North America, as well as in other regions in the Northern Hemisphere, will receive quantities of biologically available nitrogen (N) that are unprecedented over the history of life on Earth (Lamarque *et al*., 2005). This aspect of global change has the potential to constrain the accumulation of anthropogenic CO_2 in the

Earth's atmosphere by increasing ecosystem C storage, thereby slowing the pace of climate warming. Increased net primary productivity, fostered by the anthropogenic supply of growth-limiting N to plants, was thought to be one mechanism that could elicit this global biogeochemical response, although the extent of this mechanism is uncertain (Nadelhoffer *et al.,* 1999; Magnani *et al.,* 2007). Furthermore, a growing body of evidence reveals that anthropogenic N deposition can slow the microbial decay of plant detritus and increase soil C storage across a wide range of terrestrial ecosystems (Liu & Greaver, 2010; Frey *et al.*, 2015; Maaroufi *et al.,* 2015). However, the molecular and microbial mechanisms underlying this biogeochemical response are not well understood, and they are not a component of any coupled climate-biogeochemical model estimating ecosystem C storage (Thornton *et al.,* 2009; Burd *et al.,* 2015).

One plausible mechanism for reduced microbial decay under increased N deposition is a shift from basidiomycete fungal activity, some of which oxidize lignin in plant detritus and polyphenols in soil organic matter (SOM) to CO_2, to a greater degree of metabolism by bacteria and ascomycete fungi, which only partially oxidize these organic substrates (Zak *et al.,* 2011; Freedman & Zak, 2014). Such a response should increase the amount of organic matter stored in soil, as well as alter its biochemical composition. In a wide-spread forest ecosystem in eastern North America, we have accumulated evidence consistent with many aspects of this microbial response to anthropogenic N deposition. For example, forest floor turnover time, an integrated measure of microbial decay, has significantly increased under experimental N deposition (+60%), while organic matter has rapidly accumulated in both forest floor (+51%) and surface mineral soil (+18%; Table 1). Additionally, extracellular phenol oxidase (-81%) and peroxidase (-30%) enzyme activities have consistently declined under experimental N deposition, whereas the production and leaching of phenolic DOC has substantially increased (+26%; Table 1). Although experimental N deposition has not altered the abundance of fungal laccase genes that encode the phenol oxidase enzyme (Table 1), it has reduced their expression by 50% (Table 1). In contrast, bacterial laccase-like multicopper oxidase (LMCO) genes, which weakly oxidize polyphenols in plant detritus and SOM, were 26% more abundant in forest floor exposed to experimental N deposition (Freedman & Zak, 2014), suggesting that bacterial

metabolism of these substrates is more prevalent. In combination, these observations are consistent with the proposed mechanism that experimental N deposition can diminish the complete metabolism of lignin in plant detritus and other polyphenols in SOM by soil fungi, while increasing their incomplete metabolism by soil bacteria and ascomycete fungi (Freedman & Zak, 2014; Freedman *et al.,* 2016).

We initially hypothesized that such a response should both increase the amount and alter the biochemical composition of SOM produced under experimental N deposition (Thomas *et al.,* 2012). This shift from complete metabolism of plant detritus to CO_2 by fungi to incomplete decay by bacteria and ascomycete fungi impacts not only the amount, but should also alter the biochemical composition of SOM (Freedman & Zak, 2014; Thomas et al. 2012). Although the greater production of phenolic DOC under experimental N deposition is consistent with this expectation (Table 1; Pregitzer *et al.,* 2004), biochemical analyses (*i.e.,* alkaline cupric oxide oxidation) of forest floor and mineral soil revealed that neither the amount of lignin-derived molecules nor their degree of oxidation have been altered by experimental N deposition (Thomas *et al.,* 2012); these prior observations did not support our hypothesis. However, it is plausible that other types of plant- and microbial-derived organic compounds (Amelung *et al.,* 2008; Kleber, 2010; Schmidt *et al.,* 2011) have accumulated under experimental N deposition due to the changes in microbial metabolism that we have documented above. As an alternative hypothesis, it also is plausible that experimental N deposition has altered the saprotrophic microbial community in a way that reduces the extent of decay (*sensu* Whittinghill *et al.,* 2012) without causing significant changes in the biochemical composition of the accumulating organic matter, possibly due to physical occlusion by silt and clay particles (Kleber *et al.,* 2015). To further test our initial hypothesis, as well as its alternative, we used solid-state ^{13}C-NMR spectroscopy to characterize the degree of polyphenol oxidation in forest floor and SOM, as well as to characterize the abundance of other bonds that give insight into the biochemical composition of SOM (Baldock *et al.,* 1997). We further used this approach on free (fPOM) and occluded (oPOM) particulate organic matter in mineral soil to test our alternative hypothesis. Resolving our initial hypothesis from its alternative has direct implications for the amount and longevity of SOM accumulating under experimental N

deposition, and hence, the extent to which these soils will function as future sinks for anthropogenic CO_2 accumulating in the Earth's atmosphere.

Materials & Methods

Study Sites and Sampling – Beginning in 1994, we have experimentally increased atmospheric NO_3^- deposition in a series of replicate northern hardwood forest stands spanning a 500-km distance (Fig. 1). Our study deliberately spans the north-south geographic range of the sugar maple-dominated (*Acer saccharum* Marsh.) northern hardwood forests of the Upper Great Lakes and eastern North America (Braun, 1950), enabling us to generalize our experimental results across this widespread and ecologically important ecosystem. Sites are floristically and edaphically matched (> 80% sugar maple on sandy Typic Haplorthods; see Burton *et al.,* 1991), but they differ in climate along the north-south latitudinal gradient (Fig. 1). The study sites also span a gradient of atmospheric N deposition, of which NO_3^- composes ~60% of wet-plus-dry N deposition. Ambient N deposition ranges from 6.8 kg N ha^{-1} y^{-1} at Site A to 11.8 kg N ha^{-1} y^{-1} at Site D. Soils in all sites are sandy, structureless (~85% sand) spodosols, with a well-developed Oe/Oa horizon (*i.e.,* forest floor) that is permeated by a dense mat of fine roots. There are six 30-m x 30-m plots at each site, and every plot is surrounded on all sides by a 10-m wide treated buffer. Three plots at each site receive ambient atmospheric N deposition; whereas, the other three plots at each site receive ambient N deposition plus 30 kg NO_3^--N ha^{-1} y^{-1}, a rate expected by 2050 across some portions of eastern North America (Galloway *et al.,* 2004). The additional NO_3^- is delivered over the growing season in six equal applications (5 kg N ha^{-1} $month^{-1}$) of solid $NaNO_3$ pellets, which are broadcast on the forest floor.

