

2020

Nitrogen fertilization decouples roots and microbes in temperate forests: impacts on soil carbon and nitrogen cycling

Joseph E. Carrara
jocarrara@mix.wvu.edu

Follow this and additional works at: <https://researchrepository.wvu.edu/etd>



Part of the [Ecology and Evolutionary Biology Commons](#)

Recommended Citation

Carrara, Joseph E., "Nitrogen fertilization decouples roots and microbes in temperate forests: impacts on soil carbon and nitrogen cycling" (2020). *Graduate Theses, Dissertations, and Problem Reports*. 7952.
<https://researchrepository.wvu.edu/etd/7952>

This Dissertation is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Dissertation in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Dissertation has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

Nitrogen fertilization decouples roots and microbes in temperate forests: impacts on soil carbon and nitrogen cycling

Joseph E Carrara

**Dissertation submitted
to the Eberly College of Arts and Sciences
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in
Biology**

**Edward R Brzostek, Ph.D., Chair
William T Peterjohn, Ph.D.
Richard B Thomas, Ph.D.
Zachary B Freedman, Ph.D.
Jennifer S Hawkins, Ph.D.**

Department of Biology

**Morgantown, West Virginia
2020**

**Keywords: temperate forest, nitrogen, carbon, soil, arbuscular mycorrhizae,
ectomycorrhiza,**

Copyright 2020 Joseph E Carrara

Abstract

Nitrogen fertilization decouples roots and microbes in temperate forests: impacts on soil carbon and nutrient cycling

Joseph Ernest Carrara

Since the start of the industrial revolution the burning of fossil fuels has resulted in enhanced nitrogen (N) inputs into temperate forests through atmospheric deposition. As N is the limiting nutrient for tree growth across most forests, these inputs have generally enhanced above-ground biomass accumulation. However, the impacts of added N on soil carbon storage (C) are less straightforward. While the mean N response across studies is an enhancement of soil C, these results are variable with some studies reporting net C losses. The classic paradigm posits that N enhances soil C through negative effects on fungal decomposers. However, some studies report declines in decomposition without changes in fungal communities suggesting an alternate mechanism that enhances soil C. Recent research provides evidence that trees reduce C allocation belowground when N limitation is reduced and that subsequent declines in the strength of root-microbial interactions may lead to reductions in soil C cycling. In this dissertation I examine the extent to which root-microbial interactions mediate the effects of enhanced N on soil C and nutrient turnover by leveraging the long-term watershed level N fertilization experiment at the Fernow Experimental Forest, WV. Next, I examine the extent to which differences in the strength of root microbial interactions between trees that associate with ectomycorrhizal (ECM) vs arbuscular mycorrhizal (AM) fungi result in divergent soil C and nutrient cycling responses to N at the Bear Brook Watershed N fertilization experiment, ME. Finally, continuing the study at Bear Brook, I examine how root-microbial interactions in AM and ECM dominated soils recover after N fertilization ceases and the subsequent impact on soil C and nutrient turnover. First, I show that under long-term N fertilization, trees reduced belowground C allocation and that these declines were correlated with shifts in bacterial (but not fungal) community composition and declines in extracellular enzyme activities. Next, I find that microbial responses to N fertilization varied between AM and ECM soils wherein bacterial communities shifted in AM soils and fungal communities shifted in ECM soils. This change in microbial communities resulted in an enhancement of C relative to N mining enzyme activity in AM bulk soils and ECM rhizosphere soils. Finally, I show that N fertilization drove ECM trees from N mining toward N foraging by reducing root biomass and mycorrhizal colonization, and altering root morphology, and drove AM trees from mycorrhizal N foraging toward root N foraging by reducing mycorrhizal colonization while maintaining root biomass. After N fertilization ceased, ECM roots recovered, but mycorrhizal colonization remained lower in both mycorrhizal types which suggests a new root-driven nutrient acquisition steady state during initial N recovery. Overall, these results provide evidence that N fertilization can reduce soil C and nutrient cycling by driving reductions in belowground C allocation by trees that ultimately decouple root-microbial interactions. During initial recovery, ECM trees appear to reverse this by enhancing belowground C allocation to acquire N which may stimulate priming and destabilize the forest soil C sink that decades of N deposition have enhanced. The incorporation of these mechanisms into earth system models will likely reduce the uncertainty of climate predictions as N deposition patterns fluctuate in the temperate forest region.

Acknowledgements

I would like to sincerely thank my research advisor and friend Eddie Brzostek for his unwavering support in research and in life over the years it has taken to complete this work. I could not have reached this goal if not for his guidance and I look forward to continuing to work together into the future. I am also thankful to the members of my committee who have always been available and enthusiastic to help me solve problems and think of new ones that need solving. Specifically, I'd like to thank Dr. Peterjohn for the exceptional care he put into teaching me and helping me to find an identity as a scientist along the way. I thank Dr. Thomas for his patience and the advice he gave me - always with an open door and a minute to chat. I thank Dr. Hawkins for her willingness to help me dive into new research waters and her support in helping me to develop new skills. Finally, I'd like to thank Dr. Freedman whose enthusiasm to help me learn and belief in my ability has made me feel better about myself as a scientist and person. To my Mom, I could not have dreamt of this achievement without your unconditional love and support. Doug, Nick, and Jake – thank you for your kindness and enthusiastic support of me on this journey over the years. To my partner Jen, thank you for always being there at my side through the stressful parts and happy parts along this path. I immensely thank my fellow graduate students --- particularly Chris Walter, Justin Mathias, Nanette Raczka, Mark Burnham, Brooke Eastman, Shelby Isom, and Emily Bausher whose passion for science, compassion for others, and brilliance have always inspired me to be better. Additional thanks to my friends Jason Rothman and Andrew Anzel as I did this mostly to impress them. I am extremely grateful for all of the undergraduate interns who have helped to produce this work. I would like to thank the Biology Department and Core Genomics Facility staff as well as the staff at the Fernow Experimental Forest and Ivan Fernandez at Bear Brook Watershed. This research was funded by the National Science Foundation Graduate Research Program (DGE-1102689) and the Long-Term Research in Environmental Biology (LTREB) program (DEB-1119709, DEB-0417678, and DEB-1019522). Further support came from the Earl L Core Memorial Scholarship, the Professor Charles H Baer Graduate Scholarship, and the Morrissey-Ropp Scholarship.

Table of Contents

Abstract.....	ii
Acknowledgements	iii
Table of Contents.....	iv
List of Figures	vi
List of Tables.....	xi
Chapter 1. Introduction: The impacts of nitrogen deposition on temperate forest soils... 1	
1.1 The effects of elevated nitrogen deposition on soil carbon and nutrient cycling.....	2
1.2 The Fernow Experimental Forest and Bear Brook Watershed	7
1.3 Major questions of this study	9
1.4 Figures.....	10
1.5 Literature Cited.....	12
Chapter 2. Interactions among plants, bacteria, and fungi reduce extracellular enzyme activities under long-term N fertilization..... 18	
2.1 Abstract	19
2.2 Introduction	21
2.3 Methods.....	24
2.4 Results.....	33
2.5 Discussion	37
2.6 Tables and Figures.....	44
2.7 Literature Cited.....	50
Chapter 3. Differences between microbial community response to nitrogen fertilization result in unique enzyme shifts between arbuscular and ectomycorrhizal dominated stands 58	
Abstract	59
3.1 Introduction	60
3.2 Methods.....	63
3.3 Results.....	70
3.4 Discussion	73
3.5 Tables and Figures.....	79
3.6 Literature Cited.....	83
Chapter 4. Mycorrhizal type determines root-microbial responses to nitrogen fertilization and recovery..... 92	
4.1 Abstract	93
4.2 Introduction	95
4.3 Methods.....	99
4.4 Results.....	104

4.5	Discussion	107
4.6	Tables and Figures.....	113
4.7	Literature Cited.....	116
Chapter 5. Conclusions: Advancing our understanding of the role of root-microbial interactions in mediating soil C and nutrient cycling in response to long-term N deposition and recovery		125
Appendix. Supplementary Tables and Figures.....		136

List of Figures

- Figure 1-1.** Under ambient condition, plants actively send C belowground to symbiotic and saprotrophic microbes that use this C to create extracellular enzymes. These enzymes break down soil organic matter, thus releasing N for root-uptake (a). Under N fertilization, enhanced N supply likely reduces the plant C cost of N acquisition resulting in reduced belowground C allocation to microbes. With less “free C” microbial enzyme production may decline ultimately resulting in less soil organic matter turnover (b) 10
- Figure 1-2.** C allocation to rhizosphere microbes may drives decomposition to a greater degree in ECM than AM soils under ambient conditions (black arrows) and thus may be more responsive to N fertilization (yellow arrows). When soil has a high N content due to deposition, ECM trees likely rely less on the N mining ability of the rhizosphere community and acquire N in a more direct route, bypassing root-microbial interactions. In AM soils, elevated N may have a smaller impact on root-microbial interactions as turnover in AM soils is driven by free-living microbes. ... 11
- Figure 2-1.** N fertilization reduces hydrolytic and oxidative enzyme activities. Values are the overall seasonal mean enzyme activities (mean \pm SE) of (a) BG, (b) AP, (c) NAG, (d) phenol oxidase, (e) peroxidase, and (f) proteolysis for each soil fraction (i.e., bulk, rhizosphere, and organic horizon) measured in June, July, and August 2015 across all plots (n = 10 plots per watershed). Asterisks indicate significant differences in enzyme activity between watersheds within soil fractions ($p < .05$). Note difference in scale between OH vs. rhizosphere and bulk soil fractions. AA-N is amino acid nitrogen 46

Figure 2-2. N fertilization altered bacterial community composition in OH (a), bulk (c), and rhizosphere (e) soils and fungal community composition in OH (b) and bulk (d) soils, but not rhizosphere (f). All community data were obtained for each soil fraction in July 2015 (n = 10 plots per watershed). p values indicate significant differences between the N-fertilized and reference community..... 47

Figure 2-3. Bacterial, but not fungal community composition is correlated with the first NMDS axis of enzyme activity (a & b). Percent AM colonization is correlated with bacterial community composition (c), but not fungal community composition (d). Data presented are bulk soil community composition data from July 2015 (n = 10 plots per watershed). p values indicate significance of correlation..... 48

Figure 2-4. N fertilization increases (a) N mineralization and (b) nitrification in the OH but not the mineral soil. Values are the overall watershed-level mean rates (mean ± SE) measured in June, July, and August 2015 for each soil fraction (n = 10 plots per watershed). Asterisks indicate significant differences between treatments (p < .05) 49

Figure 3-1. N fertilization enhanced the ratio of simple C to nutrient enzyme activities across AM and ECM bulk and rhizosphere soils, but enhanced complex C to N only in AM bulk soils and ECM rhizosphere soils. Values are overall seasonal mean ratios (mean +SE) of (a&b) β-glucosidase: N-Acetylglucosaminidase, (c&d) β-glucosidase: acid phosphatase, (e&f) phenol oxidase: N-Acetylglucosaminidase, (g&h) peroxidase: N-Acetylglucosaminidase. Values were measured in May, July, and September across all plots (n=6 plots). Asterisks indicate p<0.05 for t-tests between N fertilized and reference plots within mycorrhizal type. Crosses indicate p<0.10..... 79

Figure 3-2. N fertilization altered enzyme profiles in AM bulk soils and ECM rhizosphere soils.

Scatterplots are NMDS1 vs NMDS2 of the enzyme profiles for (a) AM OH soils p=0.023 (b) ECM OH soils p=0.002 (c) AM bulk soils p=0.095, (d) ECM bulk soils p=0.172, (e) AM rhizosphere soils p=0.577, (f) ECM rhizosphere soils p=0.021.

Closed circles are fertilized plots, open circles are reference plots. 80

Figure 3-3. N Fertilization altered bacterial communities in AM soils and fungal communities in

ECM soils. Scatterplots are NMDS1 vs NMDS2 of the community structure for (a) AM bulk soils bacteria, (b) ECM bulk soils fungi, (c) AM rhizosphere soils bacteria, (d) ECM rhizosphere soils fungi. Closed circles are fertilized plots, open circles are reference plots. P values indicate significant differences between community

composition. 81

Figure 3-4. N fertilization reduced Basidiomycota abundance and ligninolytic gene transcription

in AM soils, but not ECM soils. Asterisks represent significant differences between reference and N fertilized values within mycorrhizal type. For (a) n=6 plots per mycorrhizal type per treatment, (b) n= 3 samples per mycorrhizal type per treatment.

Asterisk indicates p<0.05, cross indicates p<0.10. 82

Figure 4-1. Divergent responses of AM and ECM nutrient acquisition strategies to N fertilization and potential recovery trajectories. (A) We hypothesized that under N fertilization ECM roots biomass would decline, roots would become thinner and more forked, and mycorrhizal colonization would decline owing to shifts in nutrient acquisition strategy from N mining to N foraging. This would drive distinct shifts in rhizosphere enzyme activity. Conversely, AM root biomass would not decline, but mycorrhizal colonization would decline owing to shifts in mycorrhizal N foraging to root N foraging. This would result in similar shifts across rhizosphere and bulk soils. (B) As N fertilization stops, these nutrient acquisition metrics may (1) stay in their N fertilized state, (2) return to ambient state, or (3) shift to a new steady state that is not the N fertilized or ambient state..... 113

Figure 4-2. Figure 2. ECM trees reduce belowground C allocation and alter root morphology to a greater extent than AM trees under N fertilization, but partially recover during the year after fertilization ceased. Fine root biomass (FRB) values are overall seasonal mean response ratios (response ratio + propagated SE) of three measurements taken over the growing season during the last year of fertilization (2016; black bars) and during the year after fertilization ceased (2017; open bars). Percent colonization (%Col), average diameter (Diam), specific root length (SRL), and forks per gram of root (Forks) values are July response ratios measured in 2016 and 2017 presented with propagated standard errors. Asterisks indicate $p < 0.05$ for t-tests between N fertilized and reference soils within each year in AM (a) and ECM (b) soils,..... 114

Figure 4-3. AM bulk and rhizosphere soils responded the same to N fertilization and have the same enzyme shifts during the year after fertilization ceased, however ECM bulk and rhizosphere enzyme response and recovery to N varies. Values are overall mean seasonal response ratios (response ratio + propagated SE) of acid phosphatase (AP), beta-glucosidase (BG) and N-Acetylglucosaminidase in AM organic horizon (a), ECM organic horizon (b), AM bulk (c), ECM Bulk (d), AM rhizosphere (e), and ECM rhizosphere (f). Measurements were taken three times over the growing season during the last year of fertilization (2016; black bars) and during the year after fertilization ceased (2017; open bars). Asterisks indicate $p < 0.05$ for t-tests between N fertilized and reference plots during each year and within mycorrhizal type (n=6 plots)..... 115

List of Tables

Table 2-1. Fine root and mycorrhizal metrics in bulk and organic horizon soils under ambient and long-term N fertilized conditions. Mean values of all sampling dates are accompanied by standard error. Asterisks indicate significant differences between treatments ($p < 0.05$)..... 44

Table 2-2. At model steady state, fertilization reduced microbial enzyme pools by ~16% and enhanced soil C by ~3% . When root C exudation was reduced 25% on a per g root basis in the fertilized watershed, there was a further exacerbation in the reduction in microbial enzyme activity to a ~28% decline and a larger increase in soil C by ~20%. 45

Chapter 1. Introduction: The impacts of nitrogen deposition on temperate forest soils

1.1 The effects of elevated nitrogen deposition on soil carbon and nutrient cycling

The extent to which the terrestrial biosphere can sustain current rates of carbon (C) sequestration is one of the most critical questions facing society. The concentration of carbon dioxide (CO₂) in the atmosphere and its subsequent warming effect on the planet are driven by a delicate balance between processes that release CO₂ to the atmosphere (i.e., respiration, fossil fuel burning) and those that remove CO₂ from the atmosphere (i.e., photosynthesis, ocean uptake) (Schimel 1995). On land, soils are the largest pool of C exceeding that of the atmosphere and vegetation combined, and thus any changes in the fluxes associated with this pool feedback on both the global C cycle and ultimately the temperature of the Earth (Jackson *et al.*, 2017).

Since the onset of the industrial revolution, humans have perturbed this balance in several major ways. Through the burning of fossil fuels, human activity has contributed an additional ~4-7 Pg of C yr⁻¹ to the atmosphere over the last century (Schimel 1995). Most research shows that elevated CO₂ leads to higher rates of photosynthesis (Long & Ainsworth, 2004), and ultimately increased plant biomass production (McCarthy *et al.*, 2010). However, empirical evidence and stoichiometric theory suggest that this ability of plants to ramp up photosynthesis under elevated CO₂ is limited by N availability (i.e., progressive nitrogen limitation [PNL] ; (Luo *et al.*, 2006)). In support of the PNL hypothesis, research has shown that the greatest increases in biomass production occur in areas where soil N is less limiting or in species with greater N acquisition capacity (Luo *et al.*, 2006; Terrer *et al.*, 2016). Globally, N deposition continues to rise in tandem with CO₂, which appears to enhance trees capacity to capture this greenhouse gas (Mathias & Thomas, 2018), but how this will feedback on soil C storage remains unknown. As such, there is a critical need to uncover the mechanisms that mediate the soil C consequences of N deposition.

In addition to the release of CO₂, another major anthropogenic perturbation of the global C cycle stems from the acceleration of the global N cycle. These cycles are intimately linked as C uptake by vegetation is limited by N across most forest ecosystems (Reich & Oleksyn, 2004). Human activities release ~30 Tg N yr⁻¹ into the atmosphere through combustion with an additional ~120 Tg N yr⁻¹ introduced to ecosystems through fertilizer application and ~80 Tg N yr⁻¹ through agricultural N fixation (Fowler *et al.*, 2013). Across temperate forests, N is the main limiting or co-limiting nutrient in tree growth (LeBauer & Treseder, 2008). As such, increased atmospheric N deposition has generally enhanced the C sink in the vegetation of temperate forests (Quinn Thomas *et al.*, 2010). However, while, aboveground responses to N are relatively straightforward with additional N inputs relieving N limitation to tree growth (Thomas *et al.*, 2010), belowground responses on soil C dynamics are less uniform. Although a synthesis of data across a suite of N addition experiments showed soil C stocks in forests are typically enhanced by N fertilization due to declines in soil respiration (Janssens *et al.*, 2010), there exists wide variability in this response and the proximate drivers of this variability are largely unknown.

Most research aimed to explain enhancements in soil C under elevated N has focused on direct N effects on microbial community structure and function. Specifically, previous studies have documented an N induced decline in the abundance of *Basidiomycota*, a unique group of fungi capable of breaking down lignin, that often co-occurs with a loss of measured ligninolytic gene potential, transcription, and enzyme activity (Fig. 1a; Fog, 1988; Carreiro *et al.*, 2000; Saiya-Cork *et al.*, 2002; Waldrop *et al.*, 2004; Zak *et al.*, 2008). However, N induced declines in enzymes that break down soil organic matter do not always occur in tandem with declines in *Basidiomycota* abundance (DeForest *et al.* 2004, Pregitzer *et al.* 2008, Hassett *et al.* 2009), suggesting an alternate mechanism that drives N induced soil C enhancement in some forests.

One mechanism that may explain reductions in soil C turnover in response to N deposition is the decoupling of root-microbial interactions. Thus, I have created a conceptual model to illustrate how plants and microbes are dynamically coupled and how enhanced N may weaken this relationship (Fig 1-1a,b). Microbes and plants are intimately linked belowground through the exchange of plant-C for microbially acquired N in the rhizosphere (Hobbie, 2006; Paterson *et al.*, 2007; Yin *et al.*, 2013, 2014; Brzostek *et al.*, 2014). That is, plants actively allocate C belowground to free-living and symbiotic microbes in the rhizosphere that use this C to produce extracellular enzymes that break down soil organic matter and release N for plant uptake (Fig. 1-1a). In addition, mycorrhizal symbionts such as ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi use this C to create vast hyphal networks that forage for N in pore spaces that are inaccessible to roots which significantly enhances the ability of plants to access N and other nutrients (Begum *et al.*, 2019). As such, it is likely that enhanced plant available N supply due to long-term N deposition reduces the C cost of plant N acquisition, resulting in reductions in belowground C allocation by trees to obtain N. This reduction in C transfer to free living microbes and mycorrhizal fungi may feedback on microbial community structure, enzyme activities, and ultimately soil C loss and retention (Fig, 1-1b).

Following this line of reasoning, it is also likely that the relative strength of these root-microbial interactions across species drives variability in soil C and nutrient cycling responses to N fertilization. Emerging research suggests that trees that associate with ECM or AM fungi have fundamental physiological differences which contribute to unique root-microbial nutrient cycling strategies (Phillips *et al.* 2013). ECM trees have high litter C:N ratios and lower leaf mass per area, whereas AM trees have low C:N litter (Phillips *et al.*, 2013). These divergent plant traits appear to feedback on microbial decomposition wherein ECM soils tend to have higher fungal to bacterial

ratios which highlights their reliance on fungi to mine N from high C:N ECM litter (Phillips *et al.*, 2013; Cheeke *et al.*, 2017). Conversely, bacteria in AM soils rapidly mineralize low C:N AM litter leading to larger inorganic N pools (Phillips *et al.*, 2013; Midgley & Phillips, 2014; Midgley *et al.*, 2015; Cheeke *et al.*, 2017). Based on abundance in AM vs ECM species alone, this simple framework has proven robust in predicting rates of nitrate leaching, soil C storage, and N transformation rates (Phillips *et al.*, 2013; Averill *et al.*, 2014; Midgley & Phillips, 2014; Craig *et al.*, 2018). As such, it is likely that documented differences in nutrient acquisition strategies between AM and ECM trees will inform divergent responses to N additions.

Given differences in litter chemistry and nutrient acquisition strategies between AM and ECM trees, I have developed a conceptual model to capture potential differences in N response between these mycorrhizal types (Fig 1-2a.b). First, ECM trees tend to allocate a large fraction of C to microbes in the rhizosphere, to mobilize N (Brzostek *et al.*, 2013; Fernandez & Kennedy, 2015). Some of this C is transferred to ECM fungi which have higher nutrient foraging precision than ECM roots (Chen *et al.*, 2016, 2018a) and further, can synthesize extracellular enzymes that mine and degrade C and N in soil organic matter which contributes to the stimulation of decomposition in the rhizosphere (Chalot & Brun, 1998; Talbot *et al.*, 2008). As such, when N limitation is alleviated, ECM trees likely reduce C allocation to ECM fungi and microbes in the rhizosphere, effectively decreasing the rhizosphere stimulation of C turnover (Fig. 1-2a). As inorganic N availability increases in ECM stands, these fungal dominated communities that mine N may be outcompeted by microbes that scavenge inorganic N, further reducing decomposition throughout the soil matrix (Freedman & Zak, 2014).

In contrast, C and N turnover in AM stands is driven primarily by free-living microbes which do not need C from roots to mobilize N from labile soil organic matter (Phillips *et al.*, 2008).

Importantly, AM roots have higher nutrient foraging precision than AM fungi, and AM fungi lack the ability to produce extracellular enzymes (Chen *et al.*, 2016, 2018b). As such, AM fungi mainly access inorganic N by foraging which limits their influence in C turnover (Read & Perez-Moreno, 2003). Thus, in AM stands, N fertilization may have a less pronounced effect on belowground C allocation to microbes and C turnover as decomposition is primarily driven by free-living microbes whose activity is predominantly C-limited (Fig. 1-2b).

In this dissertation, I have tested these root-microbial conceptual models and the results imply that N-induced changes in forest soil C are in part controlled by changes in the strength of these root-microbial relationships. While we have advanced our mechanistic understanding of the impacts of enhanced N deposition on forest soil C and nutrient cycling, most predictive models do not account for the role of root-microbial interactions, partly because of a lack of empirical data to inform model parameters. Incorporation of root-microbial coupling into models will likely reduce model uncertainty under current rates of N deposition, and will better equip us to make climate predictions as N deposition regimes shift. For example, legislation curbing oxidized N emissions including the 1990 amendments to the Clean Air Act has resulted in a steady decline in N deposition in temperate forest since deposition peaked in the late 1990s (Du, 2016; Gilliam *et al.*, 2019). After decades of research focused on the impacts of elevated N on forest soil C dynamics that have revealed that N enhances the soil C sink, it is unclear if reductions in N deposition will reverse the underlying mechanism behind this or if these soils will continue to accumulate C at a faster rate. As such, there is a critical need to examine the capacity of soils to continue to store C as N deposition fades across the temperate forest region.

1.2 The Fernow Experimental Forest and Bear Brook Watershed

The Fernow Experimental Forest

The Fernow Experimental Forest (herein Fernow) is located in the central Appalachian Mountains near the town of Parsons, West Virginia (39.03°N, 79.67°W). Mean annual precipitation is ~ 1460 mm and mean annual temperature is 8.9°C. (Adams *et al.*, 1993). Ambient wet N deposition at the site has been declining over time from 10.4 kg N ha⁻¹ yr⁻¹ in 1989 to 5.0 kg N ha⁻¹ yr⁻¹ in 2014 (Clean Air Status and Trends Network). Soils are derived from sandstone and shale bedrock and are classified as coarse textured Inceptisols (loamy-skeletal, mixed mesic Typic Dystrochrept) of the Berks and Calvin Series (Gilliam *et al.* 1994).

The N fertilization experiment at the Fernow is comprised of a reference (24.2 ha) and a N fertilized (34.3 ha) watershed. Both watersheds have stands of similar age (~45 years old) and tree species assemblages. Trees > 2.5 cm in diameter at breast height (DBH) were cut in the N fertilized watershed in 1970 and recovered naturally until 1989 when N treatments began (Peterjohn *et al.* 1999). N is added by aerial fertilization via helicopter at a rate of 35 kg N ha⁻¹ yr⁻¹ divided among three applications annually in the form of solid pellet (NH₄)₂ SO₄ (Adams *et al.*, 1993). The N fertilized watershed has a south-facing aspect and elevation ranging from 735-860 m (Gilliam *et al.*, 2016) and N fertilization stopped in October 2019.

The Bear Brook Watershed in Maine

Bear Brook Watershed (herein Bear Brook) is located in in eastern Maine near the town of Beddington (44°52'15"N, 68°06'25"W) on the southwest slope of Lead Mountain (Wang & Fernandez 1999). Average precipitation is ~1400 mm and mean annual temperature is 5.6° C (Fernandez *et al.*, 2007). Soils are derived from a quartzite and meta-pelite bedrock locally

intruded by granite and are characterized as coarse-loamy, isotic, frigid Typic Haplorthod formed from compact basal till (Fernandez *et al.*, 2007; Patel *et al.*, 2018).

The experiment consists of two adjacent watersheds West Bear (10.8 ha), and East Bear (11.0 ha) Since 1989, West Bear was aeriually fertilized every two months between 1989 and 2016 at a rate of 25.2 kg N ha⁻¹ yr and 28.8 kg S ha⁻¹ yr⁻¹ in the form of solid pellet (NH₄)₂SO₄ making total ambient and treatment N deposition ~ 33.6 kg N ha⁻¹ (Jefts *et al.*, 2004). In the reference watershed, East Bear, the total ambient wet and dry N deposition is ~ 8.4 kg N ha⁻¹ (Fernandez & Norton, 2010).

1.3 Major questions of this study

The research undertaken in this dissertation has three broad questions:

1. (*Chapter 2*) – To what extent do plant microbial interactions mediate soil carbon and nutrient cycling responses to long-term nitrogen fertilization?
2. (*Chapter 3*) – To what extent do differences in nutrient acquisition strategies between ectomycorrhizal and arbuscular mycorrhizal trees explain variability in soil carbon and nutrient cycling responses to long-term nitrogen deposition?
3. (*Chapter 4*) – To what extent do differences in nutrient acquisition strategies between ectomycorrhizal and arbuscular mycorrhizal trees drive the trajectory of recovery as N deposition fades across the temperate forest region?

