FINAL REPORT

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Final Report

Microbial Mechanisms Enhancing Soil C Storage (DE-SC0004850)

With support from DoE BER (Role of Microbial Communities in Carbon Cycling), we have been able to study the molecular mechanisms by which experimental N deposition has decreased plant litter decay and increased the accumulation of organic matter in forest floor and surface soil (*i.e.*, soil C storage). Through a series of biogeochemical measurements and molecular analyses (*e.g.,* targeted and shotgun metatranscriptomics), we have been able to test our initial hypotheses that: *ecosystem response to anthropogenic N deposition (i.e., greater soil C storage) is governed by the environmental regulation of microbial gene expression (Hypothesis I). We also hypothesized that experimental N deposition has decreased the decay of fine roots, which has lead to organic matter accumulation in forest floor and surface mineral soil (Hypothesis II).* In our experiment, the forest floor (Oe/Oa) and surface mineral soil are permeated by a dense mat of fine roots, which are almost exclusively sugar maple. Below, we first describe the biogeochemical changes elicited by almost two decades of experimental N deposition, and then explain our progress toward understanding whether a change in microbial community composition (*i.e.,* who's there and how many), function (*i.e.,* what they are doing), or both underlie this dramatic increase in soil C storage.

We have conducted a long-term field experiment to simulate future rates of anthropogenic N deposition and understand the underlying plant and microbial processes mediating ecosystem C storage. Since 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 sites deliberately span 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 wide-spread and ecologically important ecosystem. Sites are floristically and edaphically matched (> 80% sugar maple on 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. 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. The other three plots at each site receive ambient N deposition plus 3 g NO_3^--N m^{-2} y^{-1}, a rate expected by 2050 across portions of eastern North America (Galloway et al. 2004). The additional NO_3^- is delivered over the growing season in six equal applications (0.5 g N m^{-2} $month^{-1}$) of solid $NaNO_3$ pellets, which are broadcast over the forest floor. Soil temperature and matric potential have been continuously measured since 1994, and we annually measure a wide array of ecological variables (*e.g.,* litter production, tree growth, forest floor mass, soil organic matter, etc,), which provides long-term and detailed metadata for our molecular work. To the best of our knowledge, this is the largest and longest running experimental manipulation of atmospheric N deposition in any forest ecosystem worldwide. Our experimental infrastructure is currently supported by an NSF LTREB grant (Long-Term Research in Environmental Biology) that will enable us to sustain our experimental treatments and maintain baseline data collection until 2018.

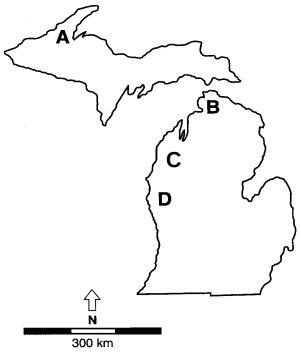

Fig. 1. Distribution of replicate sites spanning the north-south range of northern hardwood forests in the Upper Great Lakes region.

Summary of Ecosystem Response - Experimental N deposition has increased net primary productivity (NPP; +10%), but it surprisingly has not altered the production of leaf or fine-root litter (Table 1, see next page), a trend that continues to the present. Rather, greater NPP under experimental N deposition has

Table 1. Eighteen years 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 copy number, all of positive and negative responses are statistically significant.

Response to Chronic N Deposition	% Change	Citation
Plant		
Net Primary Production (NPP)	+10%	Pregitzer et al. 2008
Leaf Litter Production	0%	Pregitzer et al. 2008
Leaf Litter N Concentration	+25%	Zak et al. 2008
Fine Root Litter	0%	Burton et al. 2004
Fine Root N Concentration	0%	Burton et al. 2004
Fine Root Respiration (rate/g of root)	0%	Van Diepen et al. 2010
Extraradical AM Fungal Abundance	-43%	Burton et al. 2004, 2012
Whole Root System Respiration	0%	Burton et al. 2004, 2012
Biogeochemical		
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		
Soil Respiration	-15%	Burton et al. 2004
Active Microbial Biomass (PLFA)	-23%	DeForest et al 2004
Phenol Oxidase Activity	-33%	DeForest et al. 2004; 2005
Peroxidase Activity	-30%	DeForest et al. 2004; 2005
Basidiomycete Laccase Copy Number	-5 to -8%	Hassett et al. 2009