Forest floor and mineral soil sampling occurred in late May to early June 2013. In this way, samples from all four sites were collected during a phenologically-similar period of decay. Within each 30-m by 30-m plot, 10 random 0.1-m by 0.1-m forest floor samples (Oa/Oe horizons; ~ 4 cm thick) were collected by hand to the mineral soil surface, after removing the Oi horizon. All forest floor samples were composited within each plot and homogenized by hand in the field. Mineral soil samples were collected directly beneath forest floor samples, using a 2.5-cm diameter core extending to a depth of 10 cm. Mineral soil samples were passed through a 2-

mm sieve in the field and also were composited within each plot. All samples were kept on ice during transport to the University of Michigan, where they were stored at -20 °C. After removing any fine roots, the entire air-dried, composite forest floor sample from each plot was ground using a ball mill prior; mineral soils samples were not ground. Air-dried forest floor and mineral soil samples then were shipped directly to the Technische Universität München for organic matter fractionation and biochemical analyses.

Organic Matter Fractionation – We partitioned organic matter into five fractions based on density and size (Mueller & Kögel-Knabner, 2009). Air-dried mineral soil samples (30 g; < 2 mm) were saturated with sodium polytungstate (1.8 g/cm^3) and allowed to settle for 24 hrs. Free-floating particulate organic matter (fPOM) was removed from the solution surface by suction, and the remaining material was dispersed via ultrasonication at 440 J mL^{-1}. Occluded POM (oPOM) that was suspended in the sodium polytungstate was then separated from the mineral residue by centrifugation (30 min at 3,074 g). Sodium polytungstate was removed from fPOM and oPOM by washing each fraction with deionized water over a 20 μm sieve, thereby sorting each POM fraction into material >20 μm and <20 μm. The aforementioned protocol created five organic matter fractions based on density (< 1.8 g/cm^3) and size: fPOM >20 μm, fPOM <20 μm, oPOM >20 μm, and oPOM <20 μm; the mineral residue fraction was > 1.8 g/cm^3. These fractions differ in their extent of decay, with fPOM >20 μm exhibiting the least modification by microbial decay and oPOM <20 μm the greatest, insights gained by ^{14}C dating as well as the C:N of these fractions (von Lützow *et al.,* 2007). The mass of POM fractions (< 1.8 g/cm^3) as well as organic matter remaining (>1.8 g/cm^3) in mineral soil was quantified, and the C and N content of each fraction, was measured using a Vario EL elemental analyser (Elementar Analysensysteme, Hanau, Germany). From these data, we calculated the contribution of organic C and N in each fraction (Supplementary Table S1).

Solid state ^{13}C-NMR spectroscopy – To characterize the biochemical composition of forest floor and organic matter residing in mineral soil fractions, we employed solid state ^{13}C-NMR spectroscopy to determine the relative abundance of carboxyl, aryl, O/N-alkyl, and alkyl C (Baldlock *et al.,* 1997). By doing so, we could directly and simultaneously test our hypothesis and its alternative. The cross-polarization magic angle spinning (CPMAS) technique was applied

with a ^{13}C-resonance frequency of 50.32 MHz and a spinning speed of 6.8 kHz using a Bruker DSX 200 NMR spectrometer (Karlsruhe, Germany; *sensu* Steffens *et al.,* 2009, 2011). A ramped ^{1}H-pulse starting at 100% and decreasing to 50% of the initial power was used during a contact time of 1 ms in order to circumvent spin modulation during the Hartmann-Hahn contact. Pulse delays between 200 and 600 ms were used for all spectra. Depending on the C contents of the samples, between 2,000 and 250,000 scans were accumulated, and a line broadening between 0 and 50 Hz was applied. The ^{13}C chemical shifts were calibrated relative to tetramethylsilane (0 ppm). Relative contributions of the various C types were determined by integration of the signal intensity in their respective chemical shift regions according to Knicker *et al.,* (2005). The region from 220 to 160 ppm was assigned to aldehyde, ketone, carboxyl, and amide bonds (*i.e.,* carboxyl C). Olefinic and aromatic C were detected between 160 and 110 ppm and are noted as aryl C. Additionally, O-alkyl and N-alkyl C signals were found from 110 to 60 ppm and from 60 to 45 ppm are designated as O/N-alkyl C. Resonances of alkyl C were assigned to the region 45 to -10 ppm. We calculated alkyl to O/N-alkyl ratio for forest floor and mineral soil fractions (Baldock et al., 1997), because it is a sensitive index for the extent of decay wherein a high ratio is indicative of a lower extent of decay.

Statistical Analyses – To test our hypothesis, we used two-way ANOVA with a site by treatment interaction to compare the mean abundance of carboxyl, aryl, alkyl, O/N-alkyl C, as well as the alkyl to O/N alkyl ratio in both forest floor and bulk mineral soil. We also used the same two-way ANOVA model to test our alternative hypothesis by comparing the mass, carbon concentration, nitrogen concentration, C:N and abundance of the aforementioned organic bonds in both particulate and occluded organic matter fractions. To determine the extent of decay among fPOM and oPOM size fractions, we compared their alkyl to O/N alkyl ratios as well as C:N ratios using a three-way ANOVA, with POM fraction, site and treatment as main effects. Means were compared using a Fisher's protected LSD, and statistical significance was accepted at $\alpha = 0.05$.

Results

Experimental N deposition did not significantly alter the abundance of carboxyl, aryl, O/N-alkyl or alkyl C in forest floor (Fig. 2). Sites (main effect) differed from one another in the abundance

of carboxyl and aryl C in forest floor, but the abundance of O/N-alkyl and alkyl C did not differ among them (P = 0.10 to 0.80; data not shown). Moreover, we observed no interaction between site and treatment on the abundance of any C bond type in forest floor. In bulk mineral soil, although the abundance of carboxyl C in organic matter was significantly lower under experimental N deposition (P = 0.02; Fig. 2), we found no difference in the proportion of aryl, O/N-alkyl, or alkyl C in mineral soil exposed to ambient and experimental N deposition (Fig. 2). Site was not a significant main factor in our analysis of bulk mineral soil, and we also observed no interaction between site and treatment. Further, experimental N deposition had no effect on the alkyl to O/N-alkyl C ratio in either forest floor (0.26 *vs.* 0.26; ambient vs. experimental N deposition, respectively) or mineral soil (0.59 *vs.* 0.60), indicating a similar degree of decay in both treatments. Taken together, these observations do not support our hypothesis that a reduction of microbial decay and oxidative enzyme activity has altered the biochemical composition of organic matter accumulating under experimental N deposition. Because the abundance of aryl C did not differ between ambient and experimental N deposition, we also have no evidence to support the notion that polyphenols in forest floor and mineral soil are less oxidized under experimental N deposition.