1.4 Figures

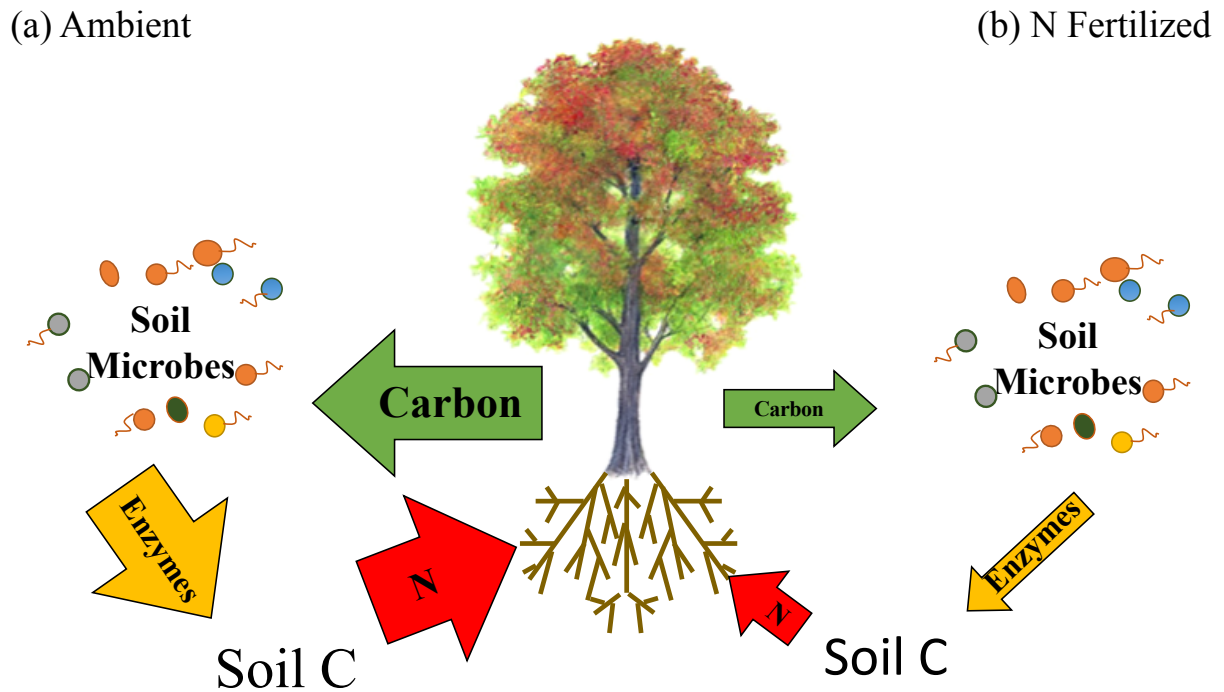


Figure 1-1. Under ambient condition, plants actively send C belowground to symbiotic and saprotrophic microbes that use this C to create extracellular enzymes. These enzymes break down soil organic matter, thus releasing N for root-uptake (a). Under N fertilization, enhanced N supply likely reduces the plant C cost of N acquisition resulting in reduced belowground C allocation to microbes. With less “free C” microbial enzyme production may decline ultimately resulting in less soil organic matter turnover (b)

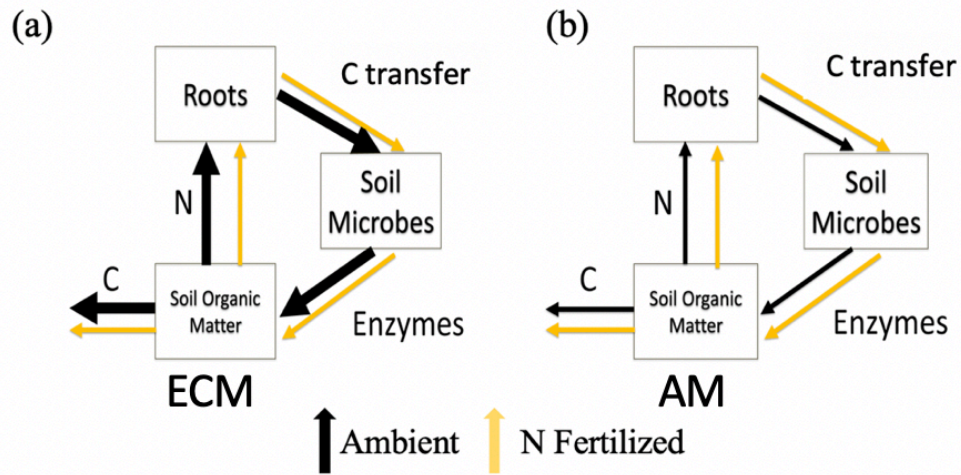


Figure 1-2. C allocation to rhizosphere microbes may drive decomposition to a greater degree in ECM than AM soils under ambient conditions (black arrows) and thus may be more responsive to N fertilization (yellow arrows). When soil has a high N content due to deposition, ECM trees likely rely less on the N mining ability of the rhizosphere community and acquire N in a more direct route, bypassing root-microbial interactions. In AM soils, elevated N may have a smaller impact on root-microbial interactions as turnover in AM soils is driven by free-living microbes.

1.5 Literature Cited

- Averill C, Turner BL, Finzi AC (2014) Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature*, **505**, 543–5.
- Begum N, Qin C, Ahanger MA et al. (2019) Role of Arbuscular Mycorrhizal Fungi in Plant Growth Regulation: Implications in Abiotic Stress Tolerance. *Frontiers in Plant Science*, **10**, 1–15.
- Brzostek ER, Greco A, Drake JE, Finzi AC (2013) Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. *Biogeochemistry*, **115**, 65–76.
- Brzostek ER, Fisher JB, Phillips RP (2014) Modeling the carbon cost of plant nitrogen acquisition: Mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. *Journal of Geophysical Research: Biogeosciences*, **119**, 1684–1697.
- Carreiro MM, Sinsabaugh RL, Repert DA, Parkhurst DF (2000) Microbial Enzyme Shifts Explain Litter Decay Responses To Simulated Nitrogen Deposition. *Ecology*, **81**, 2359–2365.
- Chalot M, Brun A (1998) Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. *FEMS Microbiology Reviews*, **22**, 21–44.
- Cheeke TE, Phillips RP, Brzostek ER, Rosling A, Bever JD, Fransson P (2017) Dominant mycorrhizal association of trees alters carbon and nutrient cycling by selecting for microbial groups with distinct enzyme function. *New Phytologist*, **214**, 432–442.
- Chen W, Koide RT, Adams TS, DeForest JL, Cheng L, Eissenstat DM (2016a) Root morphology

- and mycorrhizal symbioses together shape nutrient foraging strategies of temperate trees. *Proceedings of the National Academy of Sciences of the United States of America*, **113**, 8741–8746.
- Chen W, Eissenstat DM, Koide RT (2018a) Root diameter predicts the extramatrical hyphal exploration distance of the ectomycorrhizal fungal community. *Ecosphere*, **9**.
- Chen W, Koide RT, Eissenstat DM (2018b) Nutrient foraging by mycorrhizas: From species functional traits to ecosystem processes. *Functional Ecology*, **32**, 858–869.
- Craig ME, Turner BL, Liang C, Clay K, Johnson DJ, Phillips RP (2018) Tree mycorrhizal type predicts within-site variability in the storage and distribution of soil organic matter. *Global Change Biology*, **24**, 3317–3330.
- DeForest JL, Zak DR, Pregitzer KS, Burton AJ (2004a) Atmospheric nitrate deposition and the microbial degradation of cellobiose and vanillin in a northern hardwood forest. *Soil Biology and Biochemistry*, **36**, 965–971.
- DeForest JL, Zak DR, Pregitzer KS, Burton AJ (2004b) Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal*, **68**, 132–138.
- Du E (2016) Rise and fall of nitrogen deposition in the United States. *Proceedings of the National Academy of Sciences of the United States of America*, **113**, E3594–E3595.
- Fernandez CW, Kennedy PG (2015) Moving beyond the black-box: Fungal traits, community structure, and carbon sequestration in forest soils. *New Phytologist*, **205**, 1378–1380.
- Fog K (1988) The effect of added nitrogen on the rate of decomposition of organic matter.

Biological Reviews, **63**, 433–462.

Fowler D, Coyle M, Skiba U et al. (2013) The global nitrogen cycle in the Twentyfirst century.

Philosophical Transactions of the Royal Society B: Biological Sciences, **368**.

Freedman Z, Zak DR (2014) Atmospheric N deposition increases bacterial laccase-like multicopper oxidases: Implications for organic matter decay. *Applied and Environmental Microbiology*, **80**, 4460–4468.

Gilliam FS, Burns DA, Driscoll CT, Frey SD, Lovett GM, Watmough SA (2019) Decreased atmospheric nitrogen deposition in eastern North America: Predicted responses of forest ecosystems. *Environmental Pollution*, **244**, 560–574.

Hassett JE, Zak DR, Blackwood CB, Pregitzer KS (2009) Are basidiomycete laccase gene abundance and composition related to reduced lignolytic activity under elevated atmospheric NO₃- Deposition in a northern hardwood forest? *Microbial Ecology*, **57**, 728–739.

Hobbie EA (2006) Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. *Ecology*, **87**, 563–569.

Jackson RB, Lajtha K, Crow SE, Hugelius G, Kramer MG, Piñeiro G (2017) The Ecology of Soil Carbon: Pools, Vulnerabilities, and Biotic and Abiotic Controls. *Annual Review of Ecology, Evolution, and Systematics*, **48**, 419–445.

Janssens IA, al. et (2010) Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience*, **3**, 315–322.

LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial

- ecosystems is globally distributed. *Ecology*, **89**, 371–379.
- Long SP, Ainsworth EA (2004) What have we learned from 15 years of free-air CO₂ enrichment (FACE)? A meta-analytic review of the responses of photosynthesis , canopy properties and plant production to rising CO₂. *New Phytologist*, **165**, 351–372.
- Luo Y, Su B, Currie WS et al. (2006) Progressive Nitrogen Limitation of Ecosystem Responses to Rising Atmospheric Carbon Dioxide. *BioScience*, **54**, 731.
- Mathias JM, Thomas RB (2018) Disentangling the effects of acidic air pollution, atmospheric CO₂, and climate change on recent growth of red spruce trees in the Central Appalachian Mountains. *Global Change Biology*, **24**, 3938–3953.
- McCarthy HR, Oren R, Johnsen KH et al. (2010) Re-assessment of plant carbon dynamics at the Duke free-air CO₂ enrichment site: Interactions of atmospheric [CO₂] with nitrogen and water availability over stand development. *New Phytologist*, **185**, 514–528.
- Midgley MG, Phillips RP (2014) Mycorrhizal associations of dominant trees influence nitrate leaching responses to N deposition. 241–253.
- Midgley MG, Brzostek E, Phillips RP (2015) Decay rates of leaf litters from arbuscular mycorrhizal trees are more sensitive to soil effects than litters from ectomycorrhizal trees. *Journal of Ecology*, **103**, 1454–1463.
- Patel KF, Fernandez IJ, Nelson SJ, Gruselle M, Norton SA, Weiskittel AR (2019) Forest N Dynamics after 25 years of Whole Watershed N Enrichment: The Bear Brook Watershed in Maine. *Soil Science Society of America Journal*, **83**.
- Paterson E, Gebbing T, Abel C, Sim A, Telfer G (2007) Rhizodeposition shapes rhizosphere

- microbial community structure in organic soil. *The New phytologist*, **173**, 600–10.
- Phillips RP, ERLITZ Y, BIER R, BERNHARDT ES (2008) New approach for capturing soluble root exudates in forest soils. *Functional Ecology*, **22**, 990–999.
- Phillips RP, BRZOSTEK E, MIDGLEY MG (2013) The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist*, **199**, 41–51.
- Pregitzer KS, BURTON AJ, ZAK DR, TALHELM AF (2008) Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. *Global Change Biology*, **14**, 142–153.
- Quinn Thomas R, CANHAM CD, WEATHERS KC, GOODALE CL (2010) Increased tree carbon storage in response to nitrogen deposition in the US. *Nature Geoscience*, **3**, 13–17.
- Read DJ, PEREZ-MORENO J (2003) Mycorrhizas and nutrient cycling in ecosystems - A journey towards relevance? *New Phytologist*, **157**, 475–492.
- Reich PB, OLEKSYN J (2004) Global patterns of plant leaf N and P in relation to temperature and latitude. *Proceedings of the National Academy of Sciences*, **101**, 11001–11006.
- Saiya-Cork KR, SINSABAUGH RL, ZAK DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry*, **34**, 1309–1315.
- Talbot JM, ALLISON SD, TRESADER KK (2008) Decomposers in disguise: Mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change. *Functional Ecology*, **22**, 955–963.

Terrer C, Vicca S, Hungate BA, Phillips RP, Prentice IC (2016) Mycorrhizal association as a primary control of the CO₂ fertilization effect. *Science*, **353**, 72–74.

Waldrop MP, Zak DR, Sinsabaugh RL, Gallo M, Lauber C (2004) Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. *Ecological Applications*, **14**, 1172–1177.

Yin H, Li Y, Xiao J, Xu Z, Cheng X, Liu Q (2013) Enhanced root exudation stimulates soil nitrogen transformations in a subalpine coniferous forest under experimental warming. *Global Change Biology*, **19**, 2158–2167.

Yin H, Wheeler E, Phillips RP (2014) Root-induced changes in nutrient cycling in forests depend on exudation rates. *Soil Biology and Biochemistry*, **78**, 213–221.

Zak DR, Holmes WE, Burton AJ, Pregitzer KS, Talhelm AF (2008) Simulated atmospheric NO₃ – deposition increases organic matter by slowing decomposition. *Ecological Applications*, **18**, 2016–2027.

Chapter 2. Interactions among plants, bacteria, and fungi reduce extracellular enzyme activities under long-term N fertilization

Reprinted from

Carrara, J. E., C. A. Walter, J. S. Hawkins, W. T. Peterjohn, C. Averill, and E. R. Brzostek. 2018. Interactions among plants, bacteria, and fungi reduce extracellular enzyme activities under long-term N fertilization. *Global Change Biology* 24:2721–2734.

2.1 Abstract

Atmospheric nitrogen (N) deposition has enhanced soil carbon (C) stocks in temperate forests. Most research has posited that these soil C gains are driven primarily by shifts in fungal community composition with elevated N leading to declines in lignin degrading *Basidiomycetes*. Recent research, however, suggests that plants and soil microbes are dynamically intertwined, whereby plants send C subsidies to rhizosphere microbes to enhance enzyme production and the mobilization of N. Thus, under elevated N, trees may reduce belowground C allocation leading to cascading impacts on the ability of microbes to degrade soil organic matter through a shift in microbial species and/or a change in plant–microbe interactions. The objective of this study was to determine the extent to which couplings among plant, fungal, and bacterial responses to N fertilization alter the activity of enzymes that are the primary agents of soil decomposition. We measured fungal and bacterial community composition, root–microbial interactions, and extracellular enzyme activity in the rhizosphere, bulk, and organic horizon of soils sampled from a long-term (>25 years), whole-watershed, N fertilization experiment at the Fernow Experimental Forest in West Virginia, USA. We observed significant declines in plant C investment to fine root biomass (24.7%), root morphology, and arbuscular mycorrhizal (AM) colonization (55.9%). Moreover, we found that declines in extracellular enzyme activity were significantly correlated with a shift in bacterial community composition, but not fungal community composition. This bacterial community shift was also correlated with reduced AM fungal colonization indicating that declines in plant investment below-ground drive the response of bacterial community structure and function to N fertilization. Collectively, we find that enzyme activity responses to N fertilization are not solely driven by fungi, but instead reflect a

whole ecosystem response, whereby declines in the strength of belowground C investment to gain N cascade through the soil environment.

2.2 Introduction

Atmospheric nitrogen (N) deposition has enhanced the carbon (C) sink in both the soils and vegetation of temperate forests (Frey et al., 2014; Janssens et al., 2010; Thomas, Canham, Weathers, & Goodale, 2010). Aboveground responses to N are relatively straightforward with additional N inputs relieving N limitation to tree growth (Thomas et al., 2010). Belowground, a synthesis of data across a suite of N addition experiments showed that soil C stocks in forests are typically enhanced by N fertilization due to declines in soil respiration (Janssens et al., 2010). While this response appears robust across multiple forest types, the proximate mechanism driving these declines in soil respiration remains unclear. Given that the world's soils contain more C than the atmosphere and vegetation combined, understanding the key mechanisms driving the response of forest soil C to shifts in N input regimes is critical to predicting future rates of C sequestration (Reay, Dentener, Smith, Grace, & Feely, 2008).

Classically, shifts in fungal community composition have been viewed as the main driver of decomposition responses to enhanced N inputs (Frey, Knorr, Parrent, & Simpson, 2004; Morrison et al., 2016; Wallenstein, McNulty, Fernandez, Boggs, & Schlesinger, 2006). According to this fungal-driven view, elevated N inputs reduce the abundance of white-rot fungi (phylum *Basidiomycota*), one of the primary fungal guilds including both saprotrophs and ectomycorrhizal species that can produce oxidative enzymes capable of degrading lignin (Dix & Webster, 1995). The inability of *Basidiomycota* to compete when N limitation is removed, then leads to declines in decomposition and increased soil organic matter accumulation (Carreiro, Sinsabaugh, Repert, & Parkhurst, 2000; Fog, 1988; Waldrop, Zak, Sinsabaugh, Gallo, & Lauber, 2004; Zak, Holmes, Burton, Pregitzer, & Talhelm, 2008). In support, many studies report reduced ligninolytic enzyme activity in response to N fertilization (DeForest, Zak, Pregitzer, & Burton,

2004; Fog, 1988; Frey et al., 2014; Saiya-Cork et al. 2004, Sinsabaugh, & Zak, 2002; Zak et al., 2008). However, these enzymatic declines are not always coupled with a corresponding decline in *Basidiomycota* abundance (DeForest et al., 2004; Hassett, Zak, Blackwood, & Pregitzer, 2009). This apparent disconnect between enzyme activity and fungal community composition suggests that soil C responses to N fertilization may not solely be driven by elevated N directly inhibiting fungal growth and activity.

Emerging research suggests that enzyme activity declines in response to N fertilization may reflect an integrated ecosystem response that is driven by interactions among fungi, bacteria, and plants. Temperate forest trees allocate nearly 20% of net primary production belowground to mycorrhizae and free-living microbes in the rhizosphere which can influence microbial community composition, stimulate extracellular enzyme production and enhance plant access to N (Brzostek, Fisher, & Phillips, 2014; Hobbie, 2006; Paterson, Gebbing, Abel, Sim, & Telfer, 2007; Yin et al., 2013). When forest ecosystems are fertilized with N, trees likely reduce the C subsidies they send to rhizosphere microbes, which can alter the structure and function of not only fungal communities but also bacterial communities as well. These potential shifts in bacterial community composition are consequential given recent evidence that bacteria can synthesize enzymes to degrade simple and complex C (i.e., polyphenols, lignin) in soil organic matter (SOM; Datta et al., 2017; De Gonzalo, Colpa, Habib, & Fraaije, 2016; Jackson, Couger, Prabhakaran, Ramachandriya, & Canaan, 2017) and that shifts in bacterial function can reduce enzyme activities under N fertilization (Freedman, Upchurch, Zak, & Cline, 2016). As such, enzyme activity declines in response to N fertilization may result from parallel shifts in plant, bacterial, and fungal function; whereby reductions in plant C allocation to rhizosphere microbes indirectly alter fungal and

bacterial community composition, decrease the energy available to synthesize enzymes, and ultimately reduce the fungal and bacterial community's ability to degrade SOM.

While fungi have been shown to be important drivers of SOM increases with N fertilization (DeForest et al., 2004; Fog, 1988; Frey et al., 2014; Saiya-Cork et al., 2002; Zak et al., 2008), focusing solely on fungal responses may mask important interactions between other components of the ecosystem that can alter enzyme activity responses. Thus, the objective of this study was to examine the extent to which couplings among plant, fungal, and bacterial responses to N fertilization change extracellular enzyme activity (the proximate agent of SOM decomposition; Schimel & Weintraub, 2003) in a long-term (>25 year), watershed-scale, N fertilization experiment in West Virginia, United States. To meet this objective, we linked assays of soil enzyme activities with measurements of fungal and bacterial community composition and belowground C allocation across replicate plots in a reference and fertilized watershed, and used these measurements to parameterize a simple microbial enzyme decomposition model to examine long-term effects of N fertilization on soil C stocks.

2.3 Methods

Site description

The Fernow Experimental Forest (herein Fernow) is located in the central Appalachian Mountains near the town of Parsons, West Virginia (39.03°N, 79.67°W). Mean annual precipitation is ~1460 mm and mean annual temperature is 8.9°C. (Kochenderfer, 2006). Ambient wet N deposition at the site has been declining over time from 10.4 kg N ha⁻¹ year⁻¹ in 1989 (with an additional 4–5 kg N ha⁻¹ year⁻¹ dry deposition) to 5.0 kg N ha⁻¹ year⁻¹ in 2014 (Clean Air Status and Trends Network (Gilliam, Yurish, & Adams, 1996). Soils are derived from sandstone and shale bedrock and are classified as coarse textured Inceptisols (loamy-skeletal, mixed mesic Typic Dystrochrept) of the Berks and Calvin Series (Gilliam, Turrill, Aulick, Evans, & Adams, 1994).

To examine the impacts of N deposition on plant–microbial interactions, we sampled soils and roots from a reference watershed receiving only ambient N deposition inputs and an N-fertilized watershed at the Fernow. Both watersheds have stands of similar age (~45 years old) and tree species assemblages. Trees >2.5 cm in diameter at breast height (DBH) were cut in the N-fertilized watershed (Watershed 3) in 1970 and recovered naturally until 1989 when N treatments began (Peterjohn, Adams, & Gilliam, 2017). N is added by aerial fertilization via helicopter at a rate of 35 kg N ha⁻¹ year⁻¹ in the form of solid pellet (NH₄)₂ SO₄ (Adams, Edwards, Wood, & Kochenderfer, 1993). The N-fertilized watershed is 34.3 ha with a south-facing aspect and elevation ranging from 735 to 860 m (Gilliam et al., 2016). The reference watershed (Watershed 7) was clear cut from 1963 to 1967 (the upper half in 1963/1964 and the lower half in 1966/1977) and kept barren with herbicides until 1969 when it began natural recovery (Kochenderfer, 2006). This watershed is 24.0 ha with an east-facing aspect and elevation ranging from 731–850 m (Gilliam et al., 2016). The watersheds are dominated by arbuscular mycorrhizal associated (AM)

tree species that include tulip poplar (*Liriodendron tulipifera*), red maple (*Acer rubrum*), black cherry (*Prunus serotina*), and sugar maple (*Acer saccharum*), which together account for 59% of the basal area in the reference watershed and 72% in the N-fertilized watershed. Less abundant, ectomycorrhizal associated (ECM) tree species include sweet birch (*Betula lenta*), red oak (*Quercus rubra*), and American beech (*Fagus grandifolia*) which together account for 25% and 16% of the total basal area in the reference and N-fertilized watershed, respectively.

Soil sampling protocol

We sampled soils on three dates in June, July, and August of 2015 in order to capture early, peak, and late growing season dynamics. In each watershed, we sampled soils from an existing network of 10 randomly placed 25 × 25 m plots that have similar tree species composition (Gilliam et al., 1996). Three 10 × 10 cm organic horizon (OH) samples were collected from each plot. One OH sample was used for root metrics and the other two for measures of soil biogeochemistry. After OH removal, three 5-cm diameter soil cores were extracted from each plot to a depth of 15 cm. All samples were stored on ice and returned to West Virginia University for further processing.

Within 24 hr of sampling, mineral soil samples were separated into bulk and rhizosphere fractions. Rhizosphere soil was operationally defined as soil that adhered to roots upon removal from the soil matrix, and was carefully subsampled using forceps and homogenized (sensu Phillips & Fahey, 2005). The remaining soil was considered mineral bulk soil. After root and rhizosphere separation, the bulk and OH soil was homogenized by sieving through a 2-mm mesh. A subsample of each soil sample and fraction was stored at -80°C for assays of enzyme activity. All fine roots (<2 mm) were washed in deionized water and subsequently dried and weighed to determine standing root biomass. Subsamples of roots from each plot from the final sampling date were used to examine AM colonization and root morphology

Arbuscular mycorrhizal colonization and root morphology

To determine the extent to which N fertilization altered AM colonization, we analyzed a subsample comprising ~1/3 of all roots from the mineral and OH horizons for % AM colonization. Roots were cleared by incubating in 10% potassium hydroxide for 48–96 hr at room temperature depending on the pigmentation of the roots. Excess pigment was leached from roots by incubating in 85% ethanol for 24–48 hr. This step was necessary due to the dark pigments present in the woody tissue of our samples. Any remaining pigment was removed by soaking roots in ammonia/hydrogen peroxide solution for 15 min. Roots were prepared for staining by acidifying them in 5% hydrochloric acid, and then stained for 5 min in 0.05% trypan blue (Comas, Callahan, & Midford, 2014).

We calculated AM colonization for each sample using the grid-intersect method (Giovannetti & Mosse, 1980; McGonigle, Miller, Evans, Fairchild, & Swan, 1990). Each sample was suspended in water on a 1 × 1 cm gridded Petri dish and examined under a stereoscopic microscope. At each root-gridline intersect, colonization was determined by the presence of arbuscules or hyphae. Percent AM colonization was then calculated as the percent of total intersects examined that had visible AM structures. Given that roots with high levels of AM colonization are likely to be more involved in releasing C to rhizosphere microbes (Kaiser et al., 2015), we used the level of colonization by AM fungi as a metric of the degree to which roots and microbes were actively coupled.

We created digital images of root morphology using an LA2400 desktop scanner (WinRhizo; Regent Instruments Inc), and then used WinRhizo software to determine root length, surface area, average diameter, and number of forks of each sample. All measurements were

expressed per g of root mass and scaled to a per m² basis based on total standing root biomass (g/m²) at the plot level (Table 1-1).

Extracellular enzyme activity

For all soil fractions at each sampling date, we assayed the potential activity of hydrolytic enzymes that release nitrogen (N-Acetylglucosaminidase; NAG), phosphorus (acid phosphatase; AP), and simple carbon (β -glucosidase; BG). Given extracellular enzyme activity is the rate-limiting step of the decomposition of organic matter, we consider these measurements a metric of decomposition (Schimel & Weintraub, 2003). Soil extracellular enzyme activity has been linked to mass-loss rates of wood (Sinsabaugh et al., 1992, 1993), and soil C-mineralization (Brzostek, Greco, Drake, & Finzi, 2013; Hernández & Hobbie, 2010). After thawing, 1 g of each sample was homogenized in 50 mM sodium acetate buffer (pH 5.0). Hydrolytic enzyme activities were determined using a fluorometric microplate assay with methylumbelliferone-linked substrates. In addition, we assayed for activities of lignolytic oxidative enzymes, phenol oxidase and peroxidase, using a colorimetric microplate assay based on the hydrolysis of L-3,4-dihydroxyphenylalanine linked substrates (Saiya-Cork et al., 2002). These assays were performed under saturated substrate conditions wherein the concentration of enzyme limits activity.

We measured ambient proteolytic activity of each soil sample following a protocol adapted from Brzostek and Finzi (2011). Each subsample (2–3 g) received 10 ml of 0.05 M sodium acetate buffer (pH 5.0) and 400 μ l toluene to inhibit microbial reuptake of amino acids. Initial samples were immediately terminated by adding 3 ml of trichloroacetic acid. Incubated samples were shaken at room temperature for 4 hr prior to termination. Proteolysis was expressed as the difference between incubated and initial amino acid concentrations. Amino acid concentrations were measured following the fluorometric OPAME method (Jones, Owen, & Farrar, 2002) where

fluorescence of each sample was a function of amino acid concentration calculated from a glycine standard curve. In contrast to the other enzyme assays described above, this proteolytic enzyme assay is run at ambient substrate conditions and reflects the limitation of activity by both enzyme and substrate concentrations.

Bacterial and fungal community composition

Soil microbial DNA was extracted from samples collected in July 2015 using the Zymo soil microbe mini prep kit (Zymo Research Corporation, Irvine, CA, USA) and quantified on a Nanodrop 3300 (Thermo Scientific, Wilmington, DE, USA). Due to logistical constraints, we chose the July sampling date in order to examine microbial community composition at the peak of the growing season. The fungal ITS1 and bacterial 16S loci were chosen for amplification via polymerase chain reaction (PCR) in order to delineate microbial community composition and the corresponding shifts associated with N amendment. All PCR reactions contained 1–5 ng template DNA, 1× Mg-included Taq buffer, 0.8 mM dNTPs, 0.05 U of Taq polymerase (Denville Scientific, South Plainfield, NJ, USA), and 0.4 pM each of either ITS1-F_KYO1 (Toju, Tanabe, Yamamoto, & Sato, 2012) and ITS2 primers (Gardes & Bruns, 1993; White, Bruns, Lee, & Taylor, 1990) for fungal amplification, or S-D-Bact-0341-b-D-17 and S-D-Bact-0785-a-A-21 (Klindworth et al., 2013) for bacterial analysis. Primers were modified to include 197 unique 8 bp index sequences (for individual sample “bar-coding”) in addition to Illumina adapters and Nextera sequencing motifs (sequences available upon request). Reaction conditions were as follows: 95°C denaturation for 3 min, 30 cycles of 98°C for 20 s, 61°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. All amplification products were resolved on a 1% agarose gel, extracted with a sterile razor, and purified using the PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). Purified products were quantified via Qubit, pooled in equimolar amounts, and sequenced

in concert on the Illumina MiSeq at the West Virginia University Genomics Core Facility (Morgantown, WV).

Paired end sequences were merged and barcodes and primers trimmed using the Quantitative insights into Microbial Ecology (QIIME) pipeline, version 1.9.0 (Caporaso et al., 2010). Using a Phred score cut-off of 30, reads with less than 75% high quality base calls were excluded from further analysis. We also discarded sequences with three or more adjacent low-quality base calls. Sequences were clustered into operational taxonomic units (OTUs) using the USEARCH algorithm with an open reference OTU picking strategy using a 97% sequence similarity threshold (Edgar, 2010). Bacterial 16S sequences were clustered against the Greengenes reference database (DeSantis et al., 2006), and fungal sequences were clustered against the UNITE ITS database (Kõljalg et al., 2013). Taxonomy was assigned using the RDP × 2.2 classifier (Wang, Garrity, Tiedje, & Cole, 2007) and associated Greengenes and UNITE reference databases. To determine the effect of N fertilization on overall species composition, the top four abundant fungal phyla (*Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota* as well as ‘unclassified’) were examined for shifts in relative abundance.

To further analyze fungal and bacterial community composition, each OTU table was proportionally normalized, such that the per OTU sequence count within a sample was divided by the total number of sequences detected in a given sample generating a proportion on the interval [0,1]. This proportional normalization has been demonstrated to be sufficiently accurate when using the Bray–Curtis similarity metric (Weiss et al., 2017). We then used normalized OTU tables to calculate Bray–Curtis similarity using the `vegdist` function within the `vegan` package for R statistical software (Oksanen et al., 2015; R Core Team, 2017). Community similarity was analyzed as a function of watershed, soil horizon, and their interaction using the `adonis` function

within the vegan package. If interactions were not significant then they were removed and we only analyzed main effects.

To understand how microbial community composition related to variation in soil enzyme profiles, we created similarity matrices of soil enzymes. We calculated Bray–Curtis similarity of enzyme profiles using all enzymes using the `vegdist` function in `vegan`. We then performed nonmetric multidimensional scaling (NMDS) to generate NMDS scores for both microbial and enzymatic profiles. We tested for relationships between microbial communities and enzymatic profiles in bulk and rhizosphere soil separately by comparing the first NMDS axes of microbial and enzymatic profiles using linear regression in SAS JMP vs. 10.0.2 (SAS Institute, Cary, NC, USA). We further examined the relationship between microbial communities and enzymatic profiles across all soil horizons by comparing the first NMDS axes of microbial and enzymatic profiles using linear regression across both watersheds.

To assess the relative importance of belowground C allocation (i.e., % AM colonization) on fungal and bacterial community composition we explored whether there were correlations between % AM colonization and the first NMDS axis of bacterial and fungal community composition in bulk and rhizosphere soil separately using SAS JMP vs. 10.0.2 (SAS Institute, Cary, NC, USA).