been focused toward woody biomass production (Pregitzer et al. 2008). Although leaf litter N concentration has significantly increased under experimental N deposition (+25%; Table 1), it has not altered the N concentration of fine-root litter (Table 1). Experimental N deposition also has decreased the abundance of extraradical AM hyphae by 43%, suggesting the mycorrhizal litter production has also declined. In combination, these observations indicate that *experimental N deposition has not altered the production of plant detritus entering soil from leaves or roots*, *but it has elevated leaf litter N concentration as well as NO_3^- concentrations in both forest floor and mineral soil*. Because high leaf litter N (+25%) and inorganic N concentrations (+288%) can reduce the metabolism of lignocellulose by some basidiomycete fungi, we expected litter decay to decline and organic matter to accumulate in forest floor and surface mineral soil. Indeed, forest floor turnover time, an integrated measure of 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). These biogeochemical responses have occurred in parallel with decline in active microbial biomass (-23%), a reduction in extracellular phenol oxidase (-33%) and peroxidase (-30%) activity, and a significant increase in the production and leaching of phenolic DOC (+26%; Table 1). Additionally, we have documented a non-significant reduction (-5 to -8%) in basidiomycete laccase (*lcc*) gene copy number, which encodes the phenol oxidase enzyme, suggesting that experimental N deposition has not altered the abundance of this gene. Further, we have documented a significant decline (-15%) in soil respiration (root plus microbial) under experimental N deposition, and

because fine root biomass and respiration have been unaltered by our experimental treatment (Table 1), this response signifies an overall decline in microbial respiration. This suite of biogeochemical and microbial observations "*set the stage*" for our prior DoE research, which focused on the microbial mechanisms that have slowed decay and caused organic matter to accumulate under experimental N deposition.

Below, we present our initial hypotheses and highlight progress we have made testing them over the past 3 years. The information we have assembled with our prior DoE support provides firm evidence that the environmental regulation of microbial gene expression underlies the decline in decay and accumulation of soil organic matter under experimental N deposition, albeit the overall response of soil microbial communities is somewhat more complex than our initial hypothesis predicted; we explain in the following paragraphs.

Hypothesis I

Ecosystem response to anthropogenic N deposition (i.e., greater soil C storage) is governed by the environmental regulation of microbial gene expression

Gene Expression - Our approach toward understanding the microbial mechanisms underlying a decline in decay and accumulation of soil organic matter centered on determining whether chronic N deposition had altered the abundance, composition, and function of soil microbial communities (Zak et al. 2006). We hypothesized that chronic N deposition would down regulate the expression of fungal genes encoding lignocellulolytic enzymes, thereby reducing decay (Table 1) and opening a niche for other lignocellulolytic soil microorganisms to occupy (e.g., *Actinobacteria*). We tested our hypothesis using targeted and shotgun metatranscriptomic approaches. mRNA extracted from forest floor in all four study sites was synthesized into cDNA for fungal laccase (*lcc*), cellobiohydrolase (*cbhI*), and β tubulin genes. In our analysis, we normalize the expression of fungal genes with lignocellulolytic function to the fungal house-keeping gene, β tubulin (Edwards et al. 2011); we also supplied our collaborator, Dr. Cheryl Kuske at the Los Alamos National Lab (LANL), with cDNA for shotgun metatranscriptomic analyses.