The mass ($g_{fraction}/g_{soil)}$ of large (> 20 μm) and small (< 20 μm) oPOM in mineral soil significantly increased under experimental N deposition, which contributed to an overall increase in POM under experimental N deposition (Fig. 3). This increase was driven by the greater contribution of small oPOM, relative to large oPOM (Fig. 3). Although experimental N deposition did not significantly alter either fPOM size fraction (Fig. 3), large fPOM was 23% greater under experimental N deposition, relative to the ambient treatment. Site had a significant influence on the mass of POM fractions, but site means for POM fractions did not display any consistent trend among our study sites (*e.g.,* north to south, data not shown); site and treatment also did not interact to influence the mass of any POM fraction in mineral soil. Additionally, we experienced minimal loss of material during our size and density fractionation, wherein recovery ranged from 98.1% to 99.9%, averaging 99.1% across all samples. In combination, the results described above support our alternative hypothesis that changes in

microbial metabolism have interacted with the physical soil environment to store more organic matter in the oPOM fractions.

Consistent with our prior observations (Table 1), the C concentration in bulk mineral soil was 22% greater under experimental N deposition (15.6 *vs.* 19.1 mg C/g; ambient *vs.* experimental N deposition, respectively; $P = 0.02$), but the N concentration did not differ (0.36 *vs.* 0.28 mg N/g; $P = 0.52$). After POM fractions were removed from bulk mineral soil, the C (3.6 *vs.* 3.0 mg C/g) and N (0.36 *vs.* 0.28 mg N/g) in residual soil (>1.8 g/cm^3) did not differ between ambient and experimental N deposition. However, the concentration of both C and N substantially increased under experimental N deposition (Fig. 4) in several POM fractions. For example, the C concentration of both oPOM size fractions was significantly greater under experimental N deposition, exhibiting an 80% increase in the large oPOM fraction and a 37% increase in the small oPOM fraction (Fig. 4). Similarly, experimental N deposition increased the C concentration of large fPOM by 33%, but it had no effect on the C concentration of small fPOM (Fig. 4). The N concentration in all POM fractions displayed the same trend as C concentration (Fig. 4), but increases in N concentration under experimental N deposition were only significant for large (+84%) and small (+26%) oPOM. Although site was a significant main effect in our analyses of the C and N concentration of POM fractions, means did not exhibit a consistent trend across our study sites; site and treatment also did not interact to influence the C or N concentration of any POM fraction or residual mineral soil. It appears that experimental N deposition has increased the accumulation of both C and N in bulk mineral soil as well as occluded POM fractions, which provides further support for our alternative hypothesis. Evidence for this is the significant increase in the mass (Fig. 3) and C concentration (Fig. 4) of several POM fractions, especially small oPOM.

In Fig. 5, we calculated the mass of organic C contained in each POM fraction as well as amounts contained in bulk and residual soil (>1.8 g/cm^3). For each POM fraction, values are the product of its mass ($g_{fraction}/g_{soil}$) and C concentration (g C/g_{soil}). The total amount of C contained in small and large oPOM significantly increased under experimental N deposition by 82% and 36%, respectively (Fig. 5). Although not significantly greater, the mass of organic C contained in large fPOM also increased by 33% under experimental N deposition. Interestingly,

the difference in bulk soil C between ambient and experimental N deposition (3.5 mg C/g) is equivalent to the additive increases in the mass of organic C in large fPOM (0.9 mg C), large oPOM (0.9 mg C/g) and small oPOM (1.9 mg C/g; combined increase = 3.7 mg C/g). Clearly, the greater mass of C stored in soil under experimental N deposition results from increases in the amount of C that has accumulated in these POM fractions, especially small oPOM (Fig. 5).

The C:N ratio of bulk mineral soil (14.0 *vs.* 15.3) and residual mineral soil (10.1 *vs.* 10.7) were significantly greater under experimental N deposition (see Supplemental Table S1). Site was not a significant main effect in our analysis of C:N in bulk soil or the residual soil after POM fractions were removed; there also was no interaction between site and treatment on the C:N of bulk soil or mineral residue. Interestingly, experimental N deposition had no influence on the C:N ratio of any POM fraction (P = 0.6 to 0.8), but the C:N ratio differed dramatically among POM fractions, providing insight into their degree of decay. For example, both large fPOM (23.5) and large oPOM (27.7) had C:N ratios that were significantly greater than either small fPOM (13.4) or small oPOM (15.1), suggesting that these small size fractions have experienced a greater degree of microbial decay than the larger size fractions.

To determine if experimental N deposition altered the biochemical composition of the four POM fractions, we quantified the abundance of carboxyl, aryl, O/N-alkyl, and alkyl C contained within them (Fig. 6). Similar to our analysis of forest floor and bulk mineral soil (Fig. 2), we have no evidence to support the hypothesis that experimental N deposition has altered the biochemical composition of soil organic matter in any POM fraction (Fig 6). For example, there were no statistically significant differences in the abundance of any bond type under ambient and experimental N deposition, and this was true for both fPOM or oPOM size fractions (Fig. 6). Site was not a significant main effect in our analysis of bond type in four POM fractions, and site did not significantly interact with treatment to influence the bond type abundance in any POM fraction. The alkyl to O/N alkyl ratio did not significantly differ (P = 0.4 to 0.8) for any fPOM or oPOM density fraction under ambient and experimental N deposition. However, averaged across N deposition treatments, there were significant differences in the alkyl to O/N alkyl ratio among size and density fractions. For example, this ratio was greatest in small fPOM (0.73) and small oPOM (0.81), relative to large fPOM (0.54) and large oPOM (0.55).

These observations indicate that organic matter residing in the small fPOM and oPOM fractions has undergone a greater degree of microbial decay. Although experimental N deposition has increased the amount of C stored in bulk mineral soil (Pregitzer et al., 2008; Zak et al., 2008) and the oPOM fractions, we have no evidence whatsoever that it has altered the biochemical composition of organic matter accumulating in soil.