Net mineralization and nitrification

For all soil fractions at each sampling date, we measured the rate of net N mineralization and net nitrification within 48 hours of collection. Net N mineralization and nitrification rates were expressed as the difference in the 1 M KCl extractable pool sizes of NH_4^+ and NO_3^- between an initial sample and a sample that was aerobically incubated in the lab for 14 days at room

temperature and under constant soil moisture (Finzi, Van Breemen, & Canham, 1998). To perform the extractions, 50 ml of 1 M KCl was added to a jar containing 5 g of each soil sample. Samples were sealed and shaken for 30 min and allowed to settle for 2 hr. Samples were filtered using Pall Supor® 450 filters via syringe into 20-ml vials and stored at -20°C prior to analysis by flow injection (Lachat QuikChem® 8500 series 2).

Statistical analysis

To determine the extent to which N fertilization altered enzyme activities, N mineralization, nitrification, and root metrics, we performed a three-way analysis of variance (ANOVA) in SAS JMP vs. 10.0.2 (SAS Institute, Cary, NC, USA) and used date, watershed, and soil horizon as factors. Although we included date in our statistical models, we present growing season means in the results reflecting our focus on treatment rather than seasonal effects. Post hoc multiple comparisons were also made among watersheds, dates, and soil fractions using the Tukey–Kramer HSD test.

Modelling potential impacts of plant-microbial linkages on soil decomposition

To further examine the extent to which linkages between plants and soil microbes drive soil enzyme responses to N fertilization as well as to estimate potential impacts on soil C stocks, we used a microbial enzyme decomposition model (Allison, Wallenstein, & Bradford, 2010) that was modified in Cheng et al. (2014) to include seasonal differences in soil temperature, the inputs of root and leaf litter, and root transfers of C to the rhizosphere. For leaf and root litter inputs, we used the mean observed litterfall rate from 1998 to 2015 for each watershed (Kochenderfer, 2006; Peterjohn et al., 2017) and our own measurements of root biomass with the assumption that roots in both watersheds turnover once each year (McCormack, Adams, Smithwick, & Eissenstat, 2017). For both roots and leaves, we assumed that turnover primarily occurred during the leaf

senescence in the fall. It is important to note there are no significant differences in leaf litterfall rates between the two watersheds (WS3 = 143 g C/m²; WS7 = 146 g C/m²; Kochenderfer, 2006). Finally, we used published values of AM root exudation rates on a per g root basis (Yin, Wheeler, & Phillips, 2014) to estimate root C transfers, assuming that these inputs occurred only during the growing season. Using these model inputs, we then ran two scenarios: (1) the fertilized and control watersheds had similar root C exudation rates per g of root, and (2) N fertilization reduced exudations rates by 25%. We chose this reduction in exudation rates to be conservative as it represents around half of the decline in AM colonization we observed in the fertilized watershed. All model simulations were run for 30 year to achieve steady state.

2.4 Results

Fine root metrics

Fine root biomass and the extent of mycorrhizal symbiosis in the bulk mineral soil layer were significantly lower in the N-fertilized watershed (Table 2-1). Overall, the bulk mineral soil in the N-fertilized watershed had 24.7% less standing fine root biomass than the reference watershed. This reduction in fine root biomass was accompanied by decreases in fine root surface area, volume, and the amount of root forks per g. Additionally, N fertilization reduced AM colonization of fine roots by 55.9%. Given the inherent limitations of measuring root production using minirhizotron tubes or ingrowth cores, we use fine root biomass and AM colonization as proxies for plant C allocation to gain nutrients. While root turnover may be enhanced under N fertilization, we base the assumption that fine root biomass is synonymous with belowground C allocation to fine roots on our observations that fine root biomass was consistently lower in the fertilized than control watersheds over all three of the sequentially cored soil sampling dates during the growing season. In addition, AM fungi have been shown to act as conduits for photosynthate C to rhizosphere microbes and as such, represent an important belowground flux of C (Kaiser et al., 2015). Thus, reduced investment in AM colonization may also lead to reduced transfers of photosynthate C to the rhizosphere resulting in an overall decline in belowground C allocation.

By contrast, in the OH, standing fine root biomass was larger in the N-fertilized watershed although root biomass in the OH horizon is markedly lower than in the bulk mineral soil (Table 2-1). However, like the bulk mineral soil, AM colonization of OH roots was significantly higher in the reference watershed (72.8% colonized) than the N-fertilized watershed (60.9% colonized).

Extracellular enzyme activity

Nearly all hydrolytic enzyme activities were lower under elevated N conditions across all three soil fractions. BG activity was significantly lower in the N-fertilized watershed in both the bulk mineral (52% decrease) and OH (17% decrease) soil fractions ($p < .05$, Figure 2-1a). N fertilization significantly lowered AP activity in the bulk (12%) and rhizosphere (36%) fractions of the mineral soil ($p < .05$, Figure 2-1b). Similarly, N amendment significantly lowered NAG activity in both bulk and rhizosphere fractions of the mineral soil by 41 and 37%, respectively ($p < .05$, Figure 2-1c). However, BG activity in the rhizosphere of the mineral soil was not significantly different between watersheds. In addition, AP and NAG activities in the OH horizon did not vary by treatment.

Lignolytic oxidative enzyme activities were also generally lower in the N-fertilized watershed. Phenol oxidase activity was significantly lower in bulk (45% decrease), rhizosphere (49% decrease), and OH horizons (57% decrease) of the fertilized watershed relative to the reference watershed ($p < .05$ Figure 2-1d). Peroxidase activity was lower in the fertilized watershed in the bulk, rhizosphere, and OH soil fractions with reductions of 30, 25, and 36%, respectively ($p < .05$ Figure 2-1e). Proteolytic enzyme activity was consistently lower in each soil fraction under N fertilization, with a 48% decrease in bulk, 56% decrease in rhizosphere, and 40% decrease in OH proteolysis (Figure 2-1f).

Microbial community composition

When fungal taxonomic units were aggregated to the phylum level, there were no significant changes in the relative abundance of the four most common fungal phyla or the unclassified group in any soil horizon (Figure S2-1). When bacterial taxonomic units were aggregated to the phylum level, there were limited shifts in the relative abundance of the seven most common bacterial phyla. Relative abundance of *Actinobacteria* was higher in the OH soil of

the fertilized watershed (Figure S2-2a). Relative abundance of *Proteobacteria* was lower in the fertilized bulk soil and relative abundance of *Firmicutes* was higher in both rhizosphere and bulk soil in the fertilized watershed (Figure S2b,c).

Adonis analysis of bacterial communities revealed significant effects for watershed, soil horizon ($p < .001$), and their interaction ($p = .02$, total model $R^2 = .32$). Within watersheds, post hoc comparisons showed OH communities were different than bulk and rhizosphere communities (Figure S2-3a,b). There was no difference between bulk and rhizosphere communities (Figure S2-3a,b). Across watersheds, bacterial community composition differed in all soil fractions such that OH, rhizosphere and bulk soil exhibited unique communities in the N-fertilized watershed compared to the reference (Figure 2-2a,c,e).

Adonis analysis of fungal communities revealed significant effects of watershed and soil horizon ($p < .001$, total model $R^2 = .12$), but not their interaction. Within watersheds, post hoc comparisons of fungal communities showed OH communities were different than bulk and rhizosphere communities in both watersheds, but there was no difference between bulk and rhizosphere communities (Figure S2-3c,d). Across watersheds, fungal community composition within OH and bulk soil fractions was significantly different ($p < .01$; Figure 2-2b,d). However, there was no significant difference in fungal communities between watersheds in rhizosphere soil (Figure 2-2f).

Comparison of bacterial and enzymatic NMDS scores across all soil horizons showed a positive relationship between bacterial communities and enzyme profiles ($R^2 = .48$, $p < .01$; Figure S2-4a). Comparison of fungal and enzymatic NMDS scores across all soil horizons and both watersheds showed no significant relationship between fungal communities and enzyme profiles (Figure S2-4b). Across watersheds, comparison of bacterial and enzymatic NMDS scores showed

a negative relationship between bacterial communities and enzyme profiles in the bulk soil ($R^2 = .36$, $p < .05$; Figure 2-3a), but no significant relationship in rhizosphere soil. Comparison of fungal and enzymatic NMDS scores across watersheds showed no significant relationships in either bulk or rhizosphere soil (Figure 2-3b). Linear regression of % AM colonization and the first bacterial NMDS axis resulted in a positive linear relationship in bulk soil ($R^2 = 0.63$, $p < .001$; Figure 2-3c), but not rhizosphere soil. There were no significant linear relationships between % AM colonization and the first fungal NMDS axis in either soil fraction (Figure 2-3d).

Net N mineralization and nitrification

The only significant difference we found in rates of nitrogen cycling was that N mineralization and net nitrification in the OH were 40% and 51% higher in the fertilized watershed than the reference. N transformation rates did not significantly differ between watersheds in either the bulk or rhizosphere soil fractions (Figure 2-4a,b).

Modelling the potential impacts of plant-microbial linkages on soil decomposition

When we ran the model to steady state, we found that reductions in root biomass and root C transfers to the rhizosphere in the N-fertilized watershed reduced microbial enzyme pools by ~16% and enhanced soil C by ~3% compared to the reference watershed (Table 2-2). Even though exudation rates on a per g root basis were the same, the fertilized watershed had lower overall exudations rates at the ecosystem scale than the control watershed because of its lower root biomass (Table 2-2). When we reduced exudation rates by 25% on a per g root basis in the fertilized watershed, there was a further exacerbation in the reduction in microbial enzyme activity to a ~28% decline and a larger increase in soil C by ~20% (Table 2-2).

2.5 Discussion

Here we provide evidence that coupled interactions among plants, fungi, and bacteria play an important role in enzyme activity responses to N fertilization. For fungi, we observed distinct shifts in fungal community structure in response to N fertilization (Figure 2-2b, d), but we found no evidence for a link between these shifts and extracellular enzyme activity (Figure 2-3b). By contrast, we found that bacterial community composition shifts under N fertilization are correlated with declines in enzyme activity (Figure 2-3a) and that these compositional shifts are tightly coupled to reductions in plant C allocation to roots and mycorrhizal fungi (Figure 2-3c). Overall, these results suggest that N fertilization drives an integrated ecosystem response whereby reductions in plant C allocation to roots and AM fungi feedback on bacterial community structure and function.

While whole-watershed fertilization at the Fernow results in a pseudoreplicated experimental design (Hurlbert, 1984), we conclude that the effects we measured are driven by N fertilization, rather than pre-existing differences between these adjacent watersheds, for four main reasons. First, soil chemistry (i.e., soil pH, cation exchange capacity, nutrient content, etc.) were similar at the beginning of the experiment (Adams & Angradi, 1996). Second, the amount of N added to the watershed yearly was originally chosen in 1989 to approximately double ambient N deposition rates, but is now more than quadruple current rates and as such, it seems unlikely that this does not incur a biogeochemical response. Third, the results from this watershed study are consistent with measurements we made during the same year in a replicated N fertilization study <2 km away from these watersheds (the Fork Mountain Long-Term Soil Productivity Plots; Adams, Burger, Zelazny, & Baumgras, 2004). In this small-scale replicated study, N fertilization reduced fine root biomass and ligninolytic enzyme activity (Figure S2-5). Finally, this

work builds upon other research at the Fernow that has found the N-fertilized watershed had lower rates of litter decomposition, (Adams & Angradi, 1996), reduced understory richness (Gilliam et al., 1994, 2016; Walter, Adams, Gilliam, & Peterjohn, 2017), and altered N cycling (Adams et al., 1993; Burnham, Cumming, Adams, & Peter-john, 2017; Gilliam, Yurish, & Adams, 2001; Gilliam et al., 1996, 2016) compared to the reference watershed.

While we did observe significant declines in enzyme activity across all three soil fractions, particularly for the lignolytic enzymes (Figure 2-1a–f), these declines were not correlated with significant shifts in fungal community composition (Figure 2-3b). The lack of a clear link between enzyme declines and changes in the fungal community does not support the prevailing paradigm that white-rot *Basidiomycota*, are the dominant cause of a decline in enzyme activities following N additions (Edwards, Zak, Kellner, Eisenlord, & Pregitzer, 2011; Fog, 1988; Freedman, Romanowicz, Upchurch, & Zak, 2015; Morrison et al., 2016). It is possible that fungal enzyme activity response to N fertilization is independent of community composition (i.e., investment in enzyme activity declines with no change in community structure); however, microbial community composition has been linked to catabolic functioning and enzyme activities across N gradients and seasons (Fierer et al., 2011; Voriskova, Brabcova, Cajthaml, & Baldrian, 2014). Furthermore, our data indicate that shifts in bacterial community composition under elevated N are correlated with reduced enzyme activity at the Fernow (Figure 2-3a). While changes in bacterial community may be influenced by overall fungal community composition, these shifts in bacterial community composition are tightly coupled to %AM colonization (a metric of belowground C allocation) suggesting that plant responses to N fertilization feedback on bacterial community structure and function (Figure 2-3c). While we cannot rule out that N-induced declines in total fungal biomass led to reductions in lignolytic enzyme activity (DeForest et al., 2004; Frey et al., 2004; Treseder,

2008; Wallenstein et al., 2006), the dominance of AM trees in our plots, whose inorganic nutrient economy is largely driven by bacteria, suggests that free-living fungi may not be an important driver of N deposition responses in AM-dominated systems (Cheeke et al., 2017; Phillips, Brzostek, & Midgley, 2013).

In contrast, it appears that the declines in enzyme activity we observed appear to be the result of a cascade of ecosystem responses affecting both microbial community composition and plant C allocation belowground. The N-induced declines in fine root biomass, AM colonization, and root morphology all indicate that there was a reduction in the investment of C belowground by trees to gain nutrients (Table 2-1). While previous research has shown that below-ground C allocation is inversely correlated with N availability (Bae, Fahey, Yanai, & Fisk, 2015; Haynes & Gower, 1995; Phillips & Fahey, 2005), the tight linkage between the first bacterial NMDS axis and %AM colonization suggest that N-induced declines in belowground C allocation caused a shift in the bacterial community composition and subsequent reductions in enzyme activity (Figure 2-3a,c). This result also supports the emerging view that roots are active engineers of microbial activity (Brzostek et al., 2013; Finzi et al., 2015; Yin et al., 2014), and highlights the need to integrate root-microbial interactions into our conceptual and predictive modeling frameworks of how forest ecosystems respond to global change (Johnson, Gehring, & Jansa, 2016; Terrer, Vicca, Hungate, Phillips, & Prentice, 2016).

An additional impact of the N fertilization treatment is that it reduced soil pH (as measured in a 0.01 M calcium chloride buffer) of the upper 5 cm of mineral soil in the fertilized watershed to 3.4 compared to 3.8 in the control watershed (Peterjohn, *unpublished data*). Soil pH is an important control on microbial community diversity (Fierer et al., 2012; Kaiser et al., 2016; Lauber, Hamady, Knight, & Fierer, 2009), microbial biomass, and enzyme activities (Rousk

& Bååth, 2011; Sinsabaugh, 2010). Thus, the decline in soil pH may account for a portion of the enzyme reductions we observed under N fertilization. To address this, we assayed the sensitivity of phenol oxidase and peroxidase enzyme activity in organic horizon, bulk and rhizosphere soils from the control watershed to three different levels of pH that spanned a 1 unit shift (data not shown). Peroxidase activity was insensitive to pH (slope = -0.74 , $r^2 = .05$, $p > .05$). While phenol oxidase activity did significantly increase as pH increased (slope = 0.10 , $r^2 = 0.53$, $p < .05$), this sensitivity would only account for a 10% decline in activity. Given that we observed a 50% decline in phenol oxidase in the treatment watershed coupled with the greater overall importance of peroxidase enzymes in our study (i.e., nearly an order of magnitude higher activity), it appears that pH is an important, but secondary driver of the enzyme activity responses we observed.

Over longer time scales, the reductions in root and microbial activity we observed at the Fernow may have important implications for soil C stocks. In our model simulation, we found that feedbacks between reductions in root biomass and enzyme production have the potential to drive nearly a 3% increase in soil C stocks (Table 2-2). When these were coupled with a 25% reduction in specific root C exudation rates, soil C in the fertilized watershed increased by nearly 20% over the 30-yr simulation. This model was designed to be theoretical and as such, it is used here to show the sensitivity of the system to the perturbations in plant inputs and is not meant to be quantitatively predictive. However, this simple modeling effort provides compelling evidence that N-induced shifts in root–microbial interactions have the potential to alter not only microbial production of soil enzymes but also the size of the soil C pool. To generate the necessary data for fully integrating root-microbial interactions into more sophisticated ecosystem models, future research should couple direct measurements of belowground C allocation responses to N fertilization (e.g., total belowground C allocation, root exudation) with the resulting impacts on microbial activity.

Given the prevailing paradigm that free-living fungi drive soil enzyme activity and subsequent decomposition responses to N fertilization (Carreiro et al., 2000; Fog, 1988; Waldrop et al., 2004; Zak et al., 2008), our results raise an important question of why shifts in plant C allocation, bacterial community structure and enzyme activities were tightly coupled at the Fernow. The divergent results between the Fernow and other long-term fertilization sites may be the result of differences in ambient N deposition and tree species composition. The Fernow is in a region that has historically received some of the highest levels of N deposition in the United States (Driscoll et al., 2001), which may have enhanced bacterial dominance before treatment began in 1989. By contrast, N fertilization experiments that have linked reductions in soil C decomposition to fungal community composition and activity tend to be located in areas with lower historical N deposition rates (Edwards et al., 2011; Freedman et al., 2015; Frey et al., 2004; Morrison et al., 2016; Wallenstein et al., 2006). Second, the Fernow is predominantly dominated by AM trees; whereas most N fertilization experiments have tended to occur in ecosystems dominated by ECM trees, with ECM-dominated sites comprising ~80% of the studies included in the Janssens et al. (2010) meta-analysis. AM trees show greater growth enhancements with N addition (Thomas et al., 2010), are characterized by lower fungal to bacterial ratios (Cheeke et al., 2017), and are less dependent on plant–microbial interactions to access N than ECM trees (Brzostek, Dragoni, Brown, & Phillips, 2015). As such, in AM-dominated systems like the Fernow, bacteria may outweigh fungi in controlling soil decomposition responses to N fertilization. Additionally, evidence that ECM trees rely more on roots and rhizosphere microbes to access N than AM trees suggests that N fertilization may lead to greater declines in belowground C allocation and enzyme activity than observed at the Fernow (Brzostek et al., 2015; Yin et al., 2014). Moving forward, the equal distribution of ECM and AM trees across the temperate forest landscape highlights a critical need

to investigate ECM and AM stand responses to N fertilization within the same ecosystem (Midgley & Phillips, 2014).

Although enzyme declines were observed across all three soil fractions (Figure 2-1a–f), N cycling shifts were only observed in the OH, where rates were elevated by nearly 50% and 40% for mineralization and nitrification, respectively (Figure 2-4a,b). OH and mineral soils differ in key biogeochemical traits and this may contribute to these divergent N cycling responses. In temperate forests, OH typically have rapid N cycling rates reflecting greater organic matter content and root densities than mineral soils (Brzostek & Finzi, 2011). Shifts in the ratio of gross immobilization to gross mineralization may also play a role. Net N mineralization rates increased in the OH despite N fertilization induced declines in proteolytic and chitinolytic enzyme activity that produce N monomers for microbial uptake. While we do not have data on gross N cycling, we hypothesize that declines in gross microbial N immobilization due to reduced N demand may have outpaced declines in gross N mineralization in the fertilized watershed. Regardless of the exact mechanism, it is important to put the OH results into context: on a mass per unit area basis, the OH is less important to total ecosystem N cycling than the underlying mineral soil.

Much of the research on soil N fertilization has focused on aboveground responses such as litter input and quality or fungal community shifts to explain widely observed reductions in enzyme activities and subsequent soil decomposition (Edwards et al., 2011; Frey et al., 2004; Morrison et al., 2016; Sinsabaugh et al., 2008; Waldrop et al., 2004; Wallenstein et al., 2006). Our results provide evidence that enzyme activity declines under long-term N fertilization at the Fernow are driven by a cascade of ecosystem responses whereby reductions in belowground plant C investment lead to shifts in bacterial community structure and a decline in their ability to degrade soil organic matter. Thus, there is a need to integrate plant–microbial interactions into our

current conceptual and predictive models of N deposition impacts on temperate forests in order to forecast soil C responses to shifting N deposition regimes.

2.6 Tables and Figures

Table 2-1. Fine root and mycorrhizal metrics in bulk and organic horizon soils under ambient and long-term N fertilized conditions. Mean values of all sampling dates are accompanied by standard error. Asterisks indicate significant differences between treatments ($p < 0.05$).

Fine Root Metric	Control	+N	% Decrease
Mineral Soil			
Root Biomass (g m ⁻²)	319.23 ± 26.31	240.51 ± 48.20	24.7*
Mycorrhizal Colonization (%)	75.29 ± 5.79	33.15 ± 6.70	55.9*
Specific Root Length (cm g ⁻¹)	1240.57 ± 95.98	976.58 ± 93.93	21.3
Surface Area (cm ² g ⁻¹)	167.13 ± 8.81	125.25 ± 8.47	25.1*
Average Diameter (mm)	0.44 ± 0.022	0.43 ± 0.022	6.5
Root Volume (cm ³ g ⁻¹)	1.84 ± 0.12	1.30 ± 0.57	29.4*
Forks/g ⁻¹	2598.14 ± 351.45	2090.80 ± 182.56	19.5*
Organic Horizon			
Root Biomass (g/m ⁻²)	30.83 ± 4.07	52.13 ± 8.50	-69.1*
Mycorrhizal Colonization (%)	72.80 ± 3.66	60.95 ± 0.01	16.3*
Specific Root Length (cm g ⁻¹)	2038.69 ± 260.99	1992.99 ± 179.60	2.24
Surface Area (cm ² g ⁻¹)	51.88 ± 5.32	85.63 ± 28.22	-25.1
Average Diameter (mm)	0.44 ± 0.022	0.43 ± 0.022	4.2
Root Volume (cm ³ g ⁻¹)	1.85 ± 0.10	1.85 ± 0.17	-0.24
Forks/g ⁻¹	6026.61 ± 738.76	5967.27 ± 909.92	0.98

Table 2-2. At model steady state, fertilization reduced microbial enzyme pools by ~16% and enhanced soil C by ~3%. When root C exudation was reduced 25% on a per g root basis in the fertilized watershed, there was a further exacerbation in the reduction in microbial enzyme activity to a ~28% decline and a larger increase in soil C by ~20%.

Watershed	Exudation	Enzyme Pool	Soil C pool
		(mg cm⁻³)	(mg cm⁻³)
Control	No change	0.0136	184.73
Fertilized	No change	0.0114	190.92
	25% reduction	0.0097	221.16

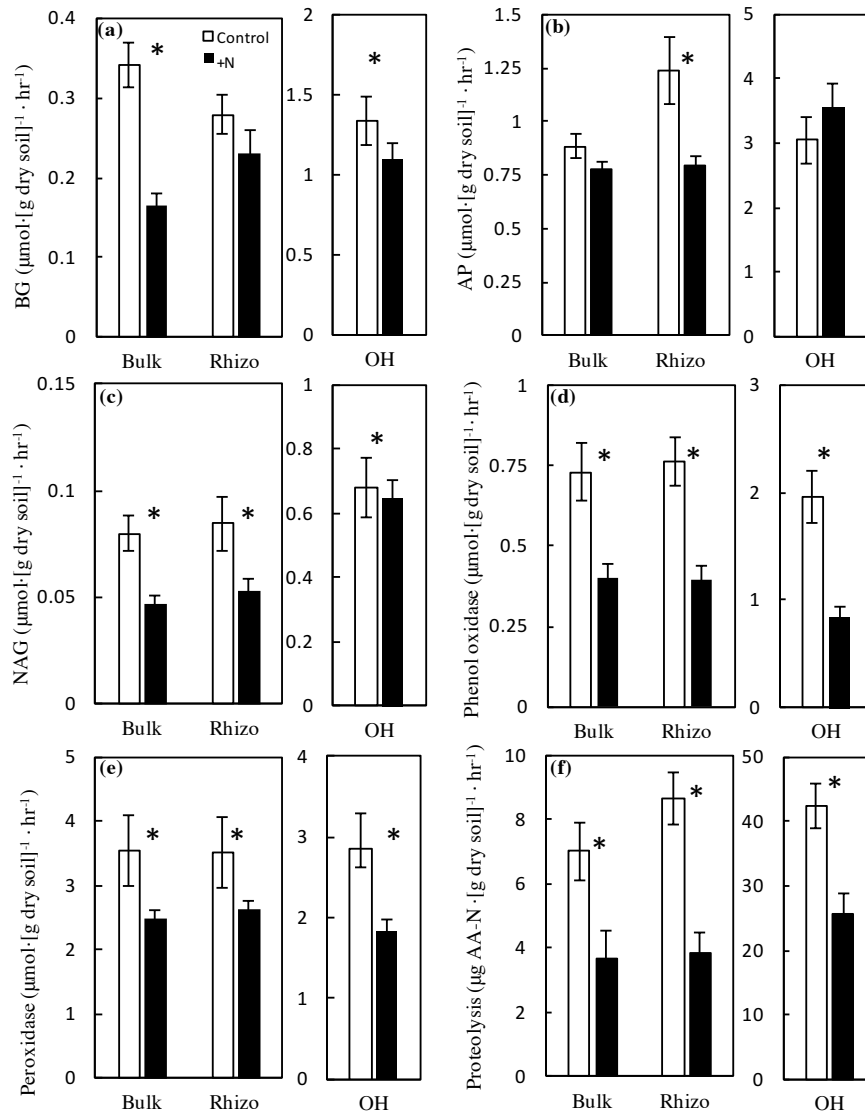


Figure 2-1. N fertilization reduces hydrolytic and oxidative enzyme activities. Values are the overall seasonal mean enzyme activities (mean \pm SE) of (a) BG, (b) AP, (c) NAG, (d) phenol oxidase, (e) peroxidase, and (f) proteolysis for each soil fraction (i.e., bulk, rhizosphere, and organic horizon) measured in June, July, and August 2015 across all plots (n = 10 plots per watershed). Asterisks indicate significant differences in enzyme activity between watersheds within soil fractions (p < .05). Note difference in scale between OH vs. rhizosphere and bulk soil fractions. AA-N is amino acid nitrogen

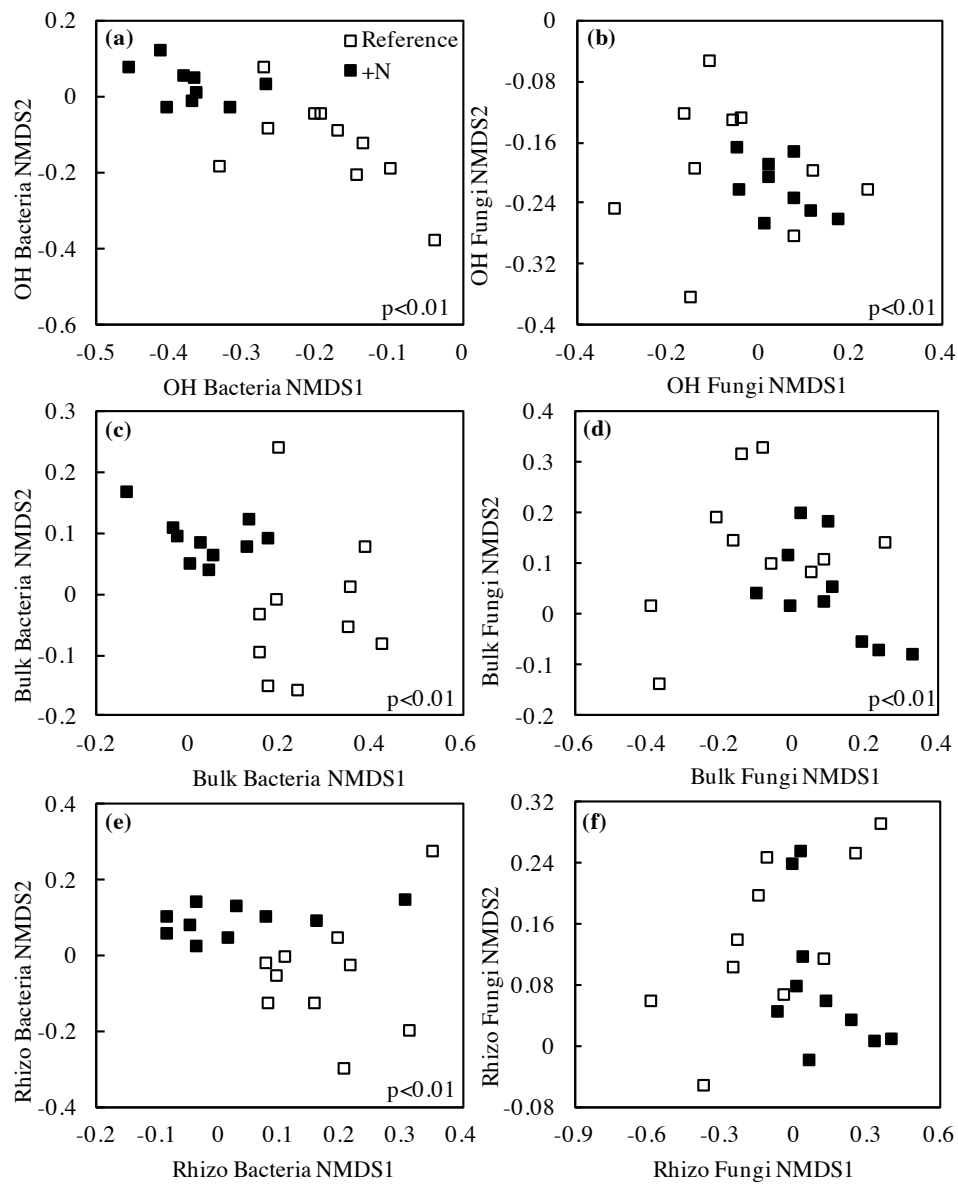


Figure 2-2. N fertilization altered bacterial community composition in OH (a), bulk (c), and rhizosphere (e) soils and fungal community composition in OH (b) and bulk (d) soils, but not rhizosphere (f). All community data were obtained for each soil fraction in July 2015 ($n = 10$ plots per watershed). p values indicate significant differences between the N-fertilized and reference community

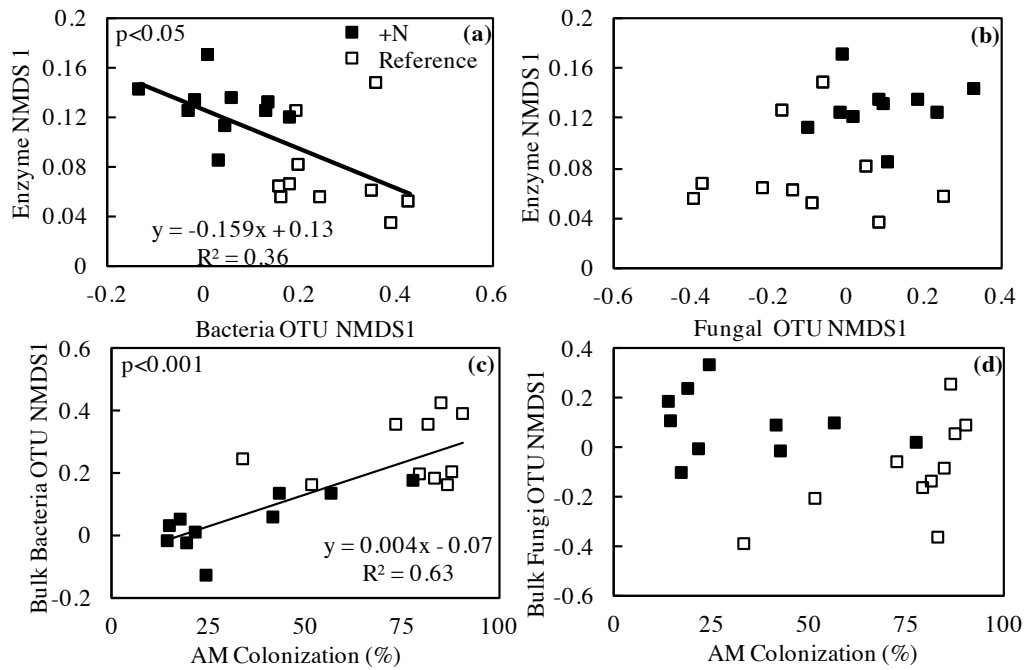


Figure 2-3. Bacterial, but not fungal community composition is correlated with the first NMDS axis of enzyme activity (a & b). Percent AM colonization is correlated with bacterial community composition (c), but not fungal community composition (d). Data presented are bulk soil community composition data from July 2015 (n = 10 plots per watershed). p values indicate significance of correlation.