In support of our hypothesis, fungal *lcc* was significantly down regulated by a factor of 2-4 under experimental N deposition, relative to its expression under ambient N deposition (Fig. 2; Edwards et al. 2011). However, we found no effect of experimental N deposition on the expression of fungal *cbhI* (Fig. 2; Edwards et al. 2011). Initially, we examined the potential for a change in fungal community composition at the most southerly of our four study sites (Site D), in which consistently lower levels of *cbhI* and *lcc* gene expression were observed over a two-year period (Edwards et al. 2011). By amplifying fungal 28S from our cDNA, we recovered 19 basidiomycete and 28 ascomycete OTUs; *Athelia, Sistotrema, Ceratobasidium* and *Ceratosebacina* taxa dominated the basidiomycete community, and Leotiomycetes dominated the ascomycete community. Moreover, experimental N deposition had *increased* the proportion of basidiomycete sequences recovered from forest floor, whereas the proportion of ascomycetes in the community was significantly lower under experimental N deposition (Edwards et al. 2011). These observations, taken together, suggest that experimental N deposition had reduced decay through a combination of lower lignolytic gene expression as well as compositional changes in the fungal community. Therefore, with our targeted

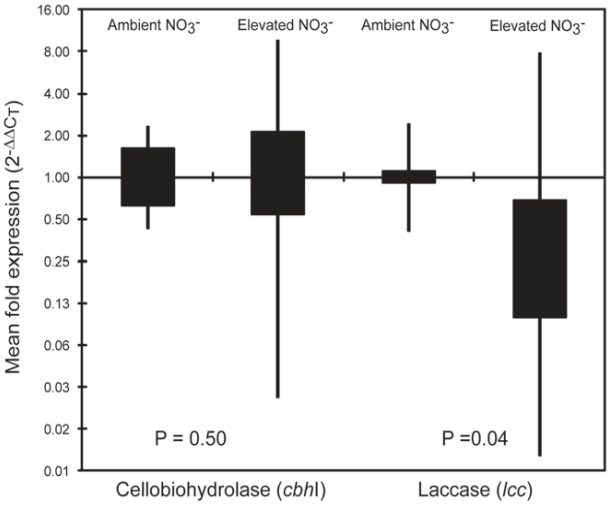

Fig. 2 Relative expression of fungal *cbhI* and *lcc* genes in the forest floor of northern hardwood forests under ambient or experimental N deposition. Boxes define the interquartile range, whiskers mark the minimum and maximum observations; relative expression (Y-axis) is log-scaled. P-values are significance of the mean difference in relative expression under ambient and experimental N deposition (Mann-Whitney U, n = 21; Edwards et al. 2011).

metatranscriptomic approach, *a decline in the expression of fungal genes is not the sole mechanism whereby experimental N deposition has caused soil organic matter to accumulate.* Rather, it appears that compositional changes in fungal communities have elicited functional responses (*e.g.,* reduced *lcc* expression) that have altered the cycling and increased the storage of soil organic matter (*i.e.,* C). Presently, the analyses of our shotgun metatranscriptomes has produced 15 million reads of non-ribosomal cDNA for each plot receiving ambient (n = 12) and experimental N deposition (n = 12); preliminary analyses have confirmed the presence of both bacterial and fungal sequences. We are 18 months into this work, and we expect to analyze these data and gain new insights over the next year.