Discussion

Although we have accumulated evidence that declines in fungal polyphenol metabolism and increased bacterial metabolisms of these substrates has lead to a significant accumulation of SOM under experimental N deposition (Table 1; Zak *et al.,* 2011; Freedman & Zak, 2014), our analyses of forest floor, mineral soil, and POM fractions using ^{13}C-NMR provides no evidence that this change in microbial metabolism has altered the biochemical composition of organic matter to any extent. Rather, biochemically equivalent SOM has accumulated under experimental N deposition in oPOM fractions, which provides support for our alternative hypothesis. Moreover, the relatively low C:N of this material, as well as the higher alkyl to O/N alkyl ratio, indicates that it has undergone substantial decay by the microbial community (Baldock et al., 1997). Because organic matter in oPOM decays at a slower pace relative to other fractions (Golchin *et al.,* 1994; Puget *et al.,* 1995; Besnard *et al.,* 1996), greater amounts of small oPOM under experimental N deposition could plausibly remain stored in soil for centuries (Rasmussen et al, 1995; McFarlane et al., 2012; Yamashita *et al.,* 2006) and perhaps longer (Mueller & Kögel-Knabner, 2009). If such a response occurs in other northern temperate forests experiencing anthropogenic N deposition, then it has important implications for the extent to which they will function as future sinks for anthropogenic CO_2 in the Earth's atmosphere.

Recently, Frey *et al.,* (2015) synthesized the results of experimental N deposition in several temperate forest ecosystems, which revealed that significant increases in SOM resulted from the slowing of microbial decay, and not from the greater production of leaf or root litter; these observations are consistent with the evidence we have accumulated in our experiment (Table 1). Importantly, the biogeochemical changes (Table 1), in our experiment appear to be a

general response of northern temperate forests to chronic anthropogenic N deposition, an aggregation of ecosystems that are a globally important sink for anthropogenic CO_2 in the Earth's atmosphere. If forests throughout the Northern Hemisphere respond in a manner similar to those in our experiment, then it is plausible that the unabated deposition of anthropogenic N will increase the amounts and longevity of organic matter stored in their soils. Such a biogeochemical response has the potential to counteract the accumulation of anthropogenic CO_2 in the atmosphere and therefore slow the pace of climate warming.

The formation of SOM is a complex process that is mediated by the amount and biochemical characteristics of detritus (*i.e.,* plant and microbial) entering soil, the metabolism of this material by saprotrophic soil microorganisms, and the physical and chemical interaction of organic decay products with mineral particles (Kögel-Knabner, 2002; Lehmann & Kleber, 2015). Associations between organic matter and soil mineral particles involve occlusion of organic compounds into aggregates at the micrometer scale and interactions with mineral surfaces at the submicron scale (Chenu and Plante, 2006; Virto et al., 2008; Kleber et al., 2015). Several recent experiments using ^{13}C and ^{15}N consistently demonstrate the transfer of both organic C and N from litter into mineral soil fractions (Hatton et al., 2012; Zeller and Dambrine, 2011; Bimüller et al., 2013; Cotrufo et al., 2015); this process is summarized in a conceptual model of C and N flow through soil mineral–organic associations by Hatton et al. (2012). Decaying plant residues enter the soil as coarse fragments that are progressively fragmented and decomposed. They are then transformed into fragments increasingly covered with fine mineral particles and form small aggregates, isolated as oPOM in our fractionation scheme. This physical transfer pathway (Cotrufo et al., 2015) is associated with a progressive accumulation of microbial residues leading to low C:N ratios (Bimüller et al., 2014), along with the progressive transfer of litter-derived materials from plant debris to microaggregates. As pointed out by Hatton et al. (2012), the progression from plant debris to aggregates induces increasing time lags prior to N reaching the dense organo-mineral associations. Mineral-stabilized organic matter is also derived from the direct association of dissolved organic matter released from decaying litter with mineral surfaces (Kaiser and Kalbitz, 2012; Cotrufo et al., 2015), resulting in organo-mineral fractions that can be isolated due to their higher density (Chenu and Plante,

2006). Our results suggest that this pathway is not affected by experimental N deposition, because the fraction isolated with a density > 1.8 g cm^{-3} (*i.e.,* residual soil) did not reveal differences in the amount of C or N contained within it (Table S1).

The results we present here amplify the importance of interactions among residual decay products and the soil mineral particles, especially silt and clay. For example, soils in our experiment are sandy (~85% sand), contain relatively small amounts of silt and clay (11 to 15%) and contain no macro-aggregates whatsoever (MacDonald *et al.,* 1991). However, despite the small proportion of fine soil particles in these soils, they had a disproportionally large effect on stabilizing organic matter under experimental N deposition. Evidence supporting this assertion is the significant increase in the amount of organic matter residing in oPOM, especially the small oPOM fraction (Fig. 3). Organic matter contained in the small oPOM is thought to be stabilized by its association with mineral particles (von Lützow *et al.,* 2007; Hatton *et al.,* 2014; Lehman & Kleber, 2015), which physically preclude attack by oxidative and hydrolytic extracellular enzymes and thereby reducing the extent of microbial decay. The higher alkyl to O/N alkyl ratio of small oPOM, together with its lower C:N, further indicate that organic matter residing in this fraction has undergone a greater degree of microbial decay than organic matter found in the large fPOM and oPOM fractions.

Although we can demonstrate significant change in fungal and bacterial organic matter metabolism under experimental N deposition (Table 1), it appears that physical and chemical interactions between decay products and mineral surfaces are driving greater soil C storage under experimental N deposition. However, we do not understand why nearly 20 years of experimental N deposition has altered the soil environment to facilitate the greater occlusion of biochemically equivalent organic matter by silt and clay particles. Regardless, this observation is consistent with a recent modeling analysis indicating that SOM has accumulated in our experiment from a reduced extent of decay, rather than a slower rate of decay (Whittinghill *et al.,* 2012). Given the rapid and substantial accumulation of SOM that we have documented under experimental N deposition, developing this understanding appears central to anticipating the extent to which anthropogenic N deposition will foster greater soil C storage in northern temperate forests.

Several lines of prior evidence indicate that fine roots are the primary source of SOM under experimental N deposition, but we have no evidence whatsoever that broad changes in either leaf or root litter biochemistry have occurred under experimental N deposition (Xia *et al.,* 2015); this result is similar to our observations of the biochemical characteristics of organic matter in forest floor and mineral soil presented here and elsewhere (Thomas et al., 2012). Foremost, fine roots in our experiment contain a 3-fold-greater lignin concentration (*i.e.,* acid-insoluble fraction) and a 2-fold-greater concentration of condensed tannins than leaf litter (Xia *et al.,* 2015). Moreover, experimental N deposition has not substantially altered the concentrations of these decay-resistant molecules in either leaf or fine root litter (Xia *et al.,* 2015). For example, the relative difference in biochemical constituents (*e.g.,* cellulose, hemicellulose, lipids, protein, soluble phenolics, tannins and lignin) between fine roots under ambient and experimental N deposition range from +6% for fine root N to -8% for condensed tannins. When combined with rates of leaf and fine root litter production (Table 1; Burton *et al.,* 2004), fine roots compose ~70% of the lignin and polyphenols entering soil, and that proportion does not differ between ambient and experimental N deposition (Xia *et al.,* 2015). Further, the biochemical composition of soil organic matter in our experiment is similar to that of fine roots, but not to leaf litter (Thomas *et al.,* 2012). Taken together, these observations indicate that fine roots are the dominant source of SOM in our experiment. Despite the change in microbial metabolism we have documented, it appears that the end products of fine root decay, which have been stabilized in oPOM, are the primary source of organic matter accumulating under experimental N deposition. Over the time scale of our experiment, it is implausible that relatively small biochemical differences (-8% to +6%) in fine root biochemistry under experimental N deposition have caused an 18% increase in SOM (Table 1). Rather, in some way, long-term experimental N deposition has increased the degree to which the products of fine root decay are stabilized by silt and clay particles, despite their relatively low abundance in the sandy soils of our experiment. Given the low amount of silt and clay in our experiment, this effect may be even more pronounced in soils of finer texture.