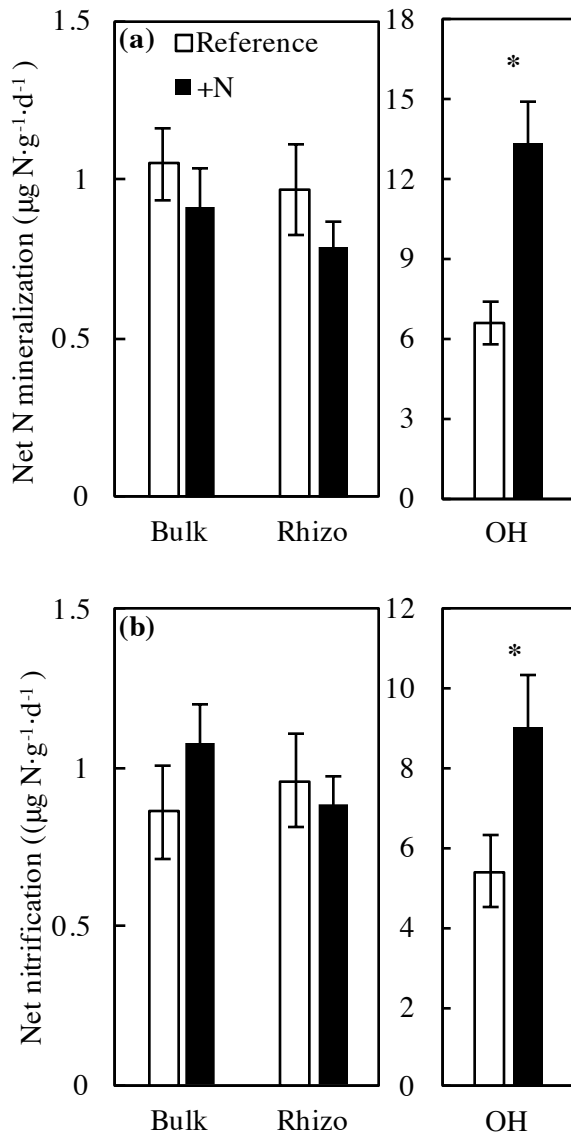


Figure 2-4. N fertilization increases (a) N mineralization and (b) nitrification in the OH but not the mineral soil. Values are the overall watershed-level mean rates (mean \pm SE) measured in June, July, and August 2015 for each soil fraction (n = 10 plots per watershed). Asterisks indicate significant differences between treatments (p < .05)

2.7 Literature Cited

- Adams MB, Edwards PJ, Wood F, Kochenderfer JN (1993) Artificial watershed acidification on the Fernow Experimental Forest, USA. *Journal of Hydrology*, 150, 505–519.
- Allison SD, Wallenstein MD, Bradford MA (2010) Soil-carbon response to warming dependent on microbial physiology. *Nature Geoscience*, 3, 336–340.
- Bae K, Fahey TJ, Yanai RD, Fisk M (2015) Soil Nitrogen Availability Affects Belowground Carbon Allocation and Soil Respiration in Northern Hardwood Forests of New Hampshire. *Ecosystems*, 18, 1179–1191.
- Brzostek ER, Finzi AC (2011) Substrate supply, fine roots, and temperature control proteolytic enzyme activity in temperate forest soils. *Ecology*, 92, 892–902.
- Brzostek ER, Greco A, Drake JE, Finzi AC (2013) Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. *Biogeochemistry*, 115, 65–76.
- Brzostek ER, Fisher JB, Phillips RP (2014) Modeling the carbon cost of plant nitrogen acquisition: Mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. *Journal of Geophysical Research: Biogeosciences*, 119, 1684–1697.
- Brzostek ER, Dragoni D, Brown ZA, Phillips RP (2015) Mycorrhizal type determines the magnitude and direction of root-induced changes in decomposition in a temperate forest. *New Phytologist*, 206, 1274–1282.
- Brzostek ER, Rebel KT, Smith KR, Phillips RP (2017) Chapter 26 – Integrating Mycorrhizas Into Global Scale Models: A Journey Toward Relevance in the Earth’s Climate System. In:

Mycorrhizal Mediation of Soil, pp. 479–499.

Caporaso JG, Kuczynski J, Stombaugh J et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335–336.

Carreiro MM, Sinsabaugh RL, Repert DA, Parkhurst DF (2000) Microbial Enzyme Shifts Explain Litter Decay Responses To Simulated Nitrogen Deposition. *Ecology*, 81, 2359–2365.

Cheeke TE, Phillips RP, Brzostek ER, Rosling A, Bever JD, Fransson P (2017) Dominant mycorrhizal association of trees alters carbon and nutrient cycling by selecting for microbial groups with distinct enzyme function. *New Phytologist*, 214, 432–442.

Cheng W, Parton WJ, Gonzalez-Meler MA et al. (2014) Synthesis and modeling perspectives of rhizosphere priming. *New Phytologist*, 201, 31–44.

Comas LH, Callahan HS, Midford PE (2014) Patterns in root traits of woody species hosting arbuscular and ectomycorrhizas: Implications for the evolution of belowground strategies. *Ecology and Evolution*, 4, 2979–2990.

DeForest JL, Zak DR, Pregitzer KS, Burton AJ (2004) Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal*, 68, 132–138.

DeSantis TZ, Hugenholtz P, Larsen N et al. (2006) Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology*, 72, 5069–5072.

Dix NJ, Webster J (1995) Colonization and Decay of Wood. In: *Fungal Ecology*, pp. 145–171. Springer Netherlands, Dordrecht.

- Driscoll CT, Lawrence GB, Bulger AJ et al. (2001) Acidic Deposition in the Northeastern United States : Sources and Inputs , Ecosystem Effects , and Management Strategies.
- Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26, 2460–2461.
- Edwards IP, Zak DR, Kellner H, Eisenlord SD, Pregitzer KS (2011) Simulated atmospheric N deposition alters fungal community composition and suppresses ligninolytic gene expression in a Northern Hardwood forest. *PLoS ONE*, 6.
- Finzi a C, Van Breemen N, Canham CD (1998) Canopy tree soil interactions within temperate forests: Species effects on soil carbon and nitrogen. *Ecological Applications*, 8, 440–446.
- Finzi AC, Abramoff RZ, Spiller KS, Brzostek ER, Darby BA, Kramer MA, Phillips RP (2015) Rhizosphere processes are quantitatively important components of terrestrial carbon and nutrient cycles. *Global Change Biology*, 21, 2082–2094.
- Fog K (1988) The effect of added nitrogen on the rate of decomposition of organic matter. *Biological Reviews*, 63, 433–462.
- Freedman ZB, Romanowicz KJ, Upchurch RA, Zak DR (2015) Differential responses of total and active soil microbial communities to long-term experimental N deposition. *Soil Biology and Biochemistry*, 90, 275–282.
- Freedman ZB, Upchurch RA, Zak DR, Cline LC (2016) Anthropogenic N Deposition Slows Decay by Favoring Bacterial Metabolism: Insights from Metagenomic Analyses. *Frontiers in Microbiology*, 7, 1–11.
- Frey SD, Knorr M, Parrent JL, Simpson RT (2004) Chronic nitrogen enrichment affects the

- structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management*, 196, 159–171.
- Frey SD, Ollinger S, Nadelhoffer K et al. (2014) Chronic nitrogen additions suppress decomposition and sequester soil carbon in temperate forests. *Biogeochemistry*, 121, 305–316.
- Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2, 113–118.
- Gilliam FS, Turrill NL, Aulick SD, Evans DK, Adams MB (1994) Herbaceous Layer and Soil Response to Experimental Acidification in a Central Appalachian Hardwood Forest. *Journal of Environmental Quality*, 23, 835–844.
- Gilliam FS, Yurish BM, Adams MB (1996) Ecosystem nutrient responses to chronic nitrogen inputs at Fernow Experimental Forest, West Virginia. *Canadian Journal of Forest Research*, 26, 196–205.
- Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist*, 84, 489–500.
- Hassett JE, Zak DR, Blackwood CB, Pregitzer KS (2009) Are basidiomycete laccase gene abundance and composition related to reduced lignolytic activity under elevated atmospheric NO₃- Deposition in a northern hardwood forest? *Microbial Ecology*, 57, 728–739.
- Haynes BE, Gower ST (1995) Belowground carbon allocation in unfertilized and fertilized red pine plantations in northern Wisconsin. *Tree Physiology*, 15, 317–325.
- Hobbie EA (2006) Carbon allocation to ectomycorrhizal fungi correlates with belowground

allocation in culture studies. *Ecology*, 87, 563–569.

Högberg MN, Högberg P, Högberg MN (2002) Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces , together with associated roots , half the dissolved organic carbon in a forest soil. *New Phytologist*, 154, 791–795.

Jones DL, Owen AG, Farrar JF (2002) Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biology and Biochemistry*, 34, 1893–1902.

Kaiser C, Kilburn MR, Clode PL et al. (2015) Exploring the transfer of recent plant photosynthates to soil microbes: Mycorrhizal pathway vs direct root exudation. *New Phytologist*, 205, 1537–1551.

Kochenderfer JN (2006) Fernow and the appalachian hardwood region. The Fernow watershed acidification study. Springer, The Netherlands, pp 17–39

Kõljalg U, Nilsson RH, Abarenkov K et al. (2013) Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, 22, 5271–5277.

Mccormack ML, Adams TS, Smithwick EAH et al. (2017) Variability in root production , phenology , and turnover rate among 12 temperate tree species. *Ecology*, 95, 2224–2235.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A New Method which Gives an Objective Measure of Colonization of Roots by Vesicular- Arbuscular Mycorrhizal Fungi. *New Phytologist*, 115, 495–501.

Midgley MG, Phillips RP (2014) Mycorrhizal associations of dominant trees influence nitrate leaching responses to N deposition. 241–253.

- Midgley MG, Phillips RP (2016) Resource stoichiometry and the biogeochemical consequences of nitrogen deposition in a mixed deciduous forest. *Ecology*, 97, 3369–3377.
- Morrison EW, Frey SD, Sadowsky JJ, van Diepen LTA, Thomas WK, Pringle A (2016) Chronic nitrogen additions fundamentally restructure the soil fungal community in a temperate forest. *Fungal Ecology*, 23, 48–57.
- Peterjohn WT, Adams MB, Gilliam FS (2017) Symptoms of Nitrogen Saturation in Two Central Appalachian Hardwood Forest Ecosystems. *Biogeochemistry*, 35, 507–522.
- Phillips RP, Fahey TJ (2005) Patterns of rhizosphere carbon flux in sugar maple (*Acer saccharum*) and yellow birch (*Betula allegheniensis*) saplings. *Global Change Biology*, 11, 983–995.
- Phillips RP, Brzostek E, Midgley MG (2013) The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist*, 199, 41–51.
- Quinn Thomas R, Canham CD, Weathers KC, Goodale CL (2010) Increased tree carbon storage in response to nitrogen deposition in the US. *Nature Geoscience*, 3, 13–17.
- Reay DS, Dentener FJ, Smith P, Grace J, Feely RA (2008) Global nitrogen deposition and carbon sinks. *Nature Geoscience*, 1, 430–437.
- Rousk J, Bååth E (2011) Growth of saprotrophic fungi and bacteria in soil. *FEMS Microbiology Ecology*, 78, 17–30.
- Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry*, 34, 1309–1315.

- Sinsabaugh RL (2010) Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry*, 42, 391–404.
- Sinsabaugh RL, Lauber CL, Weintraub MN et al. (2008) Stoichiometry of soil enzyme activity at global scale. *Ecology Letters*, 11, 1252–1264.
- Terrer C, Vicca S, Hungate BA, Phillips RP, Prentice IC (2016) Mycorrhizal association as a primary control of the CO₂ fertilization effect. *Science*, 353, 72–74.
- Toju H, Tanabe AS, Yamamoto S, Sato H (2012) High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. *PLoS ONE*, 7.
- Treseder KK (2008) Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. *Ecology Letters*, 11, 1111–1120.
- Waldrop MP, Zak DR, Sinsabaugh RL, Gallo M, Lauber C (2004) Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. *Ecological Applications*, 14, 1172–1177.
- Wallenstein MD, McNulty S, Fernandez IJ, Boggs J, Schlesinger WH (2006) Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. *Forest Ecology and Management*, 222, 459–468.
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology*, 73, 5261–5267.
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics. *PCR Protocols*, 315–322.

Yin H, Li Y, Xiao J, Xu Z, Cheng X, Liu Q (2013) Enhanced root exudation stimulates soil nitrogen transformations in a subalpine coniferous forest under experimental warming. *Global Change Biology*, 19, 2158–2167.

Yin H, Wheeler E, Phillips RP (2014) Root-induced changes in nutrient cycling in forests depend on exudation rates. *Soil Biology and Biochemistry*, 78, 213–221.

Zak DR, Holmes WE, Burton AJ, Pregitzer KS, Talhelm AF (2008) Simulated atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition. *Ecological Applications*, 18, 2016–2027.

Chapter 3. Differences between microbial community response to nitrogen fertilization result in unique enzyme shifts between arbuscular and ectomycorrhizal dominated stands

Under review and formatted for Global Change Biology

Abstract

While the effect of nitrogen (N) deposition on belowground carbon (C) cycling varies, emerging evidence shows that forest soils dominated by trees that associate with ectomycorrhizal fungi (ECM) store more C than soils dominated by trees that associate with arbuscular mycorrhizae (AM) with increasing N deposition. We hypothesized that this is due to unique nutrient cycling responses to N between AM and ECM dominated soils. ECM trees primarily obtain N through fungal mining of soil organic matter subsidized by root-C. As such we expected the largest N-induced responses of C and N cycling to occur in ECM rhizospheres and be driven by fungi. Conversely, as AM trees rely on bacterial scavengers in bulk soils to cycle N, we predicted the largest AM responses to be driven by shifts in bacteria and occur in bulk soils. To test this hypothesis, we measured microbial community composition, metatranscriptome profiles, and extracellular enzyme activity in bulk, rhizosphere, and organic horizon (OH) soils in AM and ECM dominated soils at Bear Brook Watershed in Maine, USA. After 27 years of N fertilization, fungal community composition shifted in ECM soils, whereas bacterial communities shifted in AM soils. These shifts were mirrored by enhanced C relative to N mining enzyme activities in both mycorrhizal types, but this occurred in different soil fractions. In ECM stands these shifts occurred in rhizosphere soils, but in AM stands they occurred in bulk soils. Additionally, ECM OH soils exhibited the opposite response with declines in C relative to N mining. As rhizosphere soils account for only a small portion of total soil volume relative to bulk soils, coupled with declines in C to N enzyme activity in ECM OH soils, we posit that this may partly explain why ECM soils store more C than AM soils as N inputs increase.

3.1 Introduction

Atmospheric deposition of N has fueled increases in tree growth across temperate forests (Quinn Thomas *et al.*, 2010; Averill & Waring, 2018). However, increased detrital inputs cannot fully account for the rate at which soil carbon (C) stocks increase in response to N fertilization (Pregitzer *et al.*, 2008; Zak *et al.*, 2008). Rather, most evidence points to reductions in soil C decomposition to explain this phenomenon (Fog, 1988; Zak *et al.*, 2008; Janssens & al., 2010; Edwards *et al.*, 2011; Frey *et al.*, 2014; Morrison *et al.*, 2016). While N-induced reductions in decomposition, particularly soil respiration are well-documented, the mechanism by which N reduces or alters soil microbial activity is less clear. Most research connects N induced shifts in fungal community structure and gene expression to declines in soil C decomposition (Frey *et al.*, 2004; Waldrop *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al.*, 2020). In contrast, other studies highlight shifts in bacterial community composition and function as drivers of these declines (Freedman & Zak, 2014; Freedman *et al.*, 2016a; Carrara *et al.*, 2018). While policy has driven reductions in atmospheric N deposition in some regions including the northeast US, global N deposition is expected to double over the next century (Galloway *et al.*, 2004; Reay *et al.*, 2008). As such, uncovering mechanisms that explain how N induced microbial shifts vary across forest types and horizons is paramount to informing models that predict the fate of the terrestrial C sink.

Differences between biogeochemical cycling in soils dominated by arbuscular mycorrhizal fungi (AM) or ectomycorrhizal fungi (ECM) may explain why soil C responses to N fertilization are linked to shifts in fungal community structure and function in some experiments and bacteria in others (Phillips *et al.*, 2013). Two key factors in the AM vs ECM dichotomy that may drive these differences are (1) dependence on rhizosphere stimulation of decomposition to access

nutrients, and (2) fungal vs bacterial control of decomposition. Below, we will outline how we expected these differences to influence divergent biogeochemical responses to N fertilization between mycorrhizal types.

To meet their N demand, trees that associate with ECM fungi (herein ECM trees) invest a substantial amount of C belowground to prime microbial decomposition of organic matter in the rhizosphere (Hobbie, 2006; Yin *et al.*, 2013; Brzostek *et al.*, 2014, 2015). Microbes use this C to produce extracellular enzymes that mine N from soil organic matter thus, increasing tree N supply (Phillips *et al.*, 2013; Cheeke *et al.*, 2017; Lin *et al.*, 2017). When N limitation is alleviated through fertilization, we expected ECM trees to allocate less C to microbes belowground to obtain it. We hypothesized that this reduction in C supply and increase in N would shift microbial nutrient demand in the rhizosphere and drive microbes toward producing C mining relative to N mining enzymes to maintain biomass C:N. As decomposition in ECM soils is driven by fungi that mine nutrients from soil organic matter, we hypothesized that shifts in C and N availability would drive changes in fungal community composition as competition for resources changes (Cheeke *et al.*, 2017).

Trees that associate with AM fungi (herein AM trees) invest less C belowground to rhizosphere microbes to obtain N than ECM trees and rely mainly on bacterial dominated inorganic N cycling in bulk soils to meet N demand (Averill *et al.*, 2014; Midgley & Phillips, 2014; Cheeke *et al.*, 2017). Here, bacteria scavenge and mineralize organic N leading to enhanced tree N supply (Phillips *et al.*, 2013). We hypothesized that as N fertilization alleviated N limitation in AM soils, microbes in bulk soils would become constrained by C demand. To maintain C:N microbes would then enhance C relative to N mining enzyme production in bulk soils. As bacteria drive N cycling

and decomposition in AM soils, we hypothesized that these shifts in C and N availability would result in changes in bacterial community composition.

To test these hypotheses, we established a network of AM and ECM dominated plots at the long-term (27 years) watershed scale N fertilization experiment at the Bear Brook Watershed in Maine, USA. We used an analysis of bacterial 16S ribosomal RNA and fungal 28S genes to examine N induced changes in both bacterial and fungal community structure in bulk, rhizosphere, and organic horizon soils separately. We further examined microbial function by analyzing the soil metatranscriptome and examining the presence of a suite of gene transcripts involved in carbon decomposition. In order to link genes to biogeochemistry, all of these measurements were done in tandem with measurements of extracellular enzyme activities in bulk, rhizosphere, and organic horizon soils that mineralize simple and complex C as well as N and phosphorus (P).

3.2 Methods

Study site

The Bear Brook Watershed in Maine (hereafter Bear Brook) is a unique watershed-scale fertilization experiment that has been used to examine the impact of N fertilization on biogeochemistry for 27 years. Here we advance previous research that has sought to understand the impact of N deposition on C and nutrient cycling and retention in forests by applying a mycorrhizal lens to this system. Prior research at Bear Brook has shown that N fertilization leads to increased stream water N exports, increased accumulation of N in hardwood biomass, increased foliar N concentration, and base cation depletion (Fernandez *et al.*, 2003; Elvir *et al.*, 2006; Patel *et al.*, 2019). The site is located in eastern Maine near the town of Beddington (44°52'15"N, 68°06'25"W) on the southwest slope of Lead Mountain (Wang & Fernandez 1999). The experiment consists of two adjacent watersheds West Bear (10.3 ha), and East Bear (11.0 ha). West Bear was aerially fertilized every two months between 1989 and 2016 at a rate of 25.2 kg N ha⁻¹ yr and 28.8 kg S ha⁻¹ yr⁻¹ in the form of solid pellet (NH₄)₂SO₄ compared to ambient deposition in 2016 of 1.5 kg N ha⁻¹ yr and 2.1 kg S ha⁻¹ yr⁻¹ as wet deposition (National Atmospheric Deposition Program, Greenville, ME; Jefts *et al.*, 2004; Fatemi *et al.*, 2016). As 2016 was the last year of fertilization, these measurements are among the last taken during 27 years of watershed fertilization at this site. Average precipitation is ~1400 mm and mean annual temperature is 5.6°C (Patel *et al.*, 2018). Soils are acidic, have low cation exchange capacity and base saturation, and are characterized as coarse-loamy, isotic, frigid Typic Haplorthods with an average depth of 0.9m (Norton *et al.*, 1999, Jefts *et al.*, 2004; Fernandez *et al.*, 2007).

We acknowledge that whole watershed fertilization experiments are limited to pseudo-replication as each watershed represents a treatment with only one sample (Hulbert 1984), however

prior to treatment these watersheds had similar tree species composition, soil type, and element fluxes (Norton *et al.*, 1999, Wang & Fernandez, 1999). It is noteworthy that pH is an important driver of microbial community diversity, biomass, and enzyme activity (Lauber *et al.*, 2009; Sinsabaugh, 2010; Rousk & Bååth, 2011; Fierer *et al.*, 2012; Kaiser *et al.*, 2016); however, there is little pH difference between the fertilized and reference watersheds (3.97 vs 4.01 in OH and 4.18 vs 4.28 at 5 cm depth; Jelts *et al.*, 2004) and no difference in microbial biomass (Wallenstein *et al.*, 2006).

Experimental design

In order to detect possible differences in N fertilization response between mycorrhizal types, we established a plot network of 6 AM and 6 ECM dominated 10 x 10 m plots in the lower elevation hardwood zone of both the reference and fertilized watershed (N=24 plots). Tree species were similar between watersheds with AM trees represented by red maple (*Acer rubrum*) and sugar maple (*Acer saccharum*) and ECM trees represented by American beech (*Fagus grandifolia*), grey birch (*Betula populifolia*), and yellow birch (*Betula alleghaniensis*).

To capture variability across the growing season, we sampled soils in each plot in May, July, and September of 2016. In each plot we extracted three 10 x 10 cm OH layers and homogenized them into a single sample defining this as the OH soil fraction. Next, we sampled four 5 cm diameter mineral soil cores to a depth of 15 cm beneath the OH layer and homogenized these by plot. All samples were kept on ice and transported to West Virginia University for further processing within 48-72 h. Upon return to the lab, we separated rhizosphere soil from mineral soil samples via the soil-adhesion method wherein the rhizosphere soil fraction was operationally defined as soil that remained clung to roots after modest shaking (Phillips & Fahey, 2005). Remaining mineral soil was defined as the bulk soil fraction. After removal of roots, all soils were

passed through a 2 mm sieve and stored at -80°C until further analysis. To preserve soil RNA for transcriptomic analysis during the July sampling date we sampled OH soil, and separated mineral soil into rhizosphere and bulk fractions in the field and immediately preserved in them in Lifeguard Soil Preservation Solution (MoBio, Carlsbad, CA).

Extracellular enzyme activity

To determine the extent to which N fertilization impacts microbial allocation to extracellular enzymes, we assayed the potential activity of hydrolytic enzymes that release N (N-acetylglucosaminidase; NAG), phosphorus (acid phosphatase; AP), and simple carbon (β -glucosidase; BG). In addition, we measured microbial allocation to complex C degrading oxidative enzymes phenol oxidase and peroxidase. Briefly, 1g of thawed soil was homogenized in 50 mM sodium acetate buffer (pH 5.0). Next, hydrolytic activities were determined using a fluorometric microplate assay with methylumbelliferone-linked substrates and oxidative enzymes using a colorimetric microplate assay with L-3,4-dihydroxyphenylalanine linked substrates (Saiya-Cork *et al.*, 2002). It is important to note that we measured potential enzyme activity under substrate saturated conditions. Under these conditions, the potential enzyme activities reported here can be used as a proxy of microbial enzyme pool size or microbial investment in each decomposition pathway. In order to determine the extent to which N fertilization alters microbial allocation to carbon or nutrient acquisition, we opted to express enzyme activity as ratios between C, N, and P acquiring enzymes (Midgley & Phillips, 2016). We used t-tests to determine significant differences between enzyme ratios, in order to compare fertilized and reference enzyme activities within each horizon and mycorrhizal type. T-tests were used rather than 2-way ANOVA as bulk, rhizosphere, and OH soils are ecologically distinct and considered to be unique sample groups.

To compare enzyme profiles between fertilized and reference soils at a broader resolution, we examined differences through similarity matrices. For each soil fraction within both mycorrhizal types, we calculated Bray-Curtis similarity of the five enzymes assayed using the `vegdist` function within the `vegan` package for R v 1.2.5033 (Oksanen et al., 2015; R Core Team, 2020). Next, profile similarity was compared by permutational multivariate analysis of variance (PERMANOVA) using the `adonis` function to determine if the centroids of the enzyme profiles varied significantly. For visualization of these data, we used non-metric dimensional scaling (NMDS) to generate NMDS scores of the enzymes for each soil fraction and mycorrhizal type and present the data to highlight N induced shifts in enzyme profiles.

Bacterial and fungal community composition and metatranscriptomics

Due to logistical and time constraints, all bacterial 16S, fungal 28S and metatranscriptomic data were collected during only the July sampling date. This date was chosen to capture microbial community structure and function at the peak of the growing season. To determine the bacterial and fungal community composition and gene expression in response to long-term N fertilization, DNA and RNA were simultaneously extracted for each soil fraction from ECM and AM dominated plots using the MoBio RNA Powersoil Kit supplemented with the MoBio DNA Elution Accessory Kit, following the manufacturers protocol. Samples were quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Next-Generation (NGS) amplicon-sequencing libraries were prepared using a two-step protocol, where step 1 amplifies the region of interest and step 2 adds a unique index and Illumina adapters. In step 1, each reaction contained: 5 ng of template DNA, 5 uM of F primer, 5 uM of R primer, 1X of KAPA HiFi Buffer (KAPA Biosystems, Roche Cape Town, South Africa), 0.3 mM of KAPA dNTPs, and 0.5 units KAPA HiFi HotStart DNA polymerase (Table S3-1; 28S primers from Mueller *et al.*, 2016).

Thermocycler conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 15 cycles of 98°C for 20 s, 63°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 5 min. PCR samples were purified using the AxyPrep Mag PCR Clean-up protocol (Axygen Biosciences, Union City, CA, USA) and quantified on the Qubit 2.0 Fluorometer (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). In step 2, unique index combinations were assigned to each sample (Table S3-1). Each reaction contained: 1 ng of the AxyPrep cleaned product from step 1, 5 uM of F primer, 5 uM of R primer, 1X of KAPA HiFi Buffer, 0.3 mM of KAPA dNTPs, and 0.5 units KAPA HiFi HotStart DNA polymerase. Thermocycler conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 22 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 4 min. PCR samples were purified using the AxyPrep Mag PCR Clean-up protocol (Axygen Biosciences, Union City, CA, USA) and quantified on the Qubit 2.0 Fluorometer with Qubit dsDNA HS reagents (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA). Samples were pooled in equal molar concentrations and sequenced on the Illumina MiSeq (250 bp paired-end reads) at the West Virginia University Genomics and Bioinformatics Core Facility (West Virginia University, Morgantown, WV, USA).

Sequence processing, diversity analysis, and classification was performed in Qiime2-2019.4 (Bolyen *et al.*, 2019). Low quality nucleotides, adapters, and primer sequences were trimmed and quality trimmed reads were processed using the DADA2 function to output representative sequences. DADA2 further processes the reads by removing of phiX reads, chimeric reads, and identical reads, and correcting polymerase-induced errors, and merging the forward and reverse reads, to produce a minimized representative data set. The representative data set from DADA2 was aligned for diversity analysis and phylogenetic tree reconstruction using mafft.