Community Composition & Abundance - To gain deeper insight into compositional shifts in both fungal and actinobacerial communities, and to test our hypothesis that *Actinobacteria* have become more important agents of decay, we quantified communities of these organisms under ambient and experimental N deposition (Eisenlord & Zak 2010; Entwistle et al. 2013). First, we assessed actinobacterial abundance using quantitative PCR of 16S rRNA, and we evaluated community composition using clone libraries and phylogenetic community analyses (*i.e.,* Libshuff and Unifrac). Counter to our hypothesis, experimental atmospheric N deposition had no effect on actinobacterial abundance in the forest floor (~10^7 gene copies/g); moreover, it significantly *decreased* actinobacterial abundance by 47% in surface soil. Clearly, *Actinobacteria* have not become more competitive under experimental N deposition, and, overall they responded similarly to fungi. Our analyses further revealed that experimental N deposition elicited a significant membership change in forest floor and surface soil communities, as well as significant differences in the phylogenetic structure of forest floor *Actinobacteria* (Eisenlord & Zak 2010). For example, weighted Unifrac, which measures unique branch length and relative abundance of taxa along lineages for each community (Lozupone & Knight 2005), revealed a significant difference between forest floor actinobacterial communities under ambient and experimental N deposition (P = 0.01). However, in a separate analysis, Unifrac did not detect a significant effect of experimental N deposition on actinobacterial communities in surface soil (P = 0.10), at least not at our accepted level of statistical significance. Additionally, Libshuff, a qualitative beta-diversity analysis based on the evolutionary distance, revealed that experimental N deposition significantly altered actinobacterial community composition in both forest floor (P = 0.006) and surface soil (P = 0.001). *Micromonosporaceae* and *Microbacteriaceae* families dominated the forest floor actinobacterial communities. *Micromonosporaceae* mediate lignin degradation (Mason et al. 1988; Godden et al. 1992; Eccleston et al. 2008), they occurred with high fidelity under ambient and experimental N deposition, and composed approximately 20% of forest floor *Actinobacteria* and 6% in surface soil. In the surface soil, approximately 75% of the sequences associated with this family were retrieved from the ambient treatment, whereas, in the forest floor, *Micromonosporaceae* were recovered equally from both ambient and experimental N deposition treatments. Even though this family is a small component of the surface soil actinobacterial community, experimental N deposition negatively impacted its occurrence, and perhaps its function, in lignin decomposition. Collectively, our analyses indicate that experimental N deposition had significantly and consistently altered actinobacterial community composition across a large geographic region; presently, we understand very little about the autecology and physiology of these organisms, and, at this juncture, we are unable to directly link a change in community membership with the biogeochemical response summarized in Table 1.

To further examine the community of basidiomycete and ascomycete fungi actively metabolizing plant litter into soil organic matter, we extracted rRNA from forest floor, constructed cDNA libraries, and amplified and sequenced portions of expressed fungal 28S rRNA (Entwistle et al. 2013). The active basidiomycete communities under ambient and experimental atmospheric N deposition differed significantly in composition, as measured by ∫-Libshuff (Schloss et al. 2004) and Martin's P-test (Martin 2002). However, the active ascomycete communities did not exhibit significant differences in these same community and phylogenetic metrics (Entwistle et al., 2013). Some of the most important lignin decomposers in litter are found in the "Marasmioid" clade (Matheny et al. 2006), as well as in the *Mycenaceae* and *Tricholomataceae* (Osono & Takeda 2002, Osono 2007, Valášková et al. 2007, Steffen et al. 2007, Šnajdr et al. 2011). The basidiomycete communities under experimental N deposition contained fewer OTUs associated with the "Marasmioid clade", a result consistent with our expectations of reduced representation of important lignin decomposers under experimental N deposition. In contrast, the

majority of *Mycenaceae* and *Tricholomataceae* OTUs were recovered under experimental N deposition; this result was inconsistent with our expectations as we had anticipated a decline in representation of these two families under experimental N deposition (Entwistle et al. 2013).

The data we have so far obtained clearly indicate that the expression of a fungal gene with lignolytic activity (*lcc*) has declined under experimental N deposition, and that both fungal and actinobacterial community composition has shifted in response our experimental treatment. Moreover, the decline in litter decay and rapid accumulation of soil organic matter under experimental N deposition is not the sole result of a decline in gene expression. Rather, declines in gene expression occurred in concert with changes in community membership and the phylogenetic structure of both fungal and actinobacterial communities.

Hypothesis II

Experimental N deposition has decreased the decay of fine roots, which has lead to organic matter accumulation in forest floor and surface mineral soil