The insights we have gained from solid state ^{13}C-NMR spectroscopy provide no support for the hypothesis that declines in fungal polyphenol oxidation and the greater incomplete

bacterial metabolism of these substrates has lead to a change in the biochemical composition of soil organic matter. This result is unexpected for several reasons. First, the evidence we provide above demonstrates that experimental N deposition has not altered the amount or biochemical composition of either leaf or root litter entering soil (Xia et al., 2015; Table 1). Given the consistent decline in the expression and activity of fungal extracellular enzymes that completely oxidize lignin and polyphenols in SOM (Edwards *et al.,* 2011; Freedman & Zak, 2015), one would expect to observe a greater proportion of aryl C (*i.e.,* aromatic bonds) under experimental N deposition. However, the abundance of aryl C in bulk soil, as well as in all POM fractions, was equivalent under ambient and experimental N deposition. There are two plausible alternatives that could give rise to this observation. First, the insights provided by solid state ^{13}C-NMR spectroscopy provide an accurate assessment of the biochemical characteristics of SOM under ambient and experimental N deposition, and, indeed, changes in microbial metabolism have not altered the biochemistry of SOM. Alternatively, changes in saprotrophic litter metabolism under experimental N have altered SOM biochemistry in manner that cannot be detected by this method of analysis. Presently, we are unable to determine which of these alternatives is correct and resolving them holds important insight into the mechanisms by which experimental N deposition has fostered the rapid accumulation of SOM.

One might argue that experimental N deposition has increased the abundance of arbuscular mycorrhizal fungi (AMF) and earthworms, both of which are know to aid in the formation of soil aggregates (*i.e.*, oPOM), albeit through different mechanisms. However, several pieces of evidence suggest this is not the case. The young glacially derived soils (ca. 9000 yrs) in our study are ~85% sand and contain no visible macro-aggregates (D.R. Zak, *personal observation*). Approximately 85% of trees in our study are sugar maple (*Acer saccharum* Marsh.), whose roots are infected by AMF. These fungal symbionts produce glomalin-related soil proteins, which foster soil aggregation as well as its stability over time (Rillig 2004). In contrast to this expectation, experimental N deposition has reduced the biomass of intraradical (-36%) and extraradical (-41%) AMF (Van Diepen et al., 2010), making it implausible that greater glomalin production under experimental N deposition is responsible for the increase in oPOM we document here. Moreover, glaciation has removed many of the

native earthworms across our sites, and they have yet to be invaded by either native or exotic earthworms (*e.g., Lumbricus terestris*; Tiunov *et al.,* 2006; D.R. Zak, *personal observation*). Taken together, these observations suggest that interactions between the surface of fine soil particles and organic matter decay products are the most plausible mechanism increasing oPOM under experimental N deposition.

In conclusion, we have accumulated evidence that long-term experimental N deposition has not altered the production or biochemical composition of leaf or root litter entering soil, and that the metabolic activities of lignolytic fungi have decreased, whereas the lignolytic activity of soil bacteria is potentially greater. These observations are consistent with a reduction in decay and the greater production of phenolic DOC. Despite these physiological changes and the biogeochemical responses (Table 1), the biochemical composition of forest floor and mineral soil was identical between ambient and experimental N deposition, at least as assayed by the methods used here and elsewhere (Thomas *et al.,* 2012). Rather, the accumulation of SOM has occurred primarily in oPOM, organic material that is stabilized by entrapment with silt and clay particles (Virto et al., 2008; Hatton et al., 2012). This result is unexpected and signals the importance of the interactions among physical and chemical characteristics of fine soil particles and the biochemical residues from microbial decay. We do not understand the mechanisms by which *ca.* two decades of experimental N deposition has fostered the greater occlusion of organic matter by silt and clay particles. If other forests in the Northern Hemisphere respond in similarly, then the unabated deposition of anthropogenic N has the potential to dramatically and rapidly increase soil C storage, especially in forests that occur on relatively fine-textures soil with high silt and clay contents.

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Response to Chronic N Deposition	% Change	Citation
Plant Responses		
Net Primary Production (NPP)	+10%	Pregitzer *et al.,* 2008
Leaf Litter Production	0%	Pregitzer *et al.,* 2008
Leaf Litter N Concentration	+25%	Xia *et al.,* 2015
Fine Root Litter	0%	Burton *et al.,* 2004
Fine Root Lignin Concentration	0%	Xia *et al.,* 2015
Fine Root N Concentration	+8%	Xia *et al.,* 2015
Fine Root Respiration	0%	Burton *et al.,* 2011
AM Fungal Abundance (Soil)	-41%	Van Diepen *et al.,* 2010
Whole Root System Respiration	0%	Burton *et al.,* 2011
Biogeochemical Responses		
Forest Floor Mass	+51%	Zak *et al.,* 2008
Forest Floor Turnover Time	+60%	Zak *et al.,* 2008
Soil Organic Matter Content	+18%	Zak *et al.,* 2008
Soil Solution NO_3^- Concentration	+288%	Zak *et al.,* 2008
NO_3^- Leaching	+680%	Pregitzer *et al.,* 2004
DOC Leaching	+26%	Pregitzer *et al.,* 2004
Microbial Responses		
Soil Respiration	-15%	Burton *et al.,* 2004

Table 1. Two decades of experimental N deposition have increased the storage and reduced the cycling of C in forest floor and surface mineral soil. Microbial responses are summarized for forest floor (Oe/Oa); mineral soil has exhibited similar directional trends. With the exception of laccase gene copy number, all of positive and negative responses are statistically significant ($P < 0.05$).