Bacterial 16S sequences were classified using the *silva-132-99-nb-classifier.qza* provided by Silva (Quast *et al.*, 2013; Yilmaz *et al.*, 2014). Fungal 28S sequences were classified against the large subunit database from UNITE (97% threshold) (Nilsson *et al.*, 2019). To determine the impact on N fertilization on broad-level species composition, all fungal and bacterial phyla representing more than 1% of abundance across plots were examined for significant shifts in relative abundance. To further analyze fungal and bacterial community response to N fertilization, all OTUs were normalized by dividing the number of sequences of each OTU by total sequences in each sample. Next, the OTUs were used to calculate Bray-Curtis similarity using the *vegdist* function in the *vegan* package in R. Using the *adonis* function, communities were analyzed by PERMANOVA with treatment and soil fraction as main effects for both AM and ECM dominated soils.

RNAs, extracted simultaneously with DNA from the same soil samples, were quantified using the Nanodrop 2000 spectrophotometer and quality was assessed via Agilent bioanalyzer (Agilent, Santa Clara, CA). RNAs were DNase treated with the Turbo DNA-free Kit and preserved with SUPERaseIN (Thermo Fisher Scientific), following manufacturers protocols. DNase treated RNAs were quantified on the Qubit 2.0, using the RNA Broad Range Assay. RNAs from each mycorrhizal type and each soil fraction were pooled in equal molar amounts to produce 12 total RNA pools (2 mycorrhizal types x 3 soil fractions x 2 treatments). RNA pools were submitted to the WVU Genomics and Bioinformatics core facility for Illumina library construction. Illumina sequencing libraries were generated using the ScriptSeq Complete Gold Kit (Epicentre Biotechnologies, Madison, WI), which first performs a ribosomal depletion followed by random hexamer cDNA synthesis and indexing. The adaptor-tagged RNA-Seq libraries were sequenced at the Marshall University Genomics Core Facility (Huntington, WV) on the Illumina HiSeq 2500.

To determine the impact of N fertilization on microbial transcription of genes associated with C turnover, metatranscriptome sequences were normalized by total number of sequences and queried against a suite of genes involved in the breakdown of lignin, simple carbon, and chitin using 'blastx' function in DIAMOND (v. 2.0.2 *sensu* Buchfink *et al.*, 2014; Freedman *et al.*, 2016b; Table S3-2). While community analysis was completed at the plot level, RNA was pooled within mycorrhizal type, soil fraction, and treatment for gene transcript abundance analysis. With limited statistical power, OH, bulk, and rhizosphere fractions were used as replicates to test for significant differences in transcript abundance by using t-tests between treatments within mycorrhizal type. To determine the effect N had on total ligninolytic capacity, the relative abundance values of each of the lignin degrading proteins within each treatment and mycorrhizal type was analyzed.

3.3 Results

Extracellular enzyme activity

The N response of simple C (BG) to nutrient acquiring (NAG & AP) enzyme activity ratios were similar across AM and ECM bulk and rhizosphere soils, but complex C (phenol oxidase & peroxidase) to N (N-acetylglucosaminidase) ratios varied by soil fraction between AM and ECM plots. For example, in N fertilized AM bulk and rhizosphere soils, the ratio of BG to NAG was 221 and 194% higher respectively, as compared to reference soils (Fig. 3-1 a,b; $p < 0.05$). Similarly, BG to NAG ratios were 106 and 175% higher in N fertilized ECM bulk and rhizosphere soils as compared to reference soils (Fig. 3-1 a,b; $p < 0.05$). BG to AP acquiring enzymes were higher in N fertilized AM bulk and rhizosphere soils by 148 and 142% respectively and also in ECM bulk and rhizosphere soils by 167 and 258% (Fig. 3-1 c,d; $p < 0.05$).

Examining ratios of soil enzyme activities help to determine the relative investment of microbial resources toward carbon or nutrient acquisition. N fertilization induced higher complex C to N enzyme activity ratios in only AM bulk soils and ECM rhizosphere soils. In N fertilized AM bulk soils, phenol oxidase to NAG activity ratios were 185% higher and peroxidase to NAG ratios trended 75% higher (Fig. 3-1 e,g). N fertilization had no effect on complex C to N enzyme activity ratios in ECM bulk soils. However, in N fertilized ECM rhizosphere soils, peroxidase to NAG activity ratios were 119% higher and phenol oxidase to NAG activity trended 56% higher (Fig. 3-1 f,h). N fertilization had no effect on complex C to N acquiring enzyme activity ratios in AM rhizospheres.

OH horizon soil enzyme ratios varied by mycorrhizal type. In AM OH soils, N fertilization increased the ratio of BG to AP activity by 141%, but had no significant effect on other enzyme ratios (Table S3-3). In ECM OH soils N fertilization increased the ratio of BG to AP activity by

206% (Table S3-3). Conversely, in ECM OH soils N fertilization significantly decreased the phenol oxidase to NAG activity ratio by 76% and the peroxidase to NAG ratio by 69% (Table S3-3).

Distinctive shifts in enzyme activity of AM bulk soils and ECM rhizosphere soils were further supported by PERMANOVA of Bray-Curtis similarity matrices of enzyme profiles with horizon and mycorrhizal type as factors. PERMANOVA on soil enzyme profile NMDS scores highlighted a marginal N effect in AM bulk soils ($p=0.109$), but no effect in ECM bulk soils (Fig. 3-2 c,d). Conversely, enzyme profiles were significantly different in ECM rhizospheres ($p=0.021$), but not AM rhizospheres (Fig. 3-2 e,f). In OH soils, enzyme profiles significantly shifted in both mycorrhizal types (Fig. 3-2 a,b).

Bacterial and fungal community composition and metatranscriptomics

There were only minor shifts in dominant fungal phyla across mycorrhizal types and soil fractions. When fungal taxonomy was considered at the phylum level, the three most dominant phyla across all plots were Ascomycota (~11% relative abundance), Basidiomycota (~79%) and Chytridomycota (~2 %; Table S3-4). The relative abundance of many fungal taxa were unaffected by N across the plots, however in AM bulk soils, N fertilized soils harbored ~10% fewer basidiomycetes and ~61% more ascomycetes (Table S3-4). With all soil fractions pooled, there was a marginally significant 14% decline in basidiomycetes in fertilized AM plots compared to control plots (Fig. 3-4a, $p=0.08$). Major phylum level bacterial shifts were less straightforward with limited N induced shifts occurring across mycorrhizal types and soil fractions (Table S3-5).

When considered as total community composition at the OTU level, N fertilization shifted ECM fungal community composition across soils, whereas N fertilization shifted bacterial

community composition in AM soils. Within ECM bulk and rhizosphere soils, fungal community composition differed with N fertilization (Fig. 3-3 b,d). Conversely, in AM soils, N fertilization altered bacterial community composition in bulk and rhizosphere soils (Fig. 3-3 a,c). Additionally, ECM bacterial communities differed between fertilized and control bulk soils ($p < 0.05$), but no difference was detected in ECM rhizosphere communities.

We detected limited N induced shifts in transcription of proteins associated with lignin decomposition under N fertilization across both mycorrhizal types (Table S3-2); however, there was significantly less transcription of genes encoding total lignin degrading enzymes in N fertilized AM soils compared to control when the relative abundance of all transcripts encoding lignin degrading enzymes were combined (Fig. 3-4b). Abundance of lignin degrading transcripts were pooled to better estimate total ligninolytic potential of soils rather than examining each gene individually.

3.4 Discussion

Understanding mechanisms that drive variability in soil C response to N fertilization across forest types is critical in predicting the fate of the terrestrial C sink. Here, we provide evidence of a mechanism that explains how ECM soils tend to store more C than AM soils under higher N deposition (Averill *et al.*, 2018). We show that while N induced shifts in microbial enzyme allocation toward C mining (relative to N mining) were a common response across mycorrhizal types, this occurs at a much smaller magnitude in ECM soils because it is confined to rhizosphere soils. In ECM stands, N fertilization enhanced complex C relative to N mining only in rhizosphere soils which occurred concomitantly with shifts in fungal community composition (Fig. 3-1 e-h, Fig. 3-3 b,d). These changes reflect a disruption of the rhizosphere-centric, fungal driven, organic nutrient economy of ECM trees. In AM stands, shifts toward C mining and away from N mining enzyme activity occurred in bulk soils (Fig. 3-1 e-h) which account for the majority of forest soil volume. Unlike ECM soils, these shifts were mirrored by shifts in bacterial community composition highlighting N induced changes to the bacterially driven, inorganic nutrient economy of AM trees (Fig. 3-3 a,c). N induced C losses are likely small in ECM stands, because C relative to N mining enzyme enhancements were isolated to rhizosphere soils, which account for only a small fraction of forest soil volume (Finzi *et al.*, 2015). Additionally, N induced declines in C mining relative to N mining in ECM OH soils may enhance C gains under long-term N fertilization. Based on these shifts in decomposition, ECM soils likely store more C under N fertilization than AM soils which exhibit enhanced C relative to N mining across bulk soils.

N fertilization likely reduced belowground C allocation in ECM trees to a greater extent than AM trees because they rely more heavily on organic N released by rhizosphere priming to meet their N demand (Phillips *et al.*, 2013; Brzostek *et al.*, 2015). As N supply was enhanced by

fertilization, it is probable that ECM trees shifted C allocation from belowground growth towards above-ground tissues (Litton *et al.*, 2007). This reduction in rhizosphere C availability, coupled with enhanced N supply, likely put a C constraint on soil fungi which are the dominant decomposers of high C:N ECM litter (Phillips *et al.*, 2013; Cheeke *et al.*, 2017). As such, there was a shift in fungal community composition and microbes moved toward C limitation which would restrict microbial growth. To maintain growth and biomass C:N, fungi shifted production of extracellular enzymes to match resource constraints which led to an increase in complex C relative to N mining activity by rhizosphere microbes in ECM soils (Fig. 3-1 f,h).

N fertilization responses in AM soils were the result of a more direct effect on bacterial shifts in C and N demand and were likely less influenced by declines in plant-C allocation to the rhizosphere (Brzostek *et al.*, 2015). AM litter has much lower C:N than ECM litter (Phillips *et al.*, 2013). This allows them to rely more heavily on rapid bacterial N cycling in bulk soils to meet N demand rather than sending C to prime organic N release in the rhizosphere (Midgley & Phillips, 2014). As N fertilization increased N supply in AM bulk soils, bacteria became constrained by C availability. This resulted in a shift in bacterial community composition and resource demand which led to an enhancement of C relative to N mining enzymes in bulk soils (Fig. 3-1 e,g).

Differences between AM and ECM trees in how they couple C and N cycles belowground has proven to be a powerful framework for explaining variability in biogeochemical cycles across forest ecosystems (Phillips *et al.*, 2013; Averill *et al.*, 2014, 2018; Brzostek *et al.*, 2015; Midgley *et al.*, 2015; Midgley & Phillips, 2016; Terrer *et al.*, 2016; Cheeke *et al.*, 2017). Past research has shown that (1) ECM trees prime soil decomposition to a greater extent than AM trees (Brzostek *et al.*, 2015), (2) low C:N, AM litter leads to rapid decomposition and high inorganic N availability in AM soils, and (3) decomposition is driven more by fungi in ECM soils and bacteria in AM soils

(Phillips *et al.*, 2013; Cheeke *et al.*, 2017). Here we expand on this framework by showing that these differences in belowground traits appear to drive variability in the response of soil decomposition to N fertilization and may explain why ECM soils appear to gain more soil C than AM soils under N fertilization (Midgley & Phillips, 2016; Averill *et al.*, 2018). N fertilization drove changes in ECM soils primarily through localized shifts in rhizosphere enzyme profiles that occurred in tandem with shifts in fungal communities. The increases in C mining in ECM rhizospheres were also counterbalanced by a reduction in C mining in ECM organic horizons. In AM soils, however, N fertilization led to more widespread bulk soil enzyme shifts which were mirrored by bacterial community changes. Thus, we hypothesize that differences between mycorrhizal associations in how they couple C and N cycles belowground drive who responds to enhanced N availability (i.e., fungi vs. bacteria), where this response occurs (i.e., rhizosphere vs. bulk soil), and potentially whether the system gains or loses soil C. Future research is needed to examine whether this mechanism holds across forest ecosystems, under other forms of N deposition (i.e., ammonium vs nitrate) and whether these responses operate under ambient N deposition loads.

In addition to providing a plausible mechanism to explain ECM soil C gains under elevated N deposition, these results may also shed light on why microbial community responses to N deposition vary across studies. While much research highlights shifts in fungal community composition to explain N induced decomposition shifts (Frey *et al.*, 2004; Waldrop *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al.*, 2020), others link these shifts to changes in bacterial community composition and metabolism (Freedman & Zak, 2014; Freedman *et al.*, 2016a; Carrara *et al.*, 2018). We show that fungal communities shifted in ECM soils and bacterial communities shifted in AM soils. As such, variation in microbial

responses across studies may be explained by relative mycorrhizal dominance. As these divergent microbial shifts between mycorrhizal types were apparent at such a small scale (10 x 10 m plots within a 10.3 ha watershed) it is possible that small differences in the relative abundance of AM and ECM trees can have sizable impacts on dominant microbial responses (i.e. bacterial vs fungal) to N fertilization.

Regardless of soil fraction, these shifts in enzyme activity appear to be driven by changes in microbial resource stoichiometry wherein microbes alter enzyme production as N limitation is alleviated and C limitation becomes more pronounced. This work builds on previous research that highlights the ability of microbes to alter allocation of resources to extracellular enzymes based on the relative demand of C and N to maintain growth (Schimel & Weintraub, 2003; Allison & Vitousek, 2005; Allison *et al.*, 2010; Sinsabaugh & Follstad Shah, 2012). This effect was observed in an increase in the ratio of BG to NAG activity across bulk and rhizosphere soil fractions in both mycorrhizal types which was driven primarily by a reduction in NAG activity (Fig. 3-1a,b, Table S3-6). However, the complex C to NAG activity response varied between mycorrhizal types with increases occurring in in ECM rhizospheres and in AM bulk soils (Fig. 3-1 e-h). While most studies report declines or no change in oxidative enzyme activity in response to N fertilization (Fog, 1988; Saiya-Cork *et al.*, 2002; DeForest *et al.*, 2004; Frey *et al.*, 2004, 2014; Zak *et al.*, 2008; Sinsabaugh, 2010), these distinct ECM and AM-associated increases in complex C to NAG activity ratios were partially driven by increased phenol oxidase activity in ECM rhizospheres and by increased peroxidase activity in AM bulk soils (Table S3-6). One explanation for this could be that microbes in ECM rhizospheres were pushed further toward C limitation than bulk soils due not only to N limitation alleviation, but strong declines in root-C inputs. In AM bulk soils, where saprotrophic communities are adapted to scavenging inorganic N, high inputs of N may result in

C restriction of microbial growth which may be dampened in AM rhizospheres due to some access to root-C. In either case, these mechanisms may act on soil microbes to enhance production of complex C mining enzymes in order to maintain biomass C:N.

We found little evidence that the abundance of gene transcripts involved in decomposition were correlated with microbial community composition or enzyme activity. In ECM soils, shifts in community composition of fungi were not coupled with significant shifts in the relative abundance of ligninolytic enzyme transcripts (Fig. 3-4b). In AM soils, N fertilization reduced total ligninolytic transcript abundance which occurred in tandem with a marginally significant 14.3% ($p=0.08$) decline in basidiomycete relative abundance (Fig. 3-4 a,b). Here, it is possible that long term-N fertilization suppressed fungal activity in AM soils without altering fungal community composition on a broader scale, however reductions in gene transcription were not mirrored by declines in ligninolytic enzyme activities (Deforest *et al.*, 2004). In ECM soils, it is possible that strong reductions in belowground C allocation to mycorrhizal symbionts resulted in an enhancement of free-living fungi relative to symbiotic fungi. This change could be responsible for the observed fungal community shifts in ECM soils as well as the changes in rhizosphere enzyme profiles without necessarily altering the rate of ligninolytic gene transcription across soil fractions. Regardless, we found little evidence that the relative abundance of genes involved in decomposition was a direct metric of the enzymatic potential of these soils. However, as transcriptomic data were pooled to one sample per mycorrhizal type, soil fraction, and treatment we lack the statistical power to examine changes in fungal transcription at finer scales.

Documented soil C cycling responses to N fertilization range from being driven primarily through shifts in fungi (Frey *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al.*, 2020), to declines in belowground C allocation by trees and shifts in bacteria

(Freedman & Zak, 2014; Freedman *et al.*, 2016a; Carrara *et al.*, 2018). Here we provide evidence that differences in C and nutrient cycling strategies between AM and ECM trees result in distinct fungal shifts in ECM soils and bacterial shifts in AM soils. Further, in ECM stands we find that N induced fungal shifts occur in tandem with rhizosphere-isolated enhancements in C mining relative to N mining enzyme activity, whereas the same enzyme shifts occur across AM bulk soils. These results coupled with declines in C relative to N mining in ECM OH soils provide a mechanism that may explain variability in documented microbial responses to N fertilization, and ultimately why ECM soils tend to store more C than AM soils under enhanced N. As N deposition continues to rise across much of the developing world, this mechanism can prove useful in enhancing the predictive capabilities of models that estimate the fate of the land C sink.

3.5 Tables and Figures

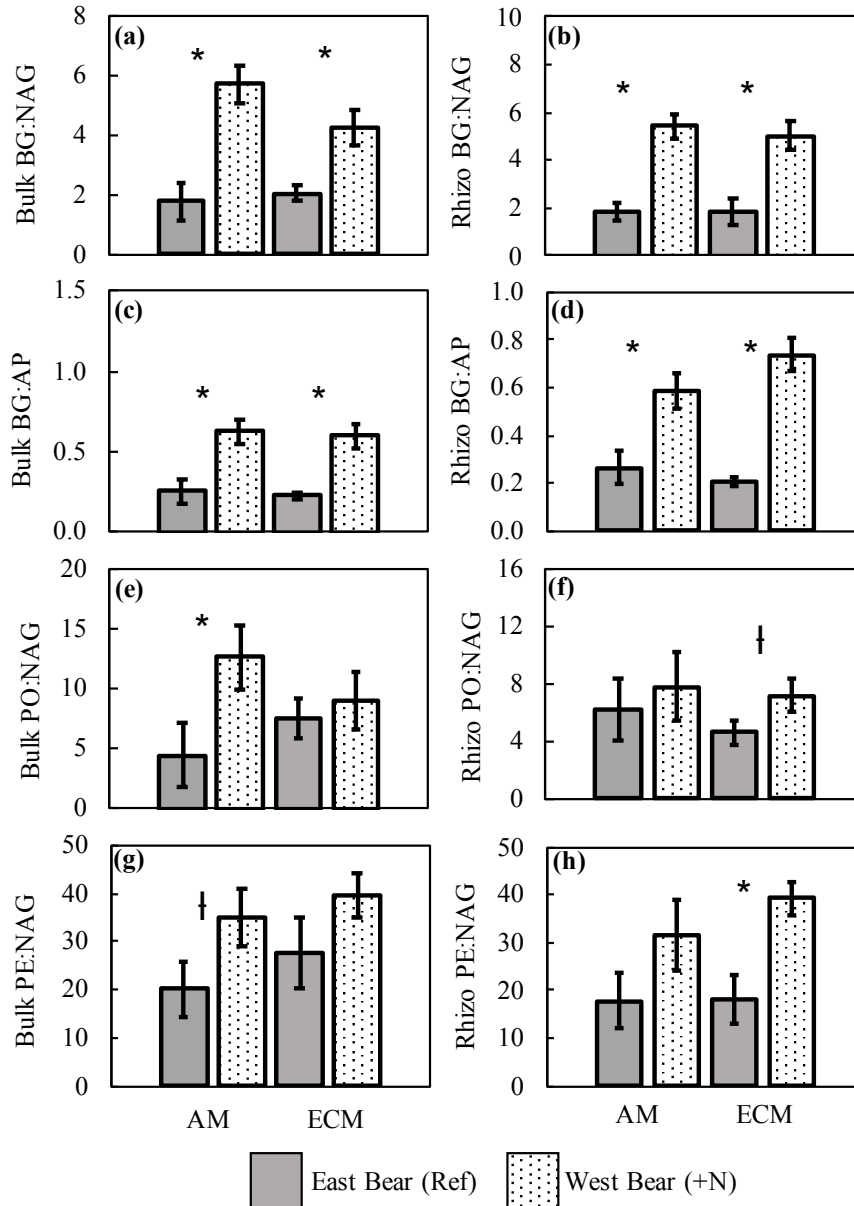


Figure 3-1. N fertilization enhanced the ratio of simple C to nutrient enzyme activities across AM and ECM bulk and rhizosphere soils, but enhanced complex C to N only in AM bulk soils and ECM rhizosphere soils. Values are overall seasonal mean ratios (mean \pm SE) of (a&b) β -glucosidase: N-Acetylglucosaminidase, (c&d) β -glucosidase: acid phosphatase, (e&f) phenol oxidase: N-Acetylglucosaminidase, (g&h) peroxidase: N-Acetylglucosaminidase. Values were measured in May, July, and September across all plots (n=6 plots). Asterisks indicate p<0.05 for t-tests between N fertilized and reference plots within mycorrhizal type. Crosses indicate p<0.10

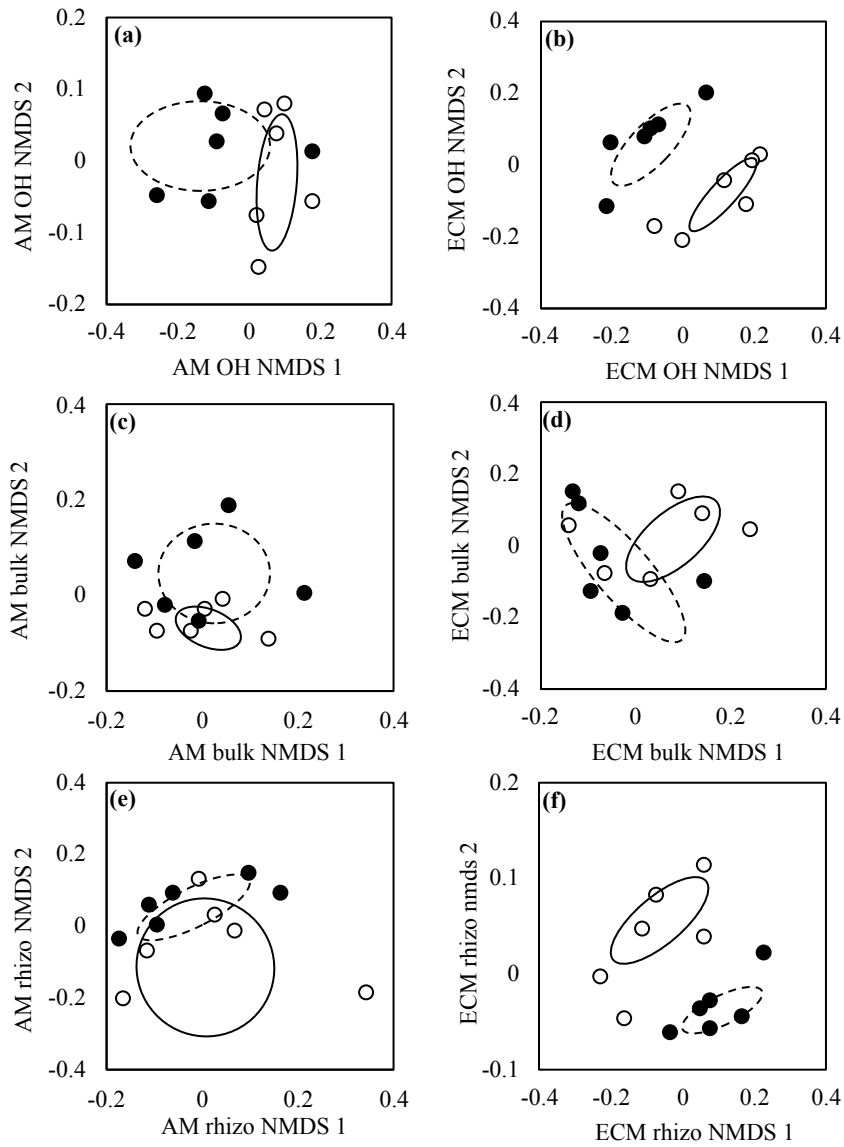


Figure 3-2. N fertilization altered enzyme profiles in AM bulk soils and ECM rhizosphere soils. Scatterplots are NMDS1 vs NMDS2 of the enzyme profiles for (a) AM OH soils $p=0.023$ (b) ECM OH soils $p=0.002$ (c) AM bulk soils $p=0.095$, (d) ECM bulk soils $p=0.172$, (e) AM rhizosphere soils $p=0.577$, (f) ECM rhizosphere soils $p=0.021$. Closed circles are fertilized plots, open circles are reference plots.

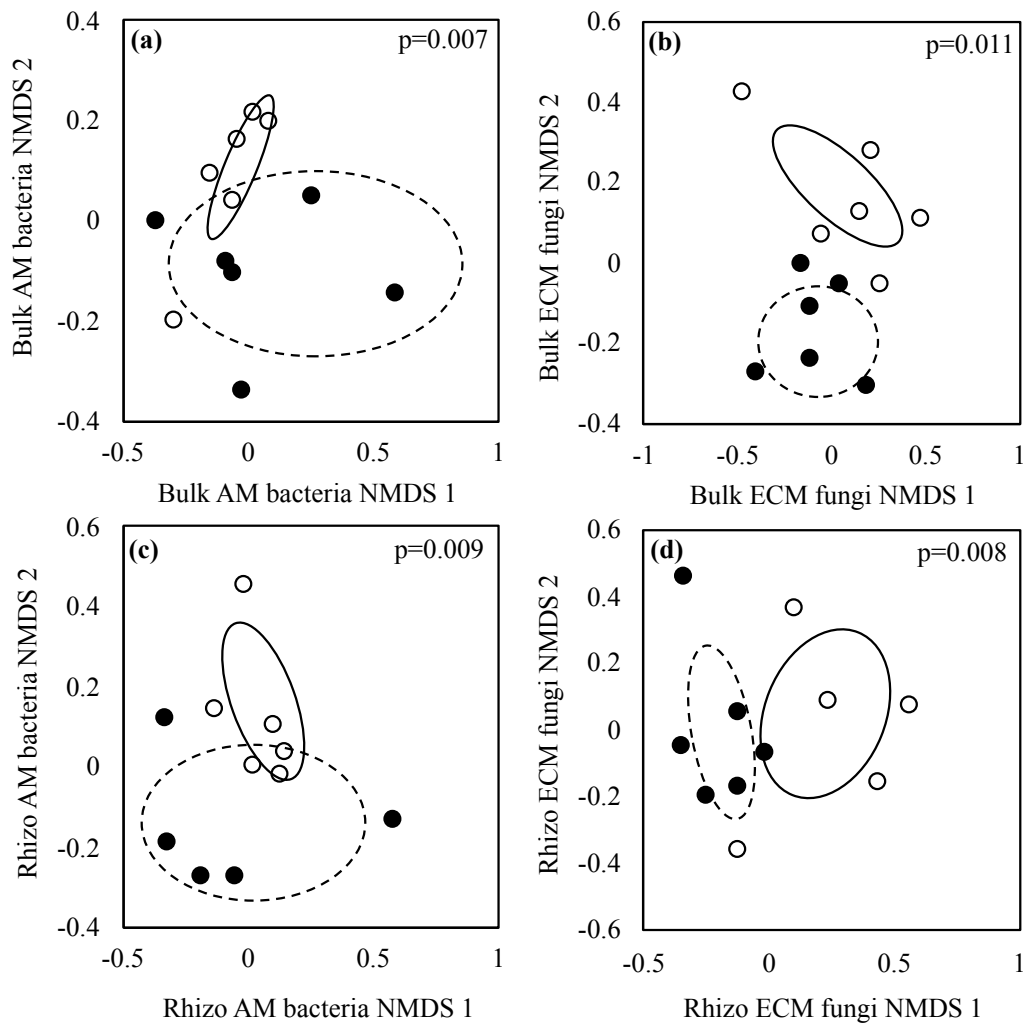


Figure 3-3. N Fertilization altered bacterial communities in AM soils and fungal communities in ECM soils. Scatterplots are NMDS1 vs NMDS2 of the community structure for (a) AM bulk soils bacteria, (b) ECM bulk soils fungi, (c) AM rhizosphere soils bacteria, (d) ECM rhizosphere soils fungi. Closed circles are fertilized plots, open circles are reference plots. P values indicate significant differences between community composition.

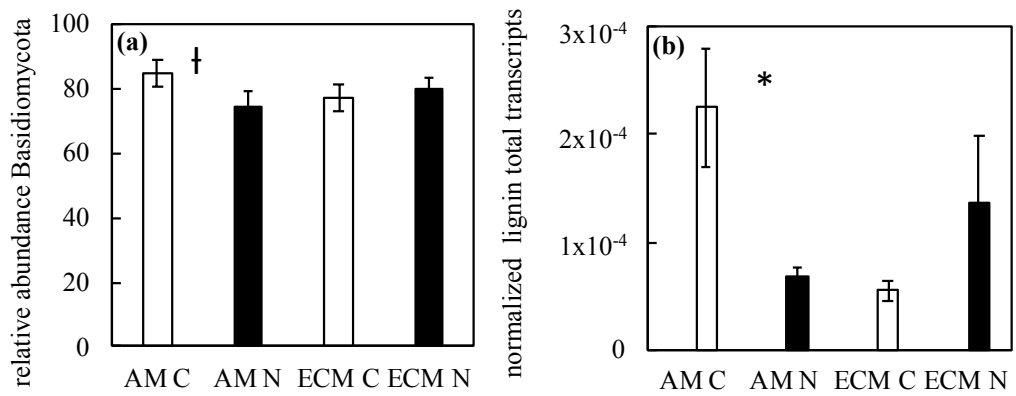


Figure 3-4. N fertilization reduced Basidiomycota abundance and ligninolytic gene transcription in AM soils, but not ECM soils. Asterisks represent significant differences between reference and N fertilized values within mycorrhizal type. For (a) n=6 plots per mycorrhizal type per treatment, (b) n= 3 samples per mycorrhizal type per treatment. Asterisk indicates p<0.05, cross indicates p<0.10.