Fine Root Biochemistry & Decay Dynamics - The forest floor (i.e., Oe/Oa horizon) and surface soil in our study sites are permeated by a dense mat of sugar maple fine roots (< 0.5 mm dia.), which, from our previous work, are a lignin-rich substrate for microbial decay (*e.g.,* 340 to 400 mg lignin/g). Moreover, fine root litter (280 g m^{-2} y^{-1}; Pregitzer et al. 2006) composes ~45% of annual detritus production in this and other forest ecosystems, making it an important substrate for microbial metabolism and soil organic matter formation. We hypothesized that a decline in fine root decay was the primary source of organic matter accumulating in forest floor and surface mineral soil; sugar maple leaves decay rapidly and have very low lignin contents (Melillo et al. 1982). To address hypothesis, we collected leaf and fine root litter, sorted fine roots by size class, and deployed leaves and fine roots in litter decay bags that will be collected during the 2012 and 2013 field seasons; the biochemical analyses of leaves and fine roots is presently underway. Our results indicate that experimental N deposition *has not altered the soluble protein, condensed tannin, or lignin concentration of leaf or root litter; therefore, altered microbial metabolism is directly responsible for reduced decay and the accumulation of organic matter.*

For example, tissue type (leaf litter versus fine roots) had larger effects on all biochemical traits than did site or experimental N deposition (Xia et al. 2015). Fine roots had greater concentrations of biochemicals associated with chemical recalcitrance than leaf litter, and this pattern persisted across sites and N deposition treatments. Leaf litter had substantially higher concentrations of non-structural carbohydrates (NSCs) and lipids ($P < 0.001$). Leaf litter also exhibited higher concentrations of cellulose and soluble phenolics than fine roots, but the magnitude of difference varied among sites (tissue × site, $P < 0.05$). In contrast, fine roots averaged 3 times higher concentrations of condensed tannins and lignin and twice the N concentration of leaf litter across all four sites ($P < 0.001$). Lignin was the most abundant of all eight biochemical components in fine root tissue, whereas cell wall polysaccharides (cellulose + hemicellulose) were the dominant constituent of leaf litter.

Because leaves and fine roots contributed comparable litter fluxes to the soil in these forests, the large biochemical differences meant that the flux of an individual biochemical class was often dominated by a single litter type. Cell-wall polysaccharides and lignin were the two largest biochemical fluxes to the soil; each of the other biochemical classes accounted for < 10% of the total litter flux. Fine roots dominated the fluxes of lignin (~ 70% of the total) and condensed tannins (~ 68%), two compounds classes that are chemically resistant to decay. Fine roots also contributed more soluble protein and N than leaves to soil across all sites ($P < 0.001$). In contrast, leaf litter contributed considerably more cellulose nonstructural carbohydrates, soluble phenolics, and lipids to the soil ($P < 0.001$). In short, leaf litter and fine roots represented fluxes of very different substrates for decomposition within these forests, but neither was altered by experimental N deposition.

After 36 months of decomposition, litter mass loss varied by site ($P = 0.025$) and also varied among fine roots, leaf litter buried in the soil, and leaf litter on the soil surface ($P < 0.001$). The buried leaf litter, incubated at the interface of the O horizon and mineral soil, had only 15.01 ± 0.04 % of initial mass remaining, whereas leaf litter incubated on the surface soil had 24.62 ± 0.04 % of initial mass remaining.

Fine roots exhibited the slowest mass loss, with more than 50% of the initial mass still remaining after three years. Experimental N deposition increased the amount of fine root mass remaining by 3-5% at sites B and D, but such pattern did not occur for fine roots at other sites nor for leaf litter, leading to a marginally significant interaction of Site × N × Tissue Type (P = 0.060). This is in line with a previous study indicating that sites B and D exhibited the most prominent surface soil carbon increase in response to experimental N deposition (Pregitzer et al. 2008).

Further, we analyzed cupric-oxide-extractable lignin-derived phenols to determine the presence, source, and relative oxidation of lignin-like compounds under ambient and experimental N deposition (Filley et al. 2008; Thomas et al. 2012). *In support of Hypothesis II, the biochemical characteristics of forest floor and surface soil organic matter, in terms of the amount and oxidation of lignin-like compounds, was almost identical to that of fine roots, not leaf litter* (Thomas et al. 2012). However, we found no effect of experimental N deposition on the amount or oxidation of lignin-like compounds in forest floor and surface mineral soil (Thomas et al. 2012), at least using the CuO-oxidation technique; this method only detects side-chain oxidation of lignin monomers and not ring clevage, which can be facilitated by different basidiomycete fungi (Filley 2003).