Active Microbial Biomass (PLFA)	-23%	DeForest *et al.*, 2004
Phenol Oxidase Activity	-81%	Freedman & Zak, 2014
Peroxidase Activity	-30%	DeForest *et al.,* 2005
Laccase Copy Number	-5 to -8%	Hassett *et al.,* 2009
Laccase Expression	-50%	Edwards *et al.,* 2011
Bacterial LMCO Copy Number	+26%	Freedman & Zak, 2014

List of Figures

Figure 1. Location of northern hardwood forest study sites in lower and upper Michigan, USA. Three plots (30 m x 30 m) in each stand receive ambient atmospheric N deposition and three plots (30 m x 30 m) receive ambient plus 30 kg N ha^{-1} y^{-1} of experimental N deposition. Treatments have been applied since 1994.

Figure 2. The abundance of C types (% of total) in forest floor and surface soil (0 to 10 cm) organic matter under ambient and experimental N deposition in a long-term study. Experimental N deposition had no effect on the proportion (%) of C types in forest floor (upper panel) and surface mineral soil (lower panel). Values are treatment means and the half length of each bar is one standard error.

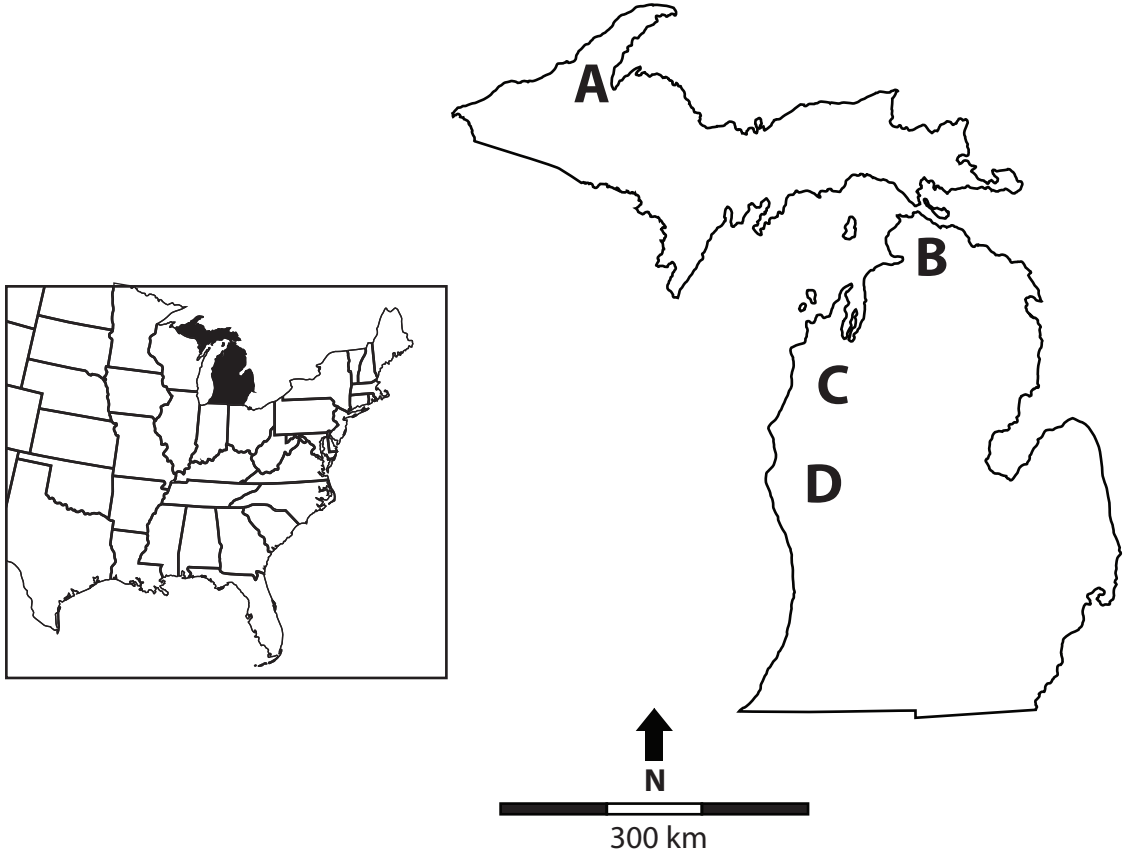
Figure 3. The percent of soil mass composed of POM fractions under ambient and experimental N deposition. Experimental N deposition has increased the mass of organic matter residing in oPOM fractions . Values are treatment means and the half length of each bar is one standard error. Asterisks indicate a significant difference ($P < 0.05$) in concentrations between treatments.

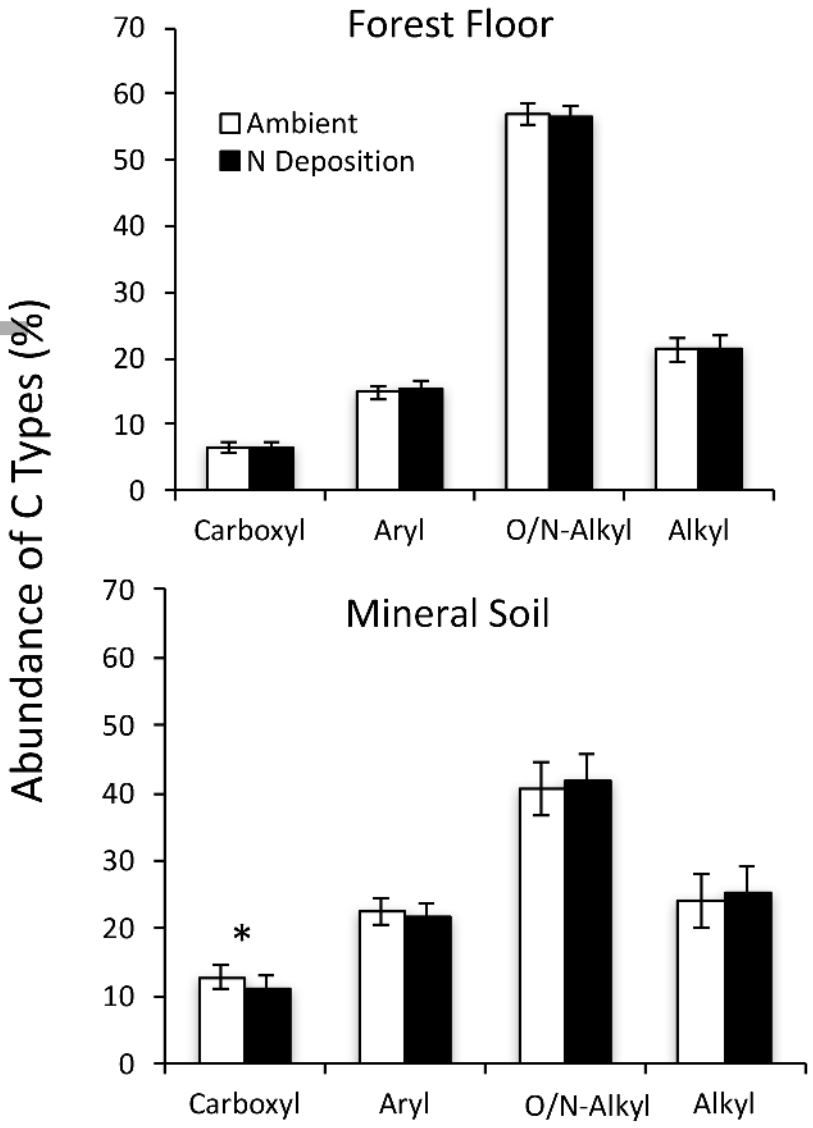
Figure 4. The concentration of organic C and N (mg/g) in soil fractions under ambient and experiential N deposition. Values are treatment means and the half length of each bar is one standard error.

Figure 5. The amount of organic C contained in bulk soil and soil fractions under ambient and experimental N deposition. Values are the product of the mass of each soil fraction and thier C concentration, providing an estimate of the mass of C (mg C/g) contained in each soil fraction.

Figure 6. The abundance of C types in fPOM and oPOM size fractions. The proportion (%) of C types in free and occluded organic matter was not altered by experimental N

deposition. Asterisks indicate a significant difference ($P < 0.05$) between treatments. Values are treatment means and the half length of each bar is one standard error.





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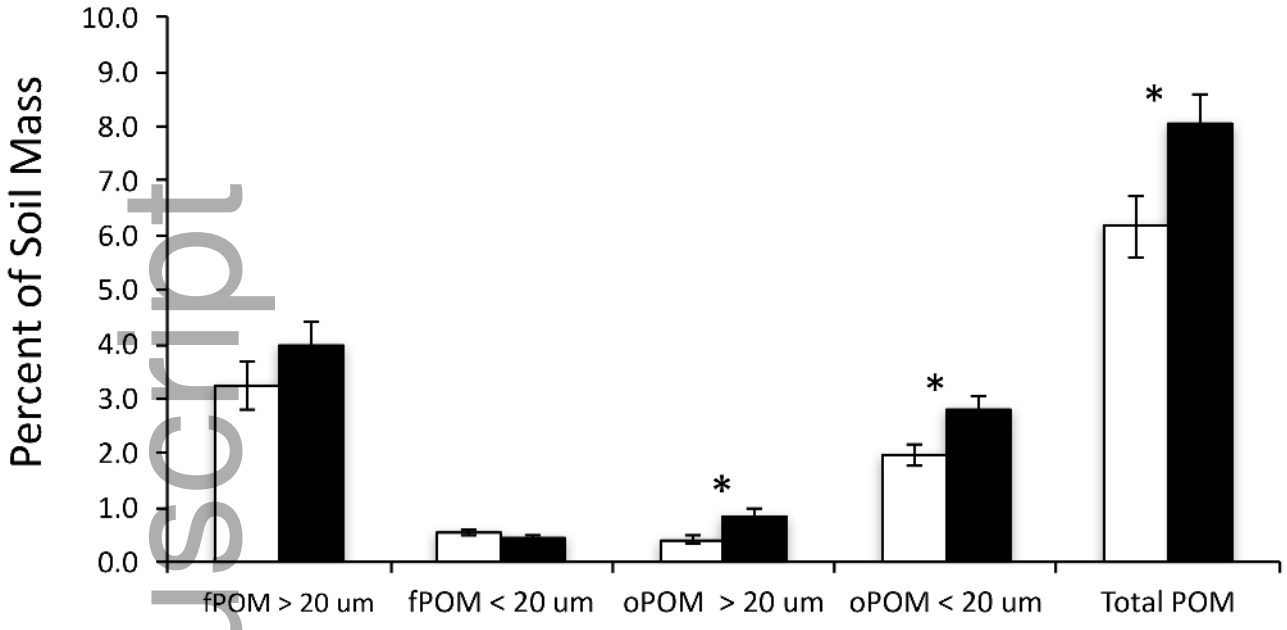

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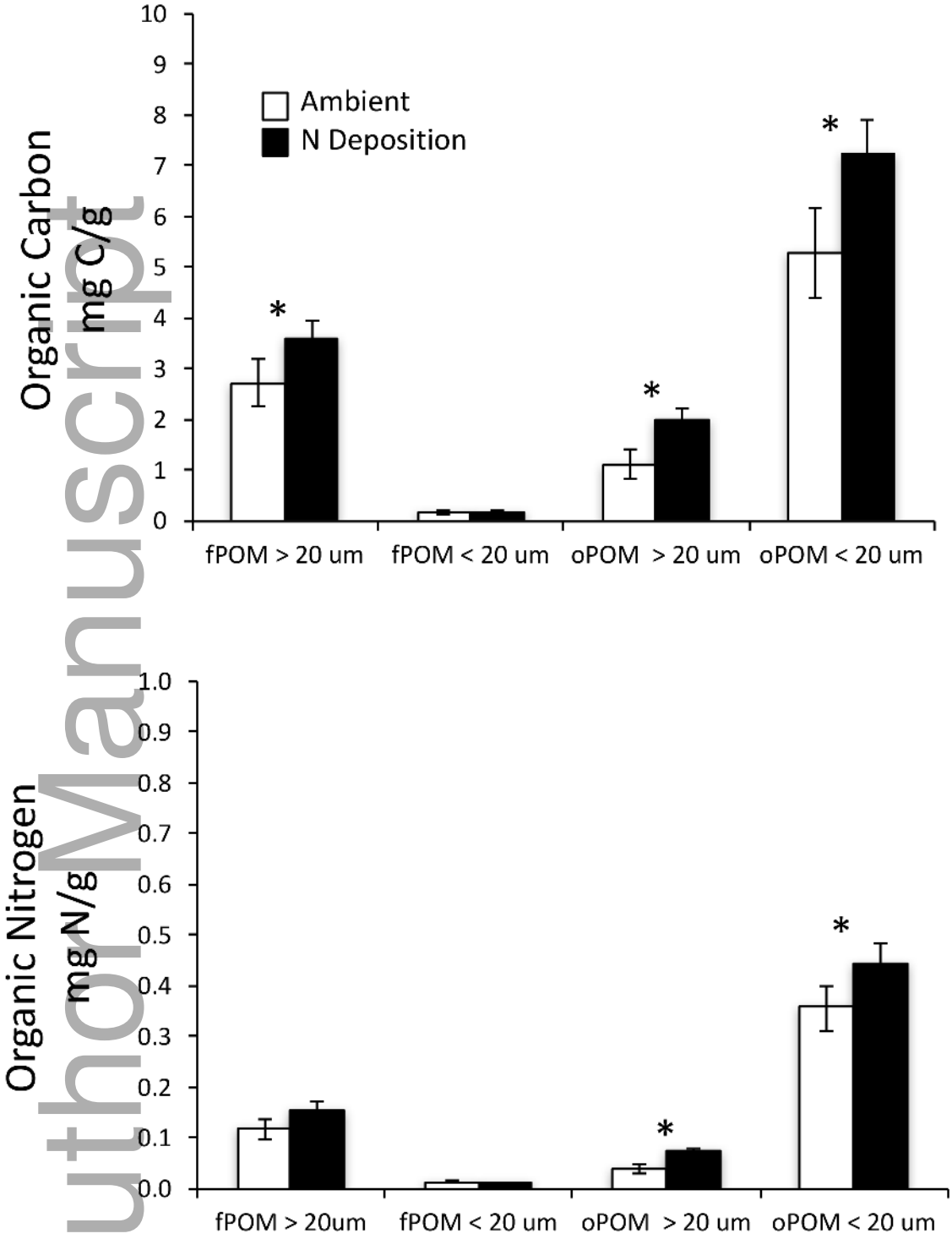

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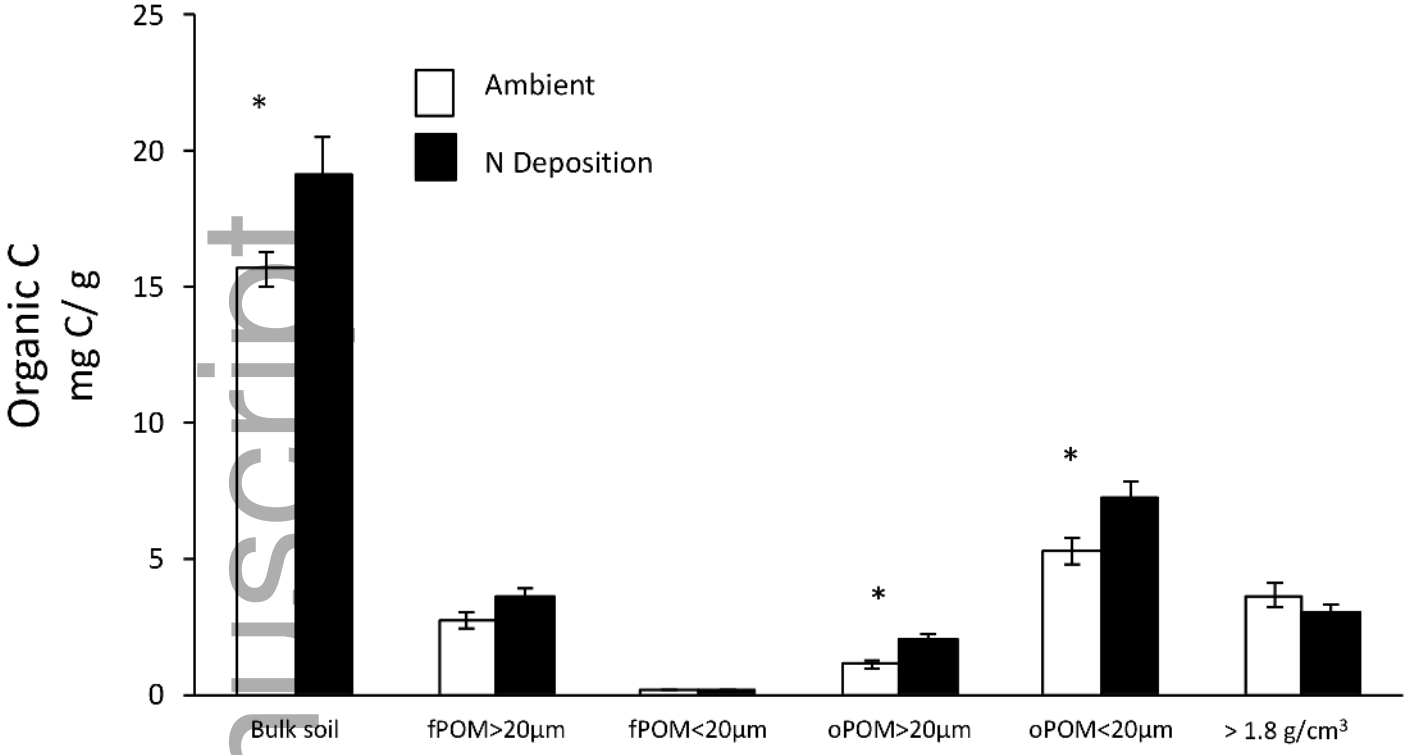

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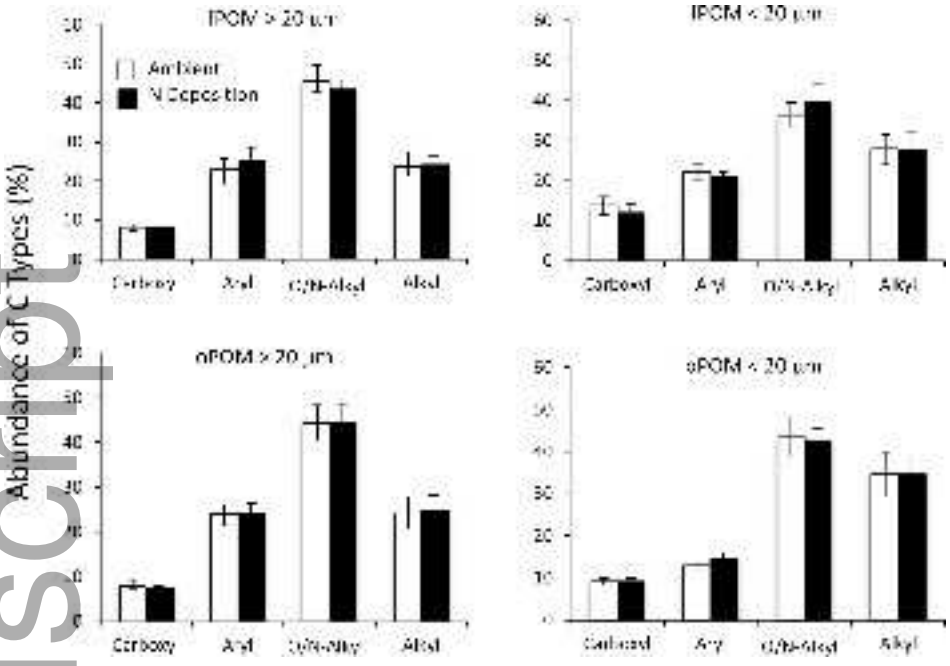

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