3.6 Literature Cited

- Allison SD, Vitousek PM (2005) Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology and Biochemistry*, **37**, 937–944.
- Allison SD, Wallenstein MD, Bradford MA (2010) Soil-carbon response to warming dependent on microbial physiology. *Nature Geoscience*, **3**, 336–340.
- Averill C, Waring B (2018a) Nitrogen limitation of decomposition and decay: How can it occur? *Global Change Biology*, **24**, 1417–1427.
- Averill C, Waring B (2018b) Nitrogen limitation of decomposition and decay: How can it occur? *Global Change Biology*, **24**, 1417–1427.
- Averill C, Turner BL, Finzi AC (2014) Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature*, **505**, 543–545.
- Averill C, Dietze MC, Bhatnagar JM (2018) Continental-scale nitrogen pollution is shifting forest mycorrhizal associations and soil carbon stocks. *Global Change Biology*, **24**, 4544–4553.
- Bolyen E, Rideout JR, Dillon MR et al. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, **37**, 852–857.
- Brzostek ER, Fisher JB, Phillips RP (2014) Modeling the carbon cost of plant nitrogen acquisition: Mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. *Journal of Geophysical Research: Biogeosciences*, **119**, 1684–1697.
- Brzostek ER, Dragoni D, Brown ZA, Phillips RP (2015) Mycorrhizal type determines the magnitude and direction of root-induced changes in decomposition in a temperate forest.

- New Phytologist*, **206**, 1274–1282.
- Buchfink B, Xie C, Huson DH (2014) Fast and sensitive protein alignment using DIAMOND. *Nature Methods*, **12**, 59–60.
- Carrara JE, Walter CA, Hawkins JS, Peterjohn WT, Averill C, Brzostek ER (2018) Interactions among plants, bacteria, and fungi reduce extracellular enzyme activities under long-term N fertilization. *Global Change Biology*, **24**, 2721–2734.
- Cheeke TE, Phillips RP, Brzostek ER, Rosling A, Bever JD, Fransson P (2017) Dominant mycorrhizal association of trees alters carbon and nutrient cycling by selecting for microbial groups with distinct enzyme function. *New Phytologist*, **214**, 432–442.
- DeForest JL, Zak DR, Pregitzer KS, Burton AJ (2004) Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. *Soil Science Society of America Journal*, **68**, 132–138.
- Edwards IP, Zak DR, Kellner H, Eisenlord SD, Pregitzer KS (2011) Simulated atmospheric N deposition alters fungal community composition and suppresses ligninolytic gene expression in a Northern Hardwood forest. *PLoS ONE*, **6**.
- Elizabeth M. Entwistle, a, b Karl J. Romanowicz, a, c William A. Argiroff, a Zachary B. Freedman, d J. Jeffrey Morris B, Donald R. Zaka C (2020) Anthropogenic N Deposition Alters the Composition of Expressed Class II Fungal Peroxidases. **84**, 1–16.
- Elvir JA, Wiersma GB, Day ME, Greenwood MS, Fernandez IJ (2006) Effects of enhanced nitrogen deposition on foliar chemistry and physiological processes of forest trees at the Bear Brook Watershed in Maine. *Forest Ecology and Management*, **221**, 207–214.

- Entwistle EM, Zak DR, Argiroff WA (2018) Anthropogenic N deposition increases soil C storage by reducing the relative abundance of lignolytic fungi. *Ecological Monographs*, **88**, 225–244.
- Fatemi FR, Fernandez IJ, Simon KS, Dail DB (2016) Nitrogen and phosphorus regulation of soil enzyme activities in acid forest soils. *Soil Biology and Biochemistry*, **98**, 171–179.
- Fernandez IJ, Rustad LE, Norton SA, Kahl JS, Cosby BJ (2003) Experimental Acidification Causes Soil Base-Cation Depletion at the Bear Brook Watershed in Maine. *Soil Science Society of America Journal*, **67**, 1909–1919.
- Fernandez IJ, Karem JE, Norton SA, Rustad LE (2007) Temperature, soil moisture, and streamflow at the Bear Brook Watershed in Maine (BBWM). *Maine Agricultural and Forest Experiment Station Technical Bulletin*, **196**.
- Fierer N, Leff JW, Adams BJ et al. (2012) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings of the National Academy of Sciences*, **109**, 21390–21395.
- Finzi AC, Abramoff RZ, Spiller KS, Brzostek ER, Darby BA, Kramer MA, Phillips RP (2015) Rhizosphere processes are quantitatively important components of terrestrial carbon and nutrient cycles. *Global Change Biology*, **21**, 2082–2094.
- Fog K (1988) The effect of added nitrogen on the rate of decomposition of organic matter. *Biological Reviews*, **63**, 433–462.
- Freedman Z, Zak DR (2014) Atmospheric N deposition increases bacterial laccase-like multicopper oxidases: Implications for organic matter decay. *Applied and Environmental*

Microbiology, **80**, 4460–4468.

Freedman ZB, Romanowicz KJ, Upchurch RA, Zak DR (2015) Differential responses of total and active soil microbial communities to long-term experimental N deposition. *Soil Biology and Biochemistry*, **90**, 275–282.

Freedman ZB, Upchurch RA, Zak DR, Cline LC (2016a) Anthropogenic N Deposition Slows Decay by Favoring Bacterial Metabolism: Insights from Metagenomic Analyses. *Frontiers in Microbiology*, **7**, 1–11.

Freedman ZB, Upchurch RA, Zak DR (2016b) Microbial potential for ecosystem N loss Is increased by experimental N deposition. *PLoS ONE*, **11**, 1–19.

Frey SD, Knorr M, Parrent JL, Simpson RT (2004) Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management*, **196**, 159–171.

Frey SD, Ollinger S, Nadelhoffer K et al. (2014) Chronic nitrogen additions suppress decomposition and sequester soil carbon in temperate forests. *Biogeochemistry*, **121**, 305–316.

Galloway JN, Dentener FJ, Capone DG et al. (2004) Nitrogen Cycles: Past, Present, and Future. *Biogeochemistry*, **70**, 153–226.

Hobbie EA (2006) Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. *Ecology*, **87**, 563–569.

Janssens IA, al. et (2010) Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience*, **3**, 315–322.

- Jefts S, Fernandez IJ, Rustad LE, Dail DB (2004) Decadal responses in soil N dynamics at the Bear Brook Watershed in Maine, USA. *Forest Ecology and Management*, **189**, 189–205.
- Kaiser K, Wemheuer B, Korolkow V et al. (2016) Driving forces of soil bacterial community structure, diversity, and function in temperate grasslands and forests. *Scientific Reports*, **6**, 1–12.
- Lauber CL, Hamady M, Knight R, Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, **75**, 5111–5120.
- Lin G, McCormack ML, Ma C, Guo D (2017) Similar below-ground carbon cycling dynamics but contrasting modes of nitrogen cycling between arbuscular mycorrhizal and ectomycorrhizal forests. *New Phytologist*, **213**, 1440–1451.
- Litton CM, Raich JW, Ryan MG (2007) Carbon allocation in forest ecosystems. *Global Change Biology*, **13**, 2089–2109.
- Midgley MG, Phillips RP (2014) Mycorrhizal associations of dominant trees influence nitrate leaching responses to N deposition. 241–253.
- Midgley MG, Phillips RP (2016) Resource stoichiometry and the biogeochemical consequences of nitrogen deposition in a mixed deciduous forest. *Ecology*, **97**, 3369–3377.
- Midgley MG, Brzostek E, Phillips RP (2015) Decay rates of leaf litters from arbuscular mycorrhizal trees are more sensitive to soil effects than litters from ectomycorrhizal trees. *Journal of Ecology*, **103**, 1454–1463.
- Morrison EW, Frey SD, Sadowsky JJ, van Diepen LTA, Thomas WK, Pringle A (2016) Chronic

- nitrogen additions fundamentally restructure the soil fungal community in a temperate forest. *Fungal Ecology*, **23**, 48–57.
- Mueller RC, Gallegos-Graves LV, Kuske CR (2016) A new fungal large subunit ribosomal RNA primer for high-throughput sequencing surveys. *FEMS Microbiology Ecology*, **92**, 1–11.
- Nilsson RH, Larsson KH, Taylor AFS et al. (2019) The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research*, **47**, D259–D264.
- Norton S, Kahl J, Fernandez I et al. (1999) The Bear Brook Watershed, Maine (BBWM), USA. *Environmental Monitoring and Assessment*, **55**, 7–51.
- Oksanen J, Blanchet GF, Kindt R et al. (2015) *vegan: Community Ecology Package*.
- Patel KF, Fernandez IJ, Nelson SJ, Gruselle M, Norton SA, Weiskittel AR (2019) Forest N Dynamics after 25 years of Whole Watershed N Enrichment: The Bear Brook Watershed in Maine. *Soil Science Society of America Journal*, **83**.
- Phillips RP, Fahey TJ (2005) Patterns of rhizosphere carbon flux in sugar maple (*Acer saccharum*) and yellow birch (*Betula allegheniensis*) saplings. *Global Change Biology*, **11**, 983–995.
- Phillips RP, Brzostek E, Midgley MG (2013) The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist*, **199**, 41–51.
- Pregitzer KS, Burton AJ, Zak DR, Talhelm AF (2008) Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. *Global Change Biology*, **14**, 142–

153.

Quast C, Pruesse E, Yilmaz P et al. (2013) The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, **41**, 590–596.

Quinn Thomas R, Canham CD, Weathers KC, Goodale CL (2010) Increased tree carbon storage in response to nitrogen deposition in the US. *Nature Geoscience*, **3**, 13–17.

R Core Team (2017) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Reay DS, Dentener FJ, Smith P, Grace J, Feely RA (2008) Global nitrogen deposition and carbon sinks. *Nature Geoscience*, **1**, 430–437.

Rousk J, Bååth E (2011) Growth of saprotrophic fungi and bacteria in soil. *FEMS Microbiology Ecology*, **78**, 17–30.

Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry*, **34**, 1309–1315.

Schimel JP, Weintraub MN (2003) The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: A theoretical model. *Soil Biology and Biochemistry*, **35**, 549–563.

Sinsabaugh RL (2010) Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry*, **42**, 391–404.

Sinsabaugh RL, Follstad Shah JJ (2012) Ecoenzymatic Stoichiometry and Ecological Theory. *Annual Review of Ecology, Evolution, and Systematics*, **43**, 313–343.

- Terrer C, Vicca S, Hungate BA, Phillips RP, Prentice IC (2016) Mycorrhizal association as a primary control of the CO₂ fertilization effect. *Science*, **353**, 72–74.
- Waldrop MP, Zak DR, Sinsabaugh RL, Gallo M, Lauber C (2004) Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. *Ecological Applications*, **14**, 1172–1177.
- Wallenstein MD, McNulty S, Fernandez IJ, Boggs J, Schlesinger WH (2006) Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. *Forest Ecology and Management*, **222**, 459–468.
- Wang Z, Fernandez I (1999) Soil Type and Forest Vegetation Influences on Forest Floor Nitrogen Dynamics at the Bear Brook Watershed in Maine (BBWM). *Environmental Monitoring and Assessment*, **55**, 221–234.
- Yilmaz P, Parfrey LW, Yarza P et al. (2014) The SILVA and “all-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Research*, **42**, 643–648.
- Yin H, Li Y, Xiao J, Xu Z, Cheng X, Liu Q (2013) Enhanced root exudation stimulates soil nitrogen transformations in a subalpine coniferous forest under experimental warming. *Global Change Biology*, **19**, 2158–2167.
- Zak DR, Holmes WE, Burton AJ, Pregitzer KS, Talhelm AF (2008) Simulated atmospheric NO₃ – deposition increases organic matter by slowing decomposition. *Ecological Applications*, **18**, 2016–2027.
- Zak DR, Argiuffo WA, Freedman ZB, Upchurch RA, Entwistle EM, Romanowicz KJ (2019) Anthropogenic N deposition, fungal gene expression, and an increasing soil carbon sink in

the Northern Hemisphere. *Ecology*, **100**, 1–8.

**Chapter 4. Mycorrhizal type determines root-microbial responses to nitrogen
fertilization and recovery**

Under review and formatted for Ecology

4.1 Abstract

Nitrogen (N) fertilization has enhanced the forest land carbon (C) sink by increasing the amount of C stored in soils. Established differences in nutrient acquisition strategies between arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) trees have been shown to influence the magnitude of this N effect on soil C. However, N deposition is declining across many temperate North American forests and little is known about how mycorrhizal-associated strategies in trees may impact recovery. To examine divergent nutrient acquisition responses to N between AM and ECM systems, we developed a conceptual framework based on the idea that N fertilization reduces the C cost of N acquisition. In this model, under N fertilization, ECM trees shift from N mining to N foraging and AM trees would shift from mycorrhizal foraging to root foraging. We expanded on this framework by hypothesizing that initial recovery occurs across a spectrum, where nutrient foraging strategies either (1) persist in their N fertilized state, (2) return to the ambient state, or (3) shift to a new steady state. We tested this framework by examining fine root biomass and morphology, mycorrhizal colonization, and soil enzyme activities in organic horizon, bulk mineral, and rhizosphere mineral soils in AM and ECM dominated plots during the last year of N fertilization and one-year post-fertilization at Bear Brook Watershed, in Maine USA. Overall, our results indicate that N fertilization disrupted the organic N mining nutrient economy of ECM trees by reducing fine root biomass and mycorrhizal colonization while altering root morphology to improve N foraging. In contrast, AM trees appeared to shift from mycorrhizal foraging toward root foraging by reducing mycorrhizal colonization while maintaining root biomass. While AM and ECM mycorrhizal colonization remained lower, rapid root recovery in ECM soils was mirrored by ECM rhizosphere and organic horizon enzyme recovery. ECM bulk and AM soil enzymes activities did not recover. Although these are short-term recovery responses, if

belowground C allocation by ECM trees continues to increase as N deposition declines, priming of soil organic matter can destabilize stored soil C in ECM stands that decades of N deposition have enhanced.

4.2 Introduction

Over the last century, human derived nitrogen (N) deposition has altered the global carbon (C) and N cycles (Galloway et al. 2004, 2008). Across observational and N fertilization studies in temperate forests, reductions in N limitation have reduce soil decomposition and increase soil C storage (Pregitzer et al. 2008, Janssens and al. 2010). Previously, most research has focused on direct N effects on microbial communities to account for these soil C gains (Frey et al. 2004, Waldrop et al. 2004, Freedman et al. 2015, Entwistle et al. 2018, Zak et al. 2019,. Zak et al. 2020). However, recent evidence has shown that as soil available N supply is enhanced, trees shift their nutrient acquisition strategy by investing less C belowground to obtain N which decouples root-microbial interactions, reduces extracellular enzyme activity, and enhances the soil C sink (Janssens et al. 2010, Carrara et al. 2018). While this research has advanced our mechanistic understanding of N impacts on soil C, N deposition is declining across many temperate forests. As such, it remains uncertain the timescale over which these root-microbial interactions will re-couple and the potential implications of this re-coupling on the stability of the “bonus” soil C from N deposition. (Gilliam et al. 2019). This uncertainty also feedbacks on our predictive understanding of the response of soil C to global change owing to a paucity of data available to inform model parametrizations of the recovery of temperate forest ecosystems from decades of N deposition.

Differences in nutrient acquisition strategies between trees that associate with AM and ECM fungi may be a key factor in predicting root-microbial responses to N deposition and recovery. Nutrient acquisition strategies are physiological and morphological mechanisms that trees use to acquire nutrients that range from priming the decomposition of soil organic matter by free-living and symbiotic microbes to mine nutrients, to foraging for nutrients with roots

(Marschener 1998, Lambers et al. 2008). ECM trees acquire N by allocating C belowground to free-living and symbiotic rhizosphere microbes to stimulate the production of extracellular enzymes that mine organic N from soil organic matter (Phillips et al. 2013, Brzostek et al. 2015). Conversely, AM trees acquire N primarily through root and hyphal foraging of inorganic N released by the rapid microbial mineralization of N-rich, AM litter (Phillips et al. 2013). These differences in nutrient acquisition strategies in turn, seem to feedback on the response of soil microbial community composition and their enzyme investment in response to excess N. Specifically, N fertilization alters fungal community composition and rhizosphere enzyme activities to a greater extent in ECM soils; whereas bacterial communities and bulk soil enzyme activities shift to a greater extent in AM soils (Midgley and Phillips 2016, Carrara et al. 2020 in review). However, the underlying changes in nutrient acquisition strategies by trees that likely drive these microbial responses, particularly how roots forage for nutrients are unknown. Adding to this knowledge gap is the uncertainty of how N induced shifts in nutrient acquisition will recover as N inputs decline. Thus, our key question is: to what extent do differences in nutrient acquisition strategies between AM and ECM trees drive belowground responses to excess N and the subsequent trajectory of recovery as N deposition fades across the temperate forest region?

To answer this question, we have developed a conceptual model to capture both the long-term response of AM and ECM nutrient acquisition strategies to N induced reductions in the C cost of N acquisition (Fig. 4-1a) and their initial recovery trajectories once N inputs cease (Fig. 4-1b). This conceptual model builds upon Gilliam et al. (2019) wherein different ecosystem properties (ex. plant diversity, soil microbial communities, C and N cycling) were predicted to have varying lag times in recovery to long-term N deposition. Here we have adapted this model to specifically focus on N-induced changes and subsequent recovery of nutrient acquisition strategies

in AM and ECM dominated soils. This model operates on the assumption that N fertilization will reduce the plant-C cost of N acquisition across both mycorrhizal types. In response to this, ECM trees will decrease belowground C allocation to obtain N as they shift from mining N in soil organic matter to foraging N. This will manifest in declines in both fine root biomass and mycorrhizal colonization. With less roots and less mycorrhizal hyphae, ECM root morphology will change to enhance foraging (i.e. roots will become thinner and more forked; Fig. 4-1a). Large declines in root-C inputs into the rhizosphere will shift microbial resource availability (Carrara et al. 2018, Carrara et al. 2020 in review) which will result in distinct changes in rhizosphere enzyme activities. By contrast, AM trees rely on N foraging by roots and fungal hyphae to meet N demand (rather than N mining; Read and Perez-Moreno 2003, Phillips et al. 2013). As fertilization enhances available N supply, AM trees will reduce belowground C allocation to mycorrhizal fungi as the C cost of these relationships begin outweigh the N benefits. However, AM trees will maintain fine root biomass and morphology in order to meet N demand because AM roots have higher foraging precision than AM fungi (Chen et al. 2016, 2018; Fig. 4-1a). That is, AM trees have been shown to proliferate fine roots in nutrient rich patches of soil at higher rates than AM fungi (Chen et al. 2016, 2018). Smaller N induced declines in belowground C allocation and a lack of root changes in AM soils suggest any N induced shifts in enzyme activity will occur across bulk and rhizosphere soils in tandem.

While this hypothetical response of AM and ECM soils to N fertilization is based on the results from observational gradient and N fertilization experiments, there is much less literature to draw on to develop hypotheses of how these systems will respond to declines in N fertilization. This is further complicated by a lack of consensus on the pace of nutrient acquisition recovery across the limited studies that do exist. For example, while one N removal study showed enhanced

mycorrhizal diversity and fruiting bodies after only four years of reduced N inputs (Boxman et al. 1995), an N fertilization study showed no mycorrhizal fruiting body recovery 47 years after N fertilization ceased ($103 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ for 15 years; Strengbom et al. 2001). Based on this uncertainty, we hypothesized that there were three possible trajectories of initial recovery for each of the root-microbial nutrient acquisition strategies outlined above: the nutrient acquisition strategy would (1) remain at the N induced altered state, (2) quickly return to the pre-N-fertilization ambient state, or (3) transition to a new steady state that is different than the N induced altered state and the pre-N-fertilization ambient state (Fig. 4-1b). This new nutrient acquisition strategy steady state may differ from ambient and N fertilized strategies in fine root biomass, morphology, and extent of mycorrhizal colonization.

We tested this conceptual framework in a network of AM and ECM dominated plots at the Bear Brook Watershed in Maine, USA. Bear Brook is a 27-year watershed-level N fertilization experiment that began in 1989 to examine the impact of acid deposition on forest biogeochemistry. The experiment now focuses on recovery as fertilization ended in 2016. We examined belowground biogeochemical responses to N additions during the last year of fertilization in 2016 and measured initial recovery in 2017, the year after fertilization ceased. To examine the impact of N fertilization and recovery on belowground C allocation, we measured fine root biomass, mycorrhizal colonization, and root morphology. To link plant response to shifts in microbial nutrient demand, we measured extracellular enzyme activity across rhizosphere, bulk, and organic horizon soils.

4.3 Methods

The Bear Brook Watershed in Maine experiment (herein Bear Brook) began in 1989 to examine the impact of acid deposition on forest health and biogeochemistry and is located on the southwest slope of Lead Mountain near Beddington, Maine (44°52'15"N, 68°06'25"W). Bear Brook is made up of two adjacent watersheds, East Bear (11.0ha) and West Bear (10.8ha) which have similar tree species composition, stand age, and soil type (Norton et al. 1999, Wang and Fernandez 1999). Soils are coarse-loamy, isotic, frigid Typic Haplorthods with an average depth of 0.9 m (Norton et al. 1999, Jefts et al. 2004, Fernandez et al. 2007). Precipitation totaled 1014 mm in 2016 and was higher in 2017 at 1086 mm as measured at the nearest weather station in Old Town, ME (National Oceanic & Atmospheric Administration). Mean annual temperature at the site is 5.06°C (Fernandez et al. 2007). Beginning in 1989, West Bear was fertilized at a rate of 25.2 kg N ha⁻¹ yr and 28.8 kg S ha⁻¹ yr⁻¹ in the form of granular (NH₄)₂SO₄. Fertilization was carried out aerially every two months ending in August 2016 after 27 years. In 2016, ambient deposition was 1.81 kg N ha⁻¹ yr and 2.11 kg S ha⁻¹ yr⁻¹. In 2017 one year after fertilization ceased, ambient deposition was 2.21 N ha⁻¹ yr and 2.61 S ha⁻¹ yr⁻¹ in 2017 (National Atmospheric Deposition Program, Greenville, ME; Jefts et al. 2004, Fatemi et al. 2016).

Experimental design:

To examine the extent to which differences in AM and ECM nutrient foraging strategies drive divergent responses to N fertilization and recovery, we established a plot network of 6 AM and 6 ECM dominated 10 x 10 m plots in in the lower elevation hardwood zone of West Bear (fertilized) and East Bear (reference) in 2016. Dominant AM trees were red maple (*Acer rubrum*) and sugar maple (*Acer saccharum*) and dominant ECM trees were American beech (*Fagus grandifolia*), grey birch (*Betula populifolia*), and yellow birch (*Betula alleghaniensis*).

To examine root-microbial responses to N fertilization and recovery across the growing season, soils were sampled in each plot three times over the growing season in the last year of N fertilization (May, July, and September 2016) and during initial recovery after N fertilization ceased in August 2016 (June, July, and September 2017). On each sampling date three 10 x 10 cm organic horizon samples were removed from beneath the litter layer and homogenized from each plot. Beneath the organic horizon, mineral soils were sampled using a 5 cm diameter core to a depth of 15 cm which were also homogenized by plot upon sampling. All soils were put on ice and transferred to West Virginia University for processing and all analyses. To determine the extent to which fine root physiology impacted soil processes, rhizosphere soil was separated from bulk mineral soils using the soil adhesion-method (*sensu* Phillips and Fahey 2005). As roots were removed from soils they were gently shaken and any soil remaining adhered to the roots was carefully removed with forceps and operationally defined as rhizosphere soil. Remaining soil was defined as bulk soil. After root removal, all soils were further homogenized by passing through a 2-mm sieve to remove all rocks and were stored at -80°C prior to analysis. In each sampling month, fine roots (<2 mm) from mineral soils were dried and scaled to g per m² to determine standing fine root biomass.

Root measurements:

Colonization:

To examine the extent to which N fertilization and recovery drove plant investment in fungal symbiosis belowground, mineral soil roots from the peak growing season sampling date (July) in 2016 and 2017 were examined for mycorrhizal colonization. To determine ECM colonization, fine roots from ECM plots were spread out on a petri dish and root tips were examined under a stereoscopic microscope. Percent ECM colonization was calculated as the

percent of total root tips that exhibited visible ECM structures including hyphal mantle and emanating mycelium (Bzdyk et al. 2019).

AM structures are not as clearly visible because they are smaller and can occur within the root. As such, AM roots were first cleared of pigment and stained to determine the presence of AM arbuscules and hyphae. Briefly, roots were cleared of pigment by soaking in 10% potassium hydroxide for 48-96 hours and excess pigment was leached from roots by incubating them in 85% ethanol for 24-48 hours. To remove any remaining pigment, roots were covered in an ammonia/hydrogen peroxide solution for 15 minutes. Finally, roots were acidified for staining in 5% hydrochloric acid and stained for 5-20 minutes in 0.06% trypan blue (Comas et al. 2014). Percent colonization of AM fungi was determined using the grid-intersect method wherein roots were spread across a 1 x 1 cm gridded petri dish and examined with a stereoscopic microscope at each intersect for the presence of arbuscules or hyphae (Giovannetti and Mosse 1980, McGonigle et al. 1990). Percent colonization was calculated as the percent of intersects that were colonized by AM structures.

Root architecture:

To examine differential responses of AM and ECM fine root structure to N fertilization and recovery, we scanned the 2016 and 2017 July root samples from the mineral soil with a LA2400 desktop scanner. These digital images were analyzed to determine root length, average diameter, and number of forks of each sample using WinRhizo software (WinRhizo, Regent Instruments Inc). We standardized these data by expressing all measurements per g of dry root biomass.

Extracellular enzyme activity

To determine the impact of N and recovery on C and nutrients turnover, we assayed the potential activity of hydrolytic enzymes that mine N (N-acetylglucosaminidase; NAG), P (acid phosphatase; AP), and simple C (β -glucosidase; BG) across all soil fractions (i.e., bulk, rhizosphere, organic horizon) and sampling dates. Briefly, 1 g of thawed soil was homogenized in 50 mM sodium acetate buffer (pH 5.0). After, we measured hydrolytic activities using a fluorometric microplate assay with methylumbelliferone-linked substrates (Saiya-Cork et al. 2002). It is important to note that as these assays are performed under substrate saturated conditions and the activities reported are potential activities and thus are a metric of relative microbial investment in nutrient or C acquisition.

Statistical analysis

To examine the response of enzyme activities to N fertilization and recovery, we performed t-tests on seasonal mean enzyme activities within each fraction and mycorrhizal type between reference and fertilized soils for 2016 (N fertilization) and 2017 (initial recovery). We chose to use t-tests rather than two-way ANOVA as we were chiefly interested in soil fraction specific responses within each mycorrhizal type which we expected would occur due to ecological differences (i.e., root density, %C) between organic horizon, rhizosphere, and bulk mineral soils (Finzi et al. 2015). To examine root responses to N fertilization and recovery, we performed t-tests between fertilized and reference July mean root-metrics within mycorrhizal types and soil fractions for 2016 and 2017. To visualize enzyme and root responses to N fertilization and recovery, we calculated effect size of N fertilization by determining the natural log of the response ratio for each metric in each year (i.e. $\ln(\text{fertilized}/\text{reference})$) (Brzostek et al. 2012). We propagated standard error of response ratios as:

Equation (1).

$$\text{S.E.} = \sqrt{\left(\frac{\text{S.E.fertilized}}{\text{mean fertilized}}\right)^2 + \left(\frac{\text{S.E.reference}}{\text{mean reference}}\right)^2}.$$

4.4 Results

Root measurements:

Colonization:

Mycorrhizal colonization was significantly lower in the fertilized watershed compared to the reference watershed for both mycorrhizal types during the last year of fertilization on 2016, and during initial recovery post-fertilization in 2017. In 2016, AM colonization was 25% lower in the fertilized AM plots than the reference plots and ECM colonization was 23% lower in the fertilized ECM plots compared to the reference plots (Fig. 4-2a,b, Table S4-1). In the year after N fertilization ceased, AM colonization remained lower in the fertilized AM plots by 35% compared to reference, and fertilized ECM plots were 32% less colonized than reference ECM plots (Fig. 4-2a,b, Table S4-1).

Root architecture:

Fine root biomass was not significantly different between fertilized and reference AM plots during the last year of fertilization, or during initial recovery. Conversely, during the last year of fertilization ECM fine root biomass was 36.9% lower in fertilized plots than reference (Fig. 4-2b, Table S4-1). In the year after N fertilization ceased there was no significant difference in fine root biomass between fertilized and reference ECM plots (Fig. 4-2b, Table S4-1).

AM root architecture was unaffected by N fertilization and remained unchanged one year post fertilization as average diameter, specific root length, and number of forks per g were not different between fertilized and reference AM plots (Fig. 4-2a, Table S4-1). ECM root architecture was altered by N fertilization, but largely recovered during initial recovery. During the last year of fertilization, ECM roots were 14.2% thinner, were 38.5% longer per g of root (specific root length),

and had 45% marginally ($p=0.056$) more forks in fertilized plots compared to references (Fig. 4-2b, Table S4-1). In the year after fertilization ceased, these differences diminished and there were no significant differences in fine root architecture between fertilized and reference ECM plots (Fig. 4-2b, Table S4-1).

Extracellular enzyme activity:

Nearly three decades of N fertilization resulted in several differences in enzyme responses between AM and ECM dominated plots. In organic horizon soils during the last year of fertilization, BG activity was 99% higher in fertilized AM plots, and 65% higher in fertilized ECM plots compared to references (Fig. 4-3a,b, Table S4-2). AP activity in the organic horizon was not different between fertilized and reference plots in either mycorrhizal type. The only difference in organic horizon enzyme activity between mycorrhizal types was that there was 143% higher NAG activity in fertilized ECM plots compared to references which was not present in AM plots (Fig. 4-3 a,b, Table S4-2). In the year after N fertilization ceased there were no significant differences in organic horizon enzyme activities in either mycorrhizal type between fertilized and reference plots (Fig. 4-3 a,b, Table S4-2).

In bulk soils, N fertilization altered enzyme activities differently between mycorrhizal types. During the last year of N fertilization, AP and NAG activity were not different in AM bulk soils, but BG activity was 27% higher in fertilized plots compared to references (Fig 4-3c, Table S4-2). During the year after N fertilization ceased, AM bulk soil AP and NAG activity were 40% and 61% lower respectively in fertilized plots compared to reference plots, but differences in BG activity were no longer significant (Fig. 4-3c, Table S4-2).

In ECM bulk soils during the last year of fertilization, AP and NAG activity were 40% and 61% lower in fertilized plots compared to reference, but there was no difference in BG activity (Fig. 4-3d, Table S4-2). In the year after N fertilization ceased, AP and NAG activities were still lower in fertilized plots compared to references (54 and 47% respectively), and there remained no difference in BG activity (Fig. 4-3d, Table S4-2).

AM rhizosphere soils responded to fertilization and recovery similarly to AM bulk soils, but ECM bulk and rhizosphere responses varied. During the last year of N fertilization, AM rhizosphere soils exhibited no differences between fertilized and reference plots in AP or NAG activity, but had higher BG activity in fertilized plots (+65%) (Fig. 4-3e, Table S4-2). In the year post fertilization, AM rhizosphere enzymes mirrored AM bulk enzyme shifts with significantly lower AP (-44%) and NAG (-79%) activities in fertilized plots compared to references and the previous enhancement of BG in fertilized plots was no longer significant (Fig. 4-3e, Table S4-2).

In ECM rhizosphere soils during the last year of fertilization, there was 44% less AP activity and 99% more BG activity in fertilized compared to reference plots with no difference in NAG activity (Fig. 4-3f, Table S4-2). In the year after N fertilization ceased, there were no significant differences in enzyme activities in ECM rhizospheres between reference and fertilized plots (Fig. 4-3f, Table S4-2).

4.5 Discussion

As forests in the eastern US recover from decades of elevated atmospheric N deposition, there is a critical need to identify mechanisms that drive soil C response to reduced N inputs in order to improve predictions of the fate of the land C sink. Here we show that differences in nutrient acquisition strategies between AM and ECM trees result in divergent root foraging responses to N fertilization and recovery which feed-back on unique extracellular enzyme shifts. Specifically, our results suggest that reductions in the C cost of N acquisition due to enhanced available N supply shifted the ECM nutrient acquisition strategy from organic N mining toward root foraging of inorganic N by reducing belowground C allocation to fine roots and mycorrhizae, but altering root morphology to enhance N foraging capacity. Conversely, N fertilization appeared to change the inorganic N foraging strategy of AM trees from being driven by mycorrhizae to being driven by roots. After fertilization stopped, ECM roots recovered in tandem with organic horizon and rhizosphere enzyme activities. However, mycorrhizal colonization remained lower in both ECM and AM soils, and AM soil enzymes did not recover which may indicate a new nutrient acquisition state in both mycorrhizal types post-fertilization. Overall, fine root recovery in ECM soils results in an initial recoupling of C and N cycling in organic horizon and rhizosphere soils. Ultimately, if this rapid recovery is coupled with a concurrent increase in inputs to rhizosphere microbes that enhance their activity then this may result in greater losses of N induced “bonus soil C” gains in ECM soils than AM soils.

Reductions in mycorrhizal colonization suggest that enhanced available N supply due to long-term N fertilization drove both AM and ECM trees to reduce C investment belowground to mycorrhizae (Fig. 4-2a). However, how AM and ECM trees optimized nutrient acquisition strategies to maintain N uptake under enhanced N availability varied. In line with our conceptual

model, the results presented here indicate that ECM trees responded to reductions in the C cost of N acquisition by reducing their reliance on mycorrhizal-driven organic N mining and shifted their N acquisition strategy towards foraging for inorganic N with their roots. Under ambient conditions, the ability of ECM hyphae to forage for N compensates for the low surface area of ECM roots compared to non-mycorrhizal roots (Chen et al. 2016, 2018). With less ECM fungi and root biomass, ECM trees enhanced root foraging capacity to maintain N uptake at high N levels through the construction of thinner, more forked roots that had higher ‘specific root length’ (length/mass) (SRL; Fig. 4-3b). Increasing SRL enhances overall surface area and is a morphological tool that has been shown to enhance nutrient uptake capability of ECM roots across climate gradients (Ostonen et al. 2013). These data highlight the plasticity of ECM roots to optimize root morphology to sustain N uptake in a high N environment despite reductions in biomass and mycorrhizal colonization.

In further support of our conceptual model, reductions in mycorrhizal colonization in AM stands without changes in root biomass or morphology indicate that N fertilization allowed AM trees to meet their N demand primarily through root uptake leading to reduced reliance on mycorrhizal foraging (Fig. 4-2a). AM trees primarily obtain N in the inorganic form from the rapid N mineralization of N rich AM litter (Phillips et al. 2013, Midgley and Phillips 2014). AM fungi assist plants in acquiring this N through the formation of vast hyphal networks that forage N from nutrient rich soil patches and pore spaces inaccessible to roots and transfer that N back to the host (Hodge et al. 2001, Govindarajulu et al. 2005, Hodge and Fitter 2010, Smith and Smith 2011, Fellbaum et al. 2012). As N fertilization enhanced the homogeneity of N throughout soil, AM roots were likely able to meet N demand with less mycorrhizal assistance in part because they have higher foraging precision than AM fungi (Chen et al. 2016, 2018). As such, AM trees reduced C

investment in mycorrhizal colonization while maintaining root biomass and morphology (Fig 4-2a). Reductions in AM fungi are common across N fertilization studies and our results suggest that this is not only driven by direct N effects on fungi, but by N induced shifts in nutrient acquisition strategy that optimize plant C investment to acquire N (Treseder 2004).

The faster recovery of ECM root biomass and morphology (Fig. 4-2b) compared to both AM and ECM root colonization (Fig 4-2a,b) may reflect differences in the threshold levels of N availability where mycorrhizal dominant strategies for N foraging are optimal. This relatively fast root recovery is consistent with those of the coniferous (ECM) Netherlands NITREX experiment which showed a recovery of fine root biomass four years after ambient N deposition was removed and soil N levels still remained elevated (Boxman et al. 1995, 1998, Persson et al. 1998). On the other hand, the slower recovery of mycorrhizal colonization may reflect that soil available N supply has yet to decline to a level where the C cost of mycorrhizae balance the N benefits, or because N fertilization reduced fungal biomass to a level that recolonization was not possible within one growing season (Frey et al. 2004, Wallenstein et al. 2006, Morrison et al. 2016). It is also possible that fungal biomass regeneration is restrained by persistently high levels of mobilized aluminum which is toxic to fungi (Firestone et al. 1983, Fernandez et al. 2003, Lawrence et al. 2015). This is in line with results from the NITREX experiment that showed ectomycorrhizal root density did not increase after four years of N deposition exclusion (Persson et al. 1998) and with results from an N fertilization experiment that showed fungal community composition and ECM fruiting body abundance had still not recovered in a Norway Spruce forest after nine years of recovery ($108 \text{ kg N ha}^{-1}\text{yr}^{-1}$ for 28 years) or even after 47 years of recovery in a Scots Pine forest ($103 \text{ kg N ha}^{-1} \text{yr}^{-1}$ for 14 years; Strengbom *et al.*, 2001). While the majority of these previous studies occurred in ECM systems, we show that AM colonization follows the same initial recovery

trajectory. The lack of a rapid recovery in AM and ECM colonization implies that both these forest types may exhibit considerable lags in returning to their pre-fertilized nutrient acquisition strategies in our conceptual model (Fig 4-1b). Coupled with evidence that mycorrhizal recovery lags at even longer timescales in other long-term N fertilization experiments, this result indicates that these systems may ultimately shift into a new steady state where nutrient acquisition is driven primarily by roots (Fig 4-1b).

In AM soils, enzyme responses seem to be driven by stoichiometric shifts in microbial resource availability associated with enhanced available N supply and not by changes in root biomass or morphology. Across AM bulk, rhizosphere, and organic horizon soils, BG activity increased (Fig 4-3c,e) which was possibly the result of enhanced microbial allocation to C acquisition in a high N environment to maintain biomass C:N (Carrara et al. 2020 in review, Sinsabaugh et al. 2002, Schimel and Weintraub 2003, Allison and Vitousek 2005, Allison et al. 2010). After N fertilization ceased, BG activity recovered to ambient levels, but new declines in AP and NAG activity were present in the previously N fertilized bulk and rhizosphere soils (Fig. 4-3c,e). While it is possible that these new declines are due to suppression of microbial activity or shifts in microbial community composition which are often observed in high N content soils (Wallenstein et al. 2006, Frey et al. 2014, Morrison et al. 2016), this also may have been driven by changes in microbial resource demand as N fertilization ceased. That is, as N induced enhancement of BG activity diminished during the year after fertilization ceased, microbes reduced AP and NAG enzyme allocation to maintain a biomass C:N:P ratio of 60:7:1 (Cleveland & Liptzin 2007).

Different responses in enzyme activities across ECM soils suggest that enzyme shifts in response to N fertilization and recovery were driven less by stoichiometry, and more by proximity

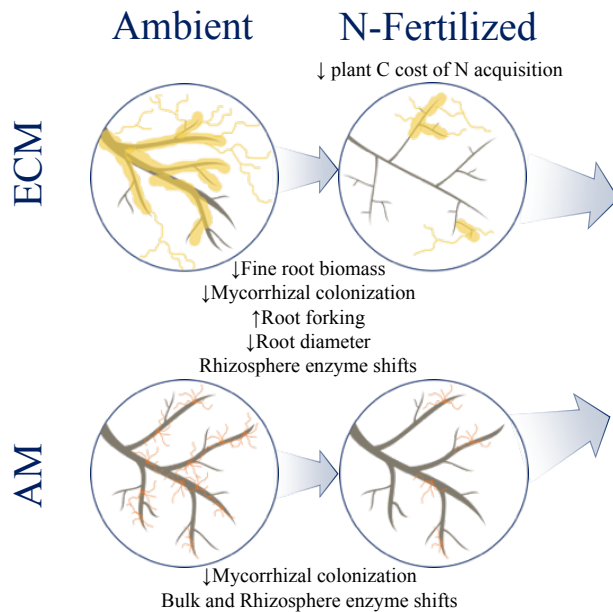
to roots or the density of roots in a given soil fraction (Brzostek et al. 2013, Yin et al. 2014). While N fertilization altered enzyme activities across all ECM soils (Fig. 4-3b,d,f), organic horizon and rhizosphere soil enzyme activities recovered after N fertilization ceased (Fig. 4-3b,f). Assuming root-C inputs increased with biomass, we posit that this enzyme recovery was due to the close proximity of rhizosphere to roots and the high root density in organic horizon soils which, coupled with declines in N availability, may have shifted microbial resource demand nearer to ambient levels. In line with this, a potential mechanism explaining why bulk soil enzyme activities remained in the N fertilized state may be that microbes in bulk soils were cut off spatially from any potential increase in root-C inputs. Further limiting bulk enzyme recovery, consistently lower mycorrhizal colonization in N fertilized soils could limit C transfer from fungal hyphae that extend into the bulk soil (Meier et al. 2015). While bulk soil enzymes did not initially recover, root proliferation and the subsequent movement and expansion of the rhizosphere and hyphosphere may homogenize root-C inputs throughout bulk soils over time which may eventually shift microbial resource demand toward ambient levels and lead to enzyme recovery.

During initial recovery, the ECM nutrient acquisition strategy seems to have reverted from N induced root foraging back to a new steady state of N acquisition without ECM fungi. With reduced competition for nutrients between ECM fungi and saprotrophic fungi, enhanced root-C inputs may lead to substantially higher priming of soil organic matter than under original ambient conditions (Averill et al. 2014). This new steady state has the potential to lead to considerable C losses from ECM forest soils which have steadily gained “bonus C” due to decades of reduced decomposition due to N deposition (Averill et al. 2018). In AM soils, the lack of enhanced C inputs during initial recovery may limit new C losses, but ultimately how these soils will recover over longer periods is unknown. While these mechanism for explaining divergent nutrient acquisition

and enzyme responses between AM and ECM trees is intriguing, we stress that it is unclear whether these shifts will persist over longer timescales or if they will continue to fluctuate until they reach a new steady state or return to the ambient state. In addition to N effects, it is likely that nutrient acquisition strategies will respond to warming induced increases in N mineralization (Contosta et al. 2011) and drought stress on roots (Rennenberg et al. 2006) as many temperate forests become warmer and drier (Allen et al. 2010, Choat et al. 2012). As such, further monitoring of biogeochemical responses to N fertilization cessation in conjunction with other climate drivers is critical to make predictions on the fate of this C sink.

Tables and Figures

a. Response to Fertilization



b. Recovery Hypotheses

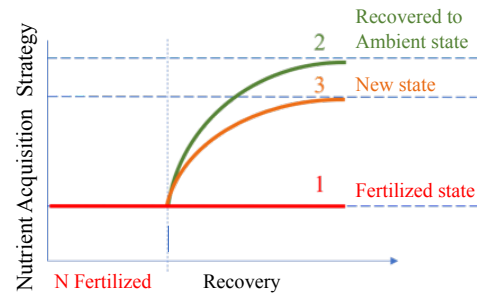


Figure 4-1. Divergent responses of AM and ECM nutrient acquisition strategies to N fertilization and potential recovery trajectories. (A) We hypothesized that under N fertilization ECM roots biomass would decline, roots would become thinner and more forked, and mycorrhizal colonization would decline owing to shifts in nutrient acquisition strategy from N mining to N foraging. This would drive distinct shifts in rhizosphere enzyme activity. Conversely, AM root biomass would not decline, but mycorrhizal colonization would decline owing to shifts in mycorrhizal N foraging to root N foraging. This would result in similar shifts across rhizosphere and bulk soils. (B) As N fertilization stops, these nutrient acquisition metrics may (1) stay in their N fertilized state, (2) return to ambient state, or (3) shift to a new steady state that is not the N fertilized or ambient state.

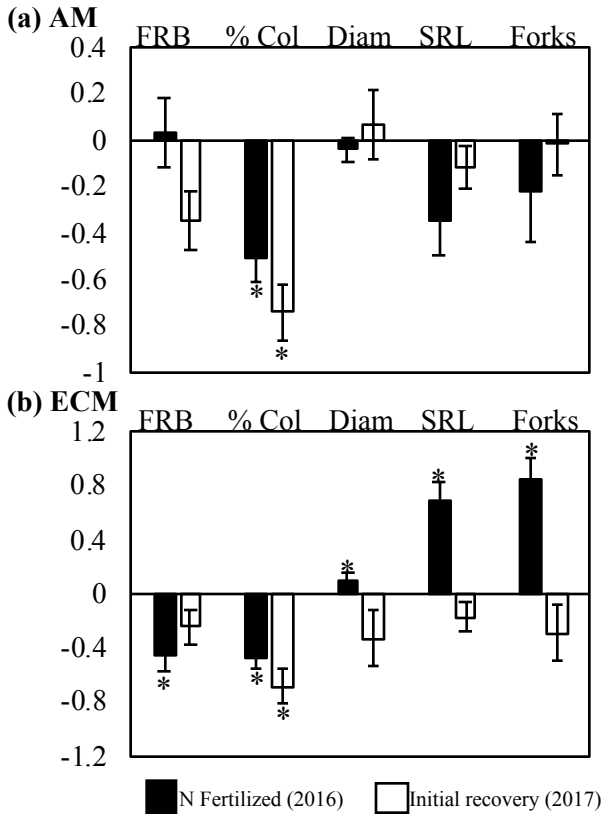


Figure 4-2. Figure 2. ECM trees reduce belowground C allocation and alter root morphology to a greater extent than AM trees under N fertilization, but partially recover during the year after fertilization ceased. Fine root biomass (FRB) values are overall seasonal mean response ratios (response ratio \pm propagated SE) of three measurements taken over the growing season during the last year of fertilization (2016; black bars) and during the year after fertilization ceased (2017; open bars). Percent colonization (%Col), average diameter (Diam), specific root length (SRL), and forks per gram of root (Forks) values are July response ratios measured in 2016 and 2017 presented with propagated standard errors. Asterisks indicate $p < 0.05$ for t-tests between N fertilized and reference soils within each year in AM (a) and ECM (b) soils,

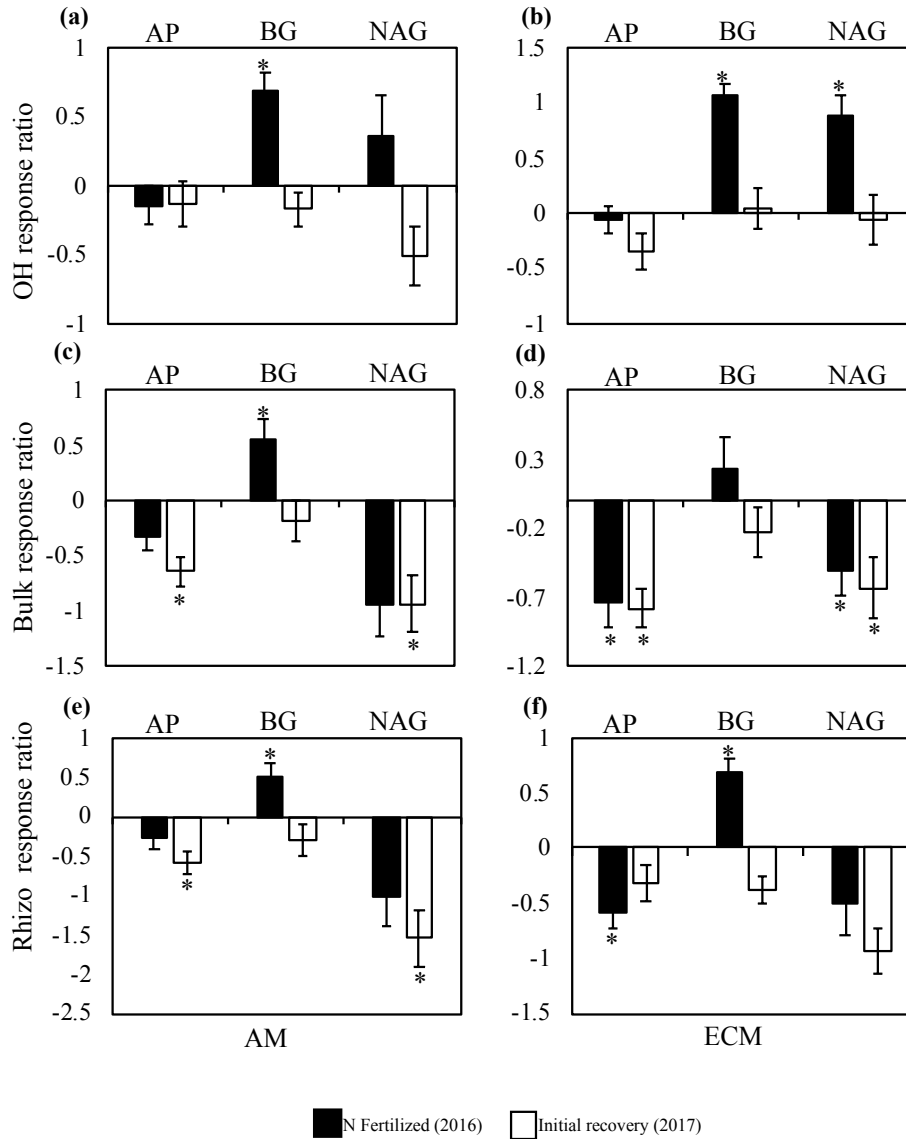


Figure 4-3. AM bulk and rhizosphere soils responded the same to N fertilization and have the same enzyme shifts during the year after fertilization ceased, however ECM bulk and rhizosphere enzyme response and recovery to N varies. Values are overall mean seasonal response ratios (response ratio \pm propagated SE) of acid phosphatase (AP), beta-glucosidase (BG) and N-acetylglucosaminidase in AM organic horizon (a), ECM organic horizon (b), AM bulk (c), ECM Bulk (d), AM rhizosphere (e), and ECM rhizosphere (f). Measurements were taken three times over the growing season during the last year of fertilization (2016; black bars) and during the year after fertilization ceased (2017; open bars). Asterisks indicate $p < 0.05$ for t-tests between N fertilized and reference plots during each year and within mycorrhizal type ($n=6$ plots).

4.6 Literature Cited

- Allen, C. D., A. K. Macalady, H. Chenchouni, D. Bachelet, N. McDowell, M. Vennetier, T. Kitzberger, A. Rigling, D. D. Breshears, E. H. (Ted. Hogg, P. Gonzalez, R. Fensham, Z. Zhang, J. Castro, N. Demidova, J. H. Lim, G. Allard, S. W. Running, A. Semerci, and N. Cobb. 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest Ecology and Management* 259:660–684.
- Allison, S. D., and P. M. Vitousek. 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology and Biochemistry* 37:937–944.
- Allison, S. D., M. D. Wallenstein, and M. A. Bradford. 2010. Soil-carbon response to warming dependent on microbial physiology. *Nature Geoscience* 3:336–340.
- Averill, C., M. C. Dietze, and J. M. Bhatnagar. 2018. Continental-scale nitrogen pollution is shifting forest mycorrhizal associations and soil carbon stocks. *Global Change Biology* 24:4544–4553.
- Averill, C., B. L. Turner, and A. C. Finzi. 2014. Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature* 505:543–5.
- Boxman, A. W., K. Blanck, T. E. Brandrud, B. A. Emmett, P. Gundersen, R. F. Hogervorst, O. J. Kjønaas, H. Persson, and V. Timmermann. 1998. Vegetation and soil biota response to experimentally-changed nitrogen inputs in coniferous forest ecosystems of the NITREX project. *Forest Ecology and Management* 101:65–79.
- Boxman, A. W., D. van Dam, H. F. G. van Dijk, R. F. Hogervorst, and C. J. Koopmans. 1995. Ecosystem responses to reduced nitrogen and sulphur inputs into two coniferous forest stands in the Netherlands. *Forest Ecology and Management* 71:7–29.
- Brzostek, E. R., J. M. Blair, J. S. Dukes, S. D. Frey, S. E. Hobbie, J. M. Melillo, R. J. Mitchell,

- E. Pendall, P. B. Reich, G. R. Shaver, A. Stefanski, M. G. Tjoelker, and A. C. Finzi. 2012. The effect of experimental warming and precipitation change on proteolytic enzyme activity: Positive feedbacks to nitrogen availability are not universal. *Global Change Biology* 18:2617–2625.
- Brzostek, E. R., D. Dragoni, Z. A. Brown, and R. P. Phillips. 2015. Mycorrhizal type determines the magnitude and direction of root-induced changes in decomposition in a temperate forest. *New Phytologist* 206:1274–1282.
- Brzostek, E. R., A. Greco, J. E. Drake, and A. C. Finzi. 2013. Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. *Biogeochemistry* 115:65–76.
- Bzdyk, R. M., J. Olchowik, M. Studnicki, J. A. Nowakowska, T. Oszako, A. Urban, and D. Hilszczańska. 2019. Ectomycorrhizal colonisation in declining oak stands on the Krotoszyn Plateau, Poland. *Forests* 10.
- Carrara, J. E., C. A. Walter, J. S. Hawkins, W. T. Peterjohn, C. Averill, and E. R. Brzostek. 2018. Interactions among plants, bacteria, and fungi reduce extracellular enzyme activities under long-term N fertilization. *Global Change Biology* 24:2721–2734.
- Chen, W., R. T. Koide, T. S. Adams, J. L. DeForest, L. Cheng, and D. M. Eissenstat. 2016. Root morphology and mycorrhizal symbioses together shape nutrient foraging strategies of temperate trees. *Proceedings of the National Academy of Sciences of the United States of America* 113:8741–8746.
- Chen, W., R. T. Koide, and D. M. Eissenstat. 2018a. Nutrient foraging by mycorrhizas: From species functional traits to ecosystem processes. *Functional Ecology* 32:858–869.
- Chen, W., R. T. Koide, and D. M. Eissenstat. 2018b. Root morphology and mycorrhizal type

- strongly influence root production in nutrient hot spots of mixed forests. *Journal of Ecology* 106:148–156.
- Choat, B., S. Jansen, T. J. Brodribb, H. Cochard, S. Delzon, R. Bhaskar, S. J. Bucci, T. S. Feild, S. M. Gleason, U. G. Hacke, A. L. Jacobsen, F. Lens, H. Maherali, J. Martínez-Vilalta, S. Mayr, M. Mencuccini, P. J. Mitchell, A. Nardini, J. Pittermann, R. B. Pratt, J. S. Sperry, M. Westoby, I. J. Wright, and A. E. Zanne. 2012. Global convergence in the vulnerability of forests to drought. *Nature* 491:752–755.
- Comas, L. H., H. S. Callahan, and P. E. Midford. 2014. Patterns in root traits of woody species hosting arbuscular and ectomycorrhizas: Implications for the evolution of belowground strategies. *Ecology and Evolution* 4:2979–2990.
- Contosta, A. R., S. D. Frey, and A. B. Cooper. 2011. Seasonal dynamics of soil respiration and N mineralization in chronically warmed and fertilized soils. *Ecosphere* 2:1–21.
- Elizabeth M. Entwistle, a, b Karl J. Romanowicz, a, c William A. Argiroff, a Zachary B. Freedman, d J. Jeffrey Morris, B., and C. Donald R. Zaka. 2020. Anthropogenic N Deposition Alters the Composition of Expressed Class II Fungal Peroxidases 84:1–16.
- Entwistle, E. M., D. R. Zak, and W. A. Argiroff. 2018. Anthropogenic N deposition increases soil C storage by reducing the relative abundance of lignolytic fungi. *Ecological Monographs* 88:225–244.
- Fatemi, F. R., I. J. Fernandez, K. S. Simon, and D. B. Dail. 2016. Nitrogen and phosphorus regulation of soil enzyme activities in acid forest soils. *Soil Biology and Biochemistry* 98:171–179.
- Fellbaum, C. R., E. W. Gachomo, Y. Beesetty, S. Choudhari, G. D. Strahan, P. E. Pfeffer, E. T. Kiers, and H. Bücking. 2012. Carbon availability triggers fungal nitrogen uptake and

- transport in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 109:2666–2671.
- Fernandez, I. J., J. E. Karem, S. A. Norton, and L. E. Rustad. 2007. Temperature, soil moisture, and streamflow at the Bear Brook Watershed in Maine (BBWM). *Maine Agricultural and Forest Experiment Station Technical Bulletin* 196.
- Fernandez, I. J., L. E. Rustad, S. A. Norton, J. S. Kahl, and B. J. Cosby. 2003. Experimental Acidification Causes Soil Base-Cation Depletion at the Bear Brook Watershed in Maine. *Soil Science Society of America Journal* 67:1909–1919.
- Finzi, A. C., R. Z. Abramoff, K. S. Spiller, E. R. Brzostek, B. A. Darby, M. A. Kramer, and R. P. Phillips. 2015. Rhizosphere processes are quantitatively important components of terrestrial carbon and nutrient cycles. *Global Change Biology* 21:2082–2094.
- Firestone, M. K., K. Killham, and J. G. McColl. 1983. Fungal toxicity of mobilized soil aluminum and manganese. *Applied and Environmental Microbiology* 46:758–761.
- Freedman, Z. B., K. J. Romanowicz, R. A. Upchurch, and D. R. Zak. 2015. Differential responses of total and active soil microbial communities to long-term experimental N deposition. *Soil Biology and Biochemistry* 90:275–282.
- Frey, S. D., M. Knorr, J. L. Parrent, and R. T. Simpson. 2004. Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management* 196:159–171.
- Frey, S. D., S. Ollinger, K. Nadelhoffer, R. Bowden, E. Brzostek, A. Burton, B. A. Caldwell, S. Crow, C. L. Goodale, A. S. Grandy, A. Finzi, M. G. Kramer, K. Lajtha, J. LeMoine, M. Martin, W. H. McDowell, R. Minocha, J. J. Sadowsky, P. H. Templer, and K. Wickings. 2014. Chronic nitrogen additions suppress decomposition and sequester soil carbon in

- temperate forests. *Biogeochemistry* 121:305–316.
- Galloway, J. N., F. J. Dentener, D. G. Capone, E. W. Boyer, R. W. Howarth, S. P. Seitzinger, G. P. Asner, C. C. Cleveland, P. A. Green, E. A. Holland, D. M. Karl, A. F. Michaels, J. H. Porter, A. R. Townsend, and C. J. Vörosmary. 2004. Nitrogen Cycles: Past, Present, and Future. *Biogeochemistry* 70:153–226.
- Galloway, J. N., A. R. Townsend, J. W. Erisman, M. Bekunda, Z. Cai, J. R. Freney, L. a Martinelli, S. P. Seitzinger, and M. a Sutton. 2008. Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions. *Science* 320:889–892.
- Gilliam, F. S., D. A. Burns, C. T. Driscoll, S. D. Frey, G. M. Lovett, and S. A. Watmough. 2019. Decreased atmospheric nitrogen deposition in eastern North America: Predicted responses of forest ecosystems. *Environmental Pollution* 244:560–574.
- Giovannetti, M., and B. Mosse. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist* 84:489–500.
- Govindarajulu, M., P. E. Pfeffer, H. Jin, J. Abubaker, D. D. Douds, J. W. Allen, H. Bücking, P. J. Lammers, and Y. Shachar-Hill. 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* 435:819–823.
- Hodge, A., C. D. Campbell, and A. H. Fitter. 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* 413:297–299.
- Hodge, A., and A. H. Fitter. 2010. Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *Proceedings of the National Academy of Sciences of the United States of America* 107:13754–13759.
- Janssens, I. A., and et al. 2010. Reduction of forest soil respiration in response to nitrogen

- deposition. *Nature Geoscience* 3:315–322.
- Jefts, S., I. J. Fernandez, L. E. Rustad, and D. B. Dail. 2004. Decadal responses in soil N dynamics at the Bear Brook Watershed in Maine, USA. *Forest Ecology and Management* 189:189–205.
- Lambers, H., J. A. Raven, G. R. Shaver, and S. E. Smith. 2008. Plant nutrient-acquisition strategies change with soil age. *Trends in Ecology and Evolution* 23:95–103.
- Lawrence, G. B., P. W. Hazlett, I. J. Fernandez, R. Ouimet, S. W. Bailey, W. C. Shortle, K. T. Smith, and M. R. Antidormi. 2015. Declining Acidic Deposition Begins Reversal of Forest-Soil Acidification in the Northeastern U.S. and Eastern Canada. *Environmental Science and Technology* 49:13103–13111.
- Marschener, H. 1998. Role of root growth, arbuscular mycorrhiza, and root exudates for the efficiency in nutrient acquisition. *Field Crops Research* 56:203–207.
- McGonigle, T. P., M. H. Miller, D. G. Evans, G. L. Fairchild, and J. A. Swan. 1990. A New Method which Gives an Objective Measure of Colonization of Roots by Vesicular-Arbuscular Mycorrhizal Fungi. *New Phytologist* 115:495–501.
- Meier, I. C., S. G. Pritchard, E. R. Brzostek, M. L. McCormack, and R. P. Phillips. 2015. The rhizosphere and hyphosphere differ in their impacts on carbon and nitrogen cycling in forests exposed to elevated CO₂. *New Phytologist* 205:1164–1174.
- Midgley, M. G., and R. P. Phillips. 2014. Mycorrhizal associations of dominant trees influence nitrate leaching responses to N deposition:241–253.
- Midgley, M. G., and R. P. Phillips. 2016. Resource stoichiometry and the biogeochemical consequences of nitrogen deposition in a mixed deciduous forest. *Ecology* 97:3369–3377.
- Morrison, E. W., S. D. Frey, J. J. Sadowsky, L. T. A. van Diepen, W. K. Thomas, and A. Pringle.

2016. Chronic nitrogen additions fundamentally restructure the soil fungal community in a temperate forest. *Fungal Ecology* 23:48–57.
- Norton, S., J. Kahl, I. Fernandez, T. Haines, L. Rustad, S. Nodvin, J. Scofield, T. Strickland, H. Erickson, P. Wigington, and J. Lee. 1999. The Bear Brook Watershed, Maine (BBWM), USA. *Environmental Monitoring and Assessment* 55:7–51.
- Ostonen, I., K. Rosenvald, H. S. Helmisaari, D. Godbold, K. Parts, V. Uri, and K. Lõhmus. 2013. Morphological plasticity of ectomycorrhizal short roots in *Betula* sp and *Picea abies* forests across climate and forest succession gradients: Its role in changing environments. *Frontiers in Plant Science* 4:1–10.
- Persson, H., K. Ahlström, and A. Clemensson-Lindell. 1998. Fine-root response to nitrogen addition and removal at Gårdsjön — results from ingrowth cores. *Root Demographics and Their Efficiencies in Sustainable Agriculture, Grasslands and Forest Ecosystems*:433–443.
- Phillips, R. P., E. Brzostek, and M. G. Midgley. 2013. The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist* 199:41–51.
- Phillips, R. P., and T. J. Fahey. 2005. Patterns of rhizosphere carbon flux in sugar maple (*Acer saccharum*) and yellow birch (*Betula allegheniensis*) saplings. *Global Change Biology* 11:983–995.
- Pregitzer, K. S., A. J. Burton, D. R. Zak, and A. F. Talhelm. 2008. Simulated chronic nitrogen deposition increases carbon storage in Northern Temperate forests. *Global Change Biology* 14:142–153.
- Read, D. J., and J. Perez-Moreno. 2003. Mycorrhizas and nutrient cycling in ecosystems - A journey towards relevance?

- Rennenberg, H., F. Loreto, A. Polle, F. Brilli, S. Fares, R. S. Beniwal, and A. Gessler. 2006. Physiological responses of forest trees to heat and drought. *Plant Biology* 8:556–571.
- Saiya-Cork, K. R., R. L. Sinsabaugh, and D. R. Zak. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. *Soil Biology and Biochemistry* 34:1309–1315.
- Schimel, J. P., and M. N. Weintraub. 2003. The implications of exoenzyme activity on microbial carbon and nitrogen limitation in soil: A theoretical model. *Soil Biology and Biochemistry* 35:549–563.
- Sinsabaugh, R. L., M. M. Carreiro, and D. A. Repert. 2002. Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. *Biogeochemistry* 60:1–24.
- Smith, S. E., and F. A. Smith. 2011. Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales. *Annual Review of Plant Biology* 62:227–250.
- Strengbom, J., A. Nordin, T. Näsholm, and L. Ericson. 2001. Slow recovery of boreal forest ecosystem following decreased nitrogen input. *Functional Ecology* 15:451–457.
- Treseder, K. K. 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO₂ in field studies. *New Phytologist* 164:347–355.
- Waldrop, M. P., D. R. Zak, R. L. Sinsabaugh, M. Gallo, and C. Lauber. 2004. Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. *Ecological Applications* 14:1172–1177.
- Wallenstein, M. D., S. McNulty, I. J. Fernandez, J. Boggs, and W. H. Schlesinger. 2006. Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term

experiments. *Forest Ecology and Management* 222:459–468.

Wang, Z., and I. Fernandez. 1999. Soil Type and Forest Vegetation Influences on Forest Floor Nitrogen Dynamics at the Bear Brook Watershed in Maine (BBWM). *Environmental Monitoring and Assessment* 55:221–234.

Yin, H., E. Wheeler, and R. P. Phillips. 2014. Root-induced changes in nutrient cycling in forests depend on exudation rates. *Soil Biology and Biochemistry* 78:213–221.

Zak, D. R., W. A. Argiroff, Z. B. Freedman, R. A. Upchurch, E. M. Entwistle, and K. J. Romanowicz. 2019. Anthropogenic N deposition, fungal gene expression, and an increasing soil carbon sink in the Northern Hemisphere. *Ecology* 100:1–8.

Chapter 5. Conclusions: Advancing our understanding of the role of root-microbial interactions in mediating soil C and nutrient cycling in response to long-term N deposition and recovery

Summary of results

My dissertation examined the role of plant-microbial interactions in mediating the impacts of long-term N deposition on temperate forest soil C and nutrient cycling and how root-microbial interactions drive initial recovery as N deposition declines. To do this I leveraged two long-term watershed level N fertilization experiments to answer three broad questions: 1) To what extent do plant microbial interactions mediate soil carbon and nutrient cycling responses to long-term nitrogen fertilization?; 2) To what extent do differences in nutrient acquisition strategies between ECM and AM trees explain variability in soil carbon and nutrient cycling responses to long-term nitrogen deposition?; and 3) To what extent do differences in nutrient acquisition strategies between ECM and AM mycorrhizal trees drive the trajectory of soil C and nutrient cycling recovery as N deposition fades across the temperate forest region?

Overall, I found evidence that N induced enhancements in soil C were not due solely to a direct negative impact on soil fungi, but rather the result of reduced root-C transfer to saprotrophic and mycorrhizal microbes which, in turn, appeared to limit extracellular enzyme production. Additionally, I found that ECM and AM dominated soils responded divergently to N fertilization wherein bacterial communities shifted to a greater extent in AM soils and fungal communities shifted to a greater extent in ECM soils which ultimately fed back on enhanced C relative to N mining enzyme activities in AM bulk soils and in ECM rhizosphere soils. Finally, I found that N fertilization appeared to shift the ECM nutrient acquisition strategy from N mining to N foraging and the AM nutrient acquisition strategy from mycorrhizal foraging toward root foraging. After N fertilization ceased, ECM roots recovered in tandem with organic horizon and rhizosphere enzyme activities whereas mycorrhizal colonization did not recover in either mycorrhizal type suggesting a new root-dominated nutrient acquisition steady state in AM and ECM soils as long-term N

deposition declines. Together, these three major results provide evidence that soil C and nutrient cycling responses to N deposition and recovery are in-part driven by changes in the strength of plant-microbial interactions rather than a direct impact of N on microbes or trees. Below, I provide more detail on how each chapter of this dissertation answers one of the broad research questions above.

Chapter 2 – To what extent do plant microbial interactions mediate soil carbon and nutrient cycling responses to long-term nitrogen fertilization?

In *Chapter 2*, I examined the extent to which couplings among plant, fungal, and bacterial responses to N fertilization alter extracellular enzyme activities (the proximate drivers of soil C loss and retention). To do this I measured fungal and bacterial community composition, mycorrhizal colonization, root biomass and morphology, and extracellular enzyme activity in the rhizosphere, bulk, and organic horizon of soils sampled from a long-term (>25 years), whole-watershed, N fertilization experiment at the Fernow Experimental Forest in West Virginia, USA. In N fertilized soils I observed declines in fine root biomass (24.7%), root morphology, and arbuscular mycorrhizal (AM) colonization (55.9%) which was likely a tree response to N induced reductions in the C cost of N acquisition. Moreover, I found that consistent declines in extracellular enzyme activity were significantly correlated with a shift in bacterial community composition, but not fungal community composition. This bacterial community shift was also correlated with reduced AM fungal colonization indicating that declines in plant C investment below-ground drive the response of bacterial community structure and function to N fertilization. Collectively, I found that enzyme activity responses to N fertilization are not solely driven by direct impacts on fungi, but instead reflect a whole ecosystem response, whereby declines in the strength of belowground C investment to gain N cascade through the soil environment.

Chapter 3 – To what extent do differences in nutrient acquisition strategies between ectomycorrhizal and arbuscular mycorrhizal trees explain variability in soil carbon and nutrient cycling responses to long-term nitrogen deposition?

In *Chapter 2*, my results revealed that N fertilization can slow soil C turnover by reducing root-C transfer to microbes belowground, and that reductions in extracellular enzymes were correlated with shifts in bacterial but not fungal community composition. As such, in *Chapter 3* my main objective was to uncover mechanisms that explain why soil C turnover declines are linked to fungal shifts in some forests (Frey *et al.*, 2004; Waldrop *et al.*, 2004; Freedman *et al.*, 2015; Entwistle *et al.*, 2018; Zak *et al.*, 2019; Entwistle *et al.*, 2020) and bacterial shifts in others (Freedman & Zak, 2014; Freedman *et al.*, 2016) and how these divergent microbial responses may drive different soil C cycling responses across forests. This led me to the hypothesis that unique nutrient cycling responses to N between AM and ECM dominated soils may account for variability in soil C response across studies. Because ECM trees obtain N primarily through fungal N mining, I expected that the largest N induced responses of C and N cycling to occur in ECM rhizospheres and be driven by fungi. Conversely, because AM trees rely on bacterial scavengers in bulk soils to cycle N, I predicted the largest AM responses to be driven by shifts in bacteria and occur in bulk soils. To test this, I measured bacterial and fungal community composition as well as a suite of extracellular enzyme activities in AM and ECM dominated reference and fertilized plots at Bear Brook Watershed, ME USA.

Confirming my hypothesis, I found that fungal community composition shifted across ECM soils, but bacterial communities shifted across AM soils. These shifts were mirrored by enhanced C relative to N mining enzyme activity in both mycorrhizal types, but this occurred in different soil fractions. In ECM stands these shifts occurred in rhizosphere soils, but in AM stands

they occurred in bulk soils. As rhizosphere soils account for only a small portion of total soil volume relative to bulk soils, coupled with a decline in C relative to N enzyme activity in ECM OH soils, I posit that this may partly explain why ECM soils appear to store more C than AM soils as N inputs increase (Averill *et al.*, 2018). It is clear that even small changes in the relative abundance of tree species across the landscape can have large implications for how forest soil C and nutrient cycling will respond to N. Predominant shifts of either soil fungi or bacteria depending on dominant mycorrhizal type enhances our understanding of the underlying mechanisms that control observed differences in AM and ECM C and nutrient cycling. In addition, these observed shifts help to confirm the existing hypothesized dichotomy of fungal driven decomposition in ECM stands and bacterial driven decomposition in AM stands. Differences in N induced changes in microbial community composition between AM and ECM soils may have additional implications for the rate of soil C accumulation in mineral-bound forms as microbial metabolism is hypothesized to be the rate-limiting driver of protected C formation (Cotrufo *et al.*, 2013). As such, changes in fungal vs bacterial communities will likely have different impacts on overall community metabolism which may result in divergent trends in mineral bound C formation and retention. Regardless, since N induced enzyme activity changes are indicative of soil C turnover, ECM soils will likely continue to gain C at a faster rate than AM soils across forests where N deposition is expected to remain high or increase.

Chapter 4 – To what extent do differences in nutrient acquisition strategies between ectomycorrhizal and arbuscular mycorrhizal trees drive the trajectory of recovery as N deposition fades across the temperate forest region?

Results from *Chapters 2-3* indicated that N fertilization can alter soil C and nutrient cycling not only through direct impacts on microbial communities, but through a decoupling of plants and

microbes belowground. Further, *Chapter 3* provided evidence that differences in nutrient cycling strategies between ECM and AM trees resulted in divergent changes in N induced enzyme activities which may in part explain variation in soil C responses to N deposition across studies. As N deposition is declining across the temperate forest region, there is a critical need to examine to what extent and over what timescales root-microbial interactions recover, and further how this recovery will shape soil C and nutrient cycling patterns in the future of temperate forests. As such in *Chapter 4* I developed a conceptual model based on known differences between ECM and AM trees, that predicted that under N fertilization ECM trees would shift in nutrient acquisition strategy from N mining to N foraging and AM trees would shift from mycorrhizal N foraging to root N foraging. As N fertilization stopped, I predicted that nutrient acquisition strategies would recover in one of three trajectories by either (1) persisting in their N fertilized state, (2) returning to the ambient state, or (3) shifting to a new steady state. To test these hypotheses I measured mycorrhizal colonization, root morphology, and enzyme activities in OH, bulk, and rhizosphere soils during the last year of N fertilization and one-year after fertilization stopped in the same ECM and AM plots measured in *Chapter 3* at Bear Brook Watershed, ME USA.

In support of my hypothesis, I found that N fertilization appeared to shift ECM trees from N mining to N foraging by reducing mycorrhizal colonization (-23%) and root biomass (-37%) while altering root morphology to enhance foraging capacity. AM trees appeared to shift from mycorrhizal foraging toward root foraging by reducing mycorrhizal colonization (-25%) while maintaining root biomass. While AM and ECM mycorrhizal colonization remained lower, rapid root recovery in ECM soils was mirrored by ECM rhizosphere and organic horizon enzyme recovery. ECM bulk and AM soil enzymes activities did not recover. Overall, it appeared that as N fertilization ceased, both ECM and AM trees shifted to a new nutrient acquisition steady-state

dominated by root foraging rather than N mining or mycorrhizal foraging. While it is possible that N induced reductions in mycorrhizal fungi may decrease soil C turnover due to reduced ECM fungal extracellular enzyme production and reduced AM hyphal exploration, it is also possible that reductions in mycorrhizal fungi can have the opposite effect. Decomposition may increase if reductions in mycorrhizae limit competition for resources with free-living microbes (Gadgil & Gadgil, 1975; Averill *et al.*, 2014). The results of *Chapter 3* showed that fungal community structure is particularly sensitive to N in ECM stands whereas bacterial communities are sensitive to N in AM stands. As such, reduced competition between mycorrhizal fungi and free-living fungi in ECM soils and bacteria in AM soils will likely have different impacts on soil C and nutrient cycling. Regardless of changes in competition, if belowground C allocation by ECM trees continues to increase as N deposition declines, priming of soil organic matter can destabilize stored soil C in ECM stands that decades of N deposition have enhanced and this priming effect may also be exacerbated by warming temperatures across this region.

Implications for models and the future

Current earth system models are tuned so that trees respond to enhanced N supply by increasing biomass uniformly above and belowground and thus, modelled N induced soil C enhancements are driven by increased aboveground inputs (Ise *et al.*, 2010; Todd-Brown *et al.*, 2013). However, the results presented herein add to a growing body of evidence that N induced reductions in the C cost of N acquisition drive reductions in belowground C allocation by trees. This appears to result in a decoupling of roots and microbes wherein reduced microbial C availability leads to declines in extracellular enzyme production which may partially account for declines in respiration and soil C gains observed across N fertilization and observational studies (Janssens & al., 2010; Averill *et al.*, 2018). Further, these results show that mycorrhizal type likely

modulates the extent of this response as belowground C allocation in ECM trees is particularly sensitive to enhanced N supply. As the incorporation of plant-microbial responses based on mycorrhizal associations have been shown to enhance the predictive capability of earth system models (Sulman *et al.*, 2019), the N response mechanisms and data presented herein are valuable in parameterizing these models to improve climate predictions.

Finally, I provide evidence that as N deposition declines, N induced shifts in nutrient acquisition by ECM and AM trees do not initially revert back to ambient conditions, but appear to move toward to a new steady state of root N foraging (rather than ECM N mining or AM mycorrhizal foraging). Specifically, my results show that this new steady is characterized by rapid ECM root recovery and enhanced C allocation belowground to fine roots. If this recovery response persists over longer time-scales, enhanced belowground C transfer during recovery may effectively ‘re-start’ the soil C priming process which was muted by enhanced N. This effect may be even more pronounced as reduced mycorrhizal colonization likely limits competition for resources with saprotrophic fungi which can restrict decomposition under ambient conditions (Gadgil & Gadgil, 1975; Averill *et al.*, 2014). Because ECM soils tend to store more C than AM soils (Craig *et al.*, 2018), and because N fertilization has enhanced this C pool to a greater extent than AM stands (Averill *et al.*, 2018), these potential soil C losses can have large implications for atmospheric C concentrations.

5.2 Literature Cited

- Averill C, Turner BL, Finzi AC (2014) Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature*, **505**, 543–545.
- Averill C, Dietze MC, Bhatnagar JM (2018) Continental-scale nitrogen pollution is shifting forest mycorrhizal associations and soil carbon stocks. *Global Change Biology*, **24**, 4544–4553.
- Cotrufo MF, Wallenstein MD, Boot CM, Deneff K, Paul E (2013) The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: Do labile plant inputs form stable soil organic matter? *Global Change Biology*, **19**, 988–995.
- Craig ME, Turner BL, Liang C, Clay K, Johnson DJ, Phillips RP (2018) Tree mycorrhizal type predicts within-site variability in the storage and distribution of soil organic matter. *Global Change Biology*, **24**, 3317–3330.
- Elizabeth M. Entwistle, a, b Karl J. Romanowicz, a, c William A. Argiroff, a Zachary B. Freedman, d J. Jeffrey Morris B, Donald R. Zaka C (2020) Anthropogenic N Deposition Alters the Composition of Expressed Class II Fungal Peroxidases. **84**, 1–16.
- Entwistle EM, Zak DR, Argiroff WA (2018) Anthropogenic N deposition increases soil C storage by reducing the relative abundance of lignolytic fungi. *Ecological Monographs*, **88**, 225–244.
- Freedman ZB, Romanowicz KJ, Upchurch RA, Zak DR (2015) Differential responses of total and active soil microbial communities to long-term experimental N deposition. *Soil Biology*

and Biochemistry, **90**, 275–282.

Frey SD, Knorr M, Parrent JL, Simpson RT (2004) Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecology and Management*, **196**, 159–171.

Gadgil RL, Gadgil PD (1975) Suppression of litter decomposition by mycorrhizal roots of *Pinus radiata*. *New Zealand Journal of Forestry Science*, **5**, 33–41.

Ise T, Litton CM, Giardina CP, Ito A (2010) Comparison of modeling approaches for carbon partitioning: Impact on estimates of global net primary production and equilibrium biomass of woody vegetation from MODIS GPP. *Journal of Geophysical Research: Biogeosciences*, **115**, 1–11.

Janssens IA, al. et (2010) Reduction of forest soil respiration in response to nitrogen deposition. *Nature Geoscience*, **3**, 315–322.

Sulman BN, Shevliakova E, Brzostek ER, Kivlin SN, Malyshev S, Menge DNL, Zhang X (2019) Diverse Mycorrhizal Associations Enhance Terrestrial C Storage in a Global Model. *Global Biogeochemical Cycles*, **33**, 501–523.

Todd-Brown KEO, Randerson JT, Post WM, Hoffman FM, Tarnocai C, Schuur EAG, Allison SD (2013) Causes of variation in soil carbon simulations from CMIP5 Earth system models and comparison with observations. *Biogeosciences*, **10**, 1717–1736.

Waldrop MP, Zak DR, Sinsabaugh RL, Gallo M, Lauber C (2004) Nitrogen deposition modifies soil carbon storage through changes in microbial enzymatic activity. *Ecological Applications*, **14**, 1172–1177.

Zak DR, Argiroff WA, Freedman ZB, Upchurch RA, Entwistle EM, Romanowicz KJ (2019)

Anthropogenic N deposition, fungal gene expression, and an increasing soil carbon sink in the Northern Hemisphere. *Ecology*, **100**, 1–8.

Appendix. Supplementary Tables and Figures

Table S3-1. 16S forward and reverse primer sequences. In step 1 of the PCR reaction, the primer sequences amplify the 16S region of the rRNA. In step 2 of the PCR reaction, primer sequences include adapter sequences, unique sample indexes, and Nextera. Primers were used on samples collected at the Bear Brook Watershed in Maine.

	Primer	Sequence
Step1 PCR	16S F	CCTACGGGNGGCNCGAGNCCTACGGGNGGCWGCAG
Step1 PCR	16S R	GACTACHVGGGTATCTAATCCNACTACHVGGGTATCTAATCC
Step1 PCR	28S - LR22R	TAGCGMACAAGTASMGTCANCTACGGGNGGCWGCAG
Step1 PCR	28S - LR3	GGTCCGTGTTTCAAGACNACTACHVGGGTATCTAATCC
Step2 PCR - Nextera Reverse	17-1	CAAGCAGAAGACGGCATAACGAGATCGTGATGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-2	CAAGCAGAAGACGGCATAACGAGATACATCGGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-3	CAAGCAGAAGACGGCATAACGAGATGCCTAAGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-4	CAAGCAGAAGACGGCATAACGAGATTGGTCAGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-5	CAAGCAGAAGACGGCATAACGAGATCACTGTGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-6	CAAGCAGAAGACGGCATAACGAGATATTGGCGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-7	CAAGCAGAAGACGGCATAACGAGATGATCTGGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-8	CAAGCAGAAGACGGCATAACGAGATTCAAGTGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-9	CAAGCAGAAGACGGCATAACGAGATCTGATCGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-10	CAAGCAGAAGACGGCATAACGAGATAAGCTAGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-11	CAAGCAGAAGACGGCATAACGAGATGTAGCCGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-12	CAAGCAGAAGACGGCATAACGAGATTACAAGGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-13	CAAGCAGAAGACGGCATAACGAGATTGACTGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-14	CAAGCAGAAGACGGCATAACGAGATGGAAGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-15	CAAGCAGAAGACGGCATAACGAGATTGACATGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-16	CAAGCAGAAGACGGCATAACGAGATACGTTGGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-17	CAAGCAGAAGACGGCATAACGAGATCGTAGCGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-18	CAAGCAGAAGACGGCATAACGAGATGTACCAGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-19	CAAGCAGAAGACGGCATAACGAGATTACGATGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-20	CAAGCAGAAGACGGCATAACGAGATGCAGGCGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-21	CAAGCAGAAGACGGCATAACGAGATCATAAGGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-22	CAAGCAGAAGACGGCATAACGAGATATGTTAGTCTCGTGGGCTCGG
Step2 PCR - Nextera Reverse	17-23	CAAGCAGAAGACGGCATAACGAGATTGCGGTGTCTCGTGGGCTCGG
Step2 PCR - Nextera Forward	15-1	CAAGCAGAAGACGGCATAACGAGATGAGGTTGTCTCGTGGGCTCGG
Step2 PCR - Nextera Forward	15-2	AATGATACGGCGACCACCGAGATCTACACATACTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-3	AATGATACGGCGACCACCGAGATCTACACACTCTTTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-4	AATGATACGGCGACCACCGAGATCTACACATGTTGTCTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-5	AATGATACGGCGACCACCGAGATCTACACATTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-6	AATGATACGGCGACCACCGAGATCTACACCCTAAGTCTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-7	AATGATACGGCGACCACCGAGATCTACACCGAGGCTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-8	AATGATACGGCGACCACCGAGATCTACACCTTTGCTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-9	AATGATACGGCGACCACCGAGATCTACACGAAATGTCTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-10	AATGATACGGCGACCACCGAGATCTACACGCTCAATCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-11	AATGATACGGCGACCACCGAGATCTACACGGACTTTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-12	AATGATACGGCGACCACCGAGATCTACACGTTGGATCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-13	AATGATACGGCGACCACCGAGATCTACACTAAGCTTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-14	AATGATACGGCGACCACCGAGATCTACACTCTCGGTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-15	AATGATACGGCGACCACCGAGATCTACACTCTTTCGTCGGCAGCGTC
Step2 PCR - Nextera Forward	15-16	AATGATACGGCGACCACCGAGATCTACACTTTGTCTCGTCGGCAGCGTC

Table S3-2. N fertilization resulted in variable, but limited effects on the relative abundance of gene transcripts involved in the breakdown of lignin, simple C, and chitin (N). Values are the normalized transcript abundance (Mean \pm SE) for lignin, ligE (lignin enantioselective glutathione S-transferase, involved in the cleavage of b-aryl ether compounds during lignin decomposition; Morel et al 2009), Lip (lipid peroxidase), vp1 (versatile peroxidase), mnp (manganese peroxidase), lcc.ascomycota (ascomycota laccase), lcc.basidiomycota (basidiomycota laccase), glx (glyoxalase), cbh1 (cellobiohydrolase), and exc (chitinase). Data presented were measured in July 2016 at the Bear Brook Watershed in Maine (n=3 samples per mycorrhizal type and treatment). Significant differences in ratios between the reference and fertilized watershed within mycorrhizal type are presented in bold ($p < 0.05$).

Process	Gene	AM control	AM +N	ECM Control	ECM +N
lignin degrading	lignin	8.0E-05 \pm 1.9E-05	2.5E-05\pm 3.3E-06	2.2E-05 \pm 3.8E-06	4.9E-05 \pm 2.2E-05
	ligE	5.6E-06 \pm 2.0E-06	3.3E-06 \pm 3.2E-07	2.1E-06 \pm 4.0E-07	5.3E-06\pm 1.7E-06
	Lip	8.0E-07 \pm 4.0E-07	4.7E-07 \pm 3.1E-07	4.2E-07 \pm 2.2E-07	1.3E-06 \pm 6.5E-07
	vp1	8.3E-07 \pm 4.0E-07	5.5E-07 \pm 3.9E-07	3.6E-07 \pm 2.0E-07	4.1E-07 \pm 2.2E-07
	mnp	8.0E-07 \pm 4.0E-07	4.7E-07 \pm 3.1E-07	4.2E-07 \pm 2.2E-07	1.2E-06 \pm 6.1E-07
	lcc.ascomycota	6.1E-05 \pm 1.4E-05	1.8E-05\pm 2.1E-06	1.4E-05 \pm 2.5E-06	3.4E-05 \pm 1.7E-05
	lcc.basidiomycota	7.6E-05 \pm 2.1E-05	1.9E-05\pm 1.9E-06	1.5E-05 \pm 2.5E-06	4.5E-05 \pm 2.4E-05
simple C degrading	glx	8.3E-06 \pm 1.3E-06	5.8E-06 \pm 1.3E-06	8.1E-06 \pm 1.8E-06	4.3E-06 \pm 1.1E-06
	cbh1	3.6E-06 \pm 1.6E-06	4.7E-06 \pm 2.8E-06	3.6E-06 \pm 1.3E-06	6.1E-06 \pm 3.3E-06
chitin (N) degrading	exc	4.6E-05 \pm 1.5E-05	1.1E-05\pm 5.3E-06	8.7E-06 \pm 3.0E-06	2.8E-05 \pm 1.5E-05

Table S3-3. Mean enzyme activity ratios across May, July, and September 2016 at the Bear Brook Watershed in Maine. Each value represents the mean enzyme activity ratio of 6 plots per mycorrhizal type and treatment across three sampling dates (n=18) for acid phosphatase (AP), β -glucosidase (BG), and N-acetylglucosaminidase (NAG), phenol oxidase (PO), and peroxidase (PE). Mean values are accompanied by standard error. Significant differences in ratios between the reference and fertilized watershed within mycorrhizal type and soil fraction are presented in bold ($p<0.05$) and by asterisk ($p<0.10$).

Mycotype	Treatment	Fraction	BG:NAG	PO:NAG	PE:NAG	BG:AP	NAG:AP
AM	Control	OH	1.06± 0.11	2.42± 0.53	1.71± 0.49	0.26± 0.03	0.25± 0.03
AM	N	OH	2.34± 0.73	2.18± 1.34	1.76± 1.12	0.62± 0.09	0.39± 0.09
AM	Control	Bulk	1.78± 0.66	4.41± 2.68	20.03± 5.89	0.25± 0.07	0.21± 0.02
AM	N	Bulk	5.71± 0.66	12.61± 2.68	35.00± 5.89*	0.62± 0.07	0.11± 0.02
AM	Control	Rhizosphere	1.84± 0.40	6.18± 2.17	17.73± 5.79	0.26± 0.02	0.23± 0.08
AM	N	Rhizosphere	5.42± 0.51	7.82± 2.44	31.60± 7.43	0.59± 0.06	0.11± 0.01
ECM	Control	OH	1.41± 0.25	4.50± 1.52	1.46± 0.35	0.25± 0.03	0.21± 0.04
ECM	N	OH	1.52± 0.12	1.04± 0.32	0.45± 0.15	0.77± 0.03	0.52± 0.04
ECM	Control	Bulk	2.05± 0.27	7.53± 1.69	27.75± 7.41	0.22± 0.02	0.12± 0.02
ECM	N	Bulk	4.25± 0.60	9.00± 2.40	39.54± 4.50	0.60± 0.07	0.14± 0.01
ECM	Control	Rhizosphere	1.82± 0.57	4.60± 0.79	17.97± 5.01	0.21± 0.03	0.16± 0.04
ECM	N	Rhizosphere	5.03± 0.63	7.17± 1.15*	39.30± 3.44	0.74± 0.07	0.16± 0.02

Table S3-4. Basidiomycete abundance was lower and ascomycete abundance higher in N fertilized AM bulk soils compared to reference. There were no other significant shifts in the three most abundant fungal phyla. Values presented are relative abundance (Mean \pm SE). Data presented were measured in July 2016 at the Bear Brook Watershed in Maine (n=6 plots per mycorrhizal type, fraction, and treatment). Significant differences in ratios between the reference and fertilized watershed within mycorrhizal type and soil fraction are presented in bold (p<0.05).

Mycotype	Treatment	Fraction	Ascomycota	Basidiomycota	Chytridomycota
AM	Control	OH	19.91 \pm 7.72	76.47 \pm 8.65	0.74 \pm 0.21
AM	N	OH	13.92 \pm 3.33	72.13 \pm 7.47	2.87 \pm 1.45
AM	Control	Bulk	2.83 \pm 1.24	92.97 \pm 