Linking Molecular Responses to Biogeochemical Dynamics - As a complement to our molecular analyses of soil microbial communities, we used a well-established biogeochemical model (TRACE; Currie et al. 1999) to evaluate whether greater soil C storage under experimental N deposition resulted from a slower decomposition *rate* (Fig. 3; upper panel) or a reduced *extent* of decay (Fig. 3; lower panel). Using data from published forest litter decomposition studies, we determined that, on average, experimental N deposition decreased lignin decay rates by 30% and increased cellulose decay by 9% (Whittinghill et al. 2012). *Incorporating these responses in decay rates into TRACE, which was calibrated using field data from all sites, did not reproduce the greater soil C storage we have observed under experimental N deposition*. However, when the *extent* of decomposition was decreased in TRACE (*e.g.,* a greater amount of litter entering long-lived soil organic matter; Fig. 3; lower panel), the model reproduced greater soil C storage by increasing the amount of litter entering the humus pool, therein accurately predicting greater soil C storage in under experimental N deposition. Our analyses indicate that a decreased *extent* of litter decay as humus is formed, *rather than slower rates of litter decay*, is likely responsible for the accumulation of organic matter, and hence greater soil C storage under experimental N deposition. In combination our other work, our modeling analyses further support the idea that experimental N deposition has fundamentally altered the biochemical processes by which saprotrophic microbial communities are processing plant litter into soil organic matter, a response that plausibly arises from a change in fine root decay.

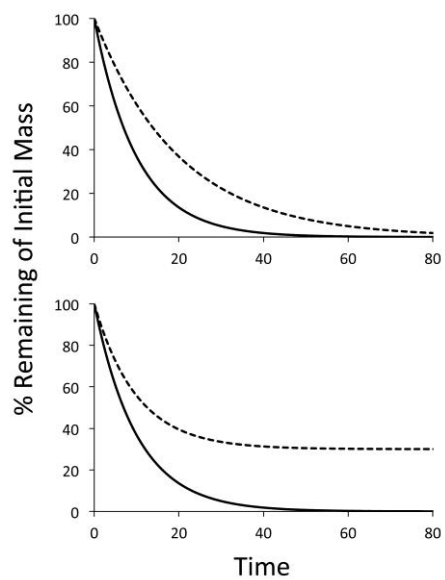

Fig. 3. A decline in the rate constant (upper panel) versus a decrease in the extent (lower panel) of decay under experimental N deposition. Solid lines are ambient and dashed lines are experimental N deposition. In the lower panel, the rate constant for decay is unchanged, but the extent of decay has been reduced, which allows organic matter to accumulate in this conceptual and mathematical model.

Summary of DoE Support - Since the start of our DoE BER project, we have been able to demonstrate that enhanced soil C storage under chronic N deposition has occurred along with a reduction in *lcc* expression, and, moreover, with changes in basidiomycete, ascomycete, and *Actinobacteria* community composition. A lower *extent* of fine root decay, rather than decay *rate*, appears to be an important mechanism increasing soil C storage; however, this response has not apparently altered the amount of lignin-like compounds in forest floor and surface soil (Xia et al. 2015), nor their relative degree of oxidation (Thomas et al. 2012). In combination, our results support the idea that chronic N deposition has directly altered the composition and function of saprotrophic communities

in soil, thereby fundamentally altering the manner in which saprotrophic communities are processing plant litter, likely fine roots, into soil organic matterIn 2011, Dr. Cheryl Kuske (Los Alamos National Lab; LANL) was awarded a "sister grant" from DoE BER to analyze the shot metatranscriptome in all of our study sites. We have supplied her with cDNA, and this complementary project now underway will provide key insights into the overall metabolic response of the soil community exposed to experimental N deposition. Over the course of this grant, we have involved 3 postdoctoral scholars, 4 doctoral students, 4 master's students, and tens of undergraduate students. Our efforts have culminated in 16 peer-reviewed publications, three master's theses, and one doctoral dissertation.

Publications from Microbial Mechanisms Enhancing Soil C Storage (DE-SC0004850)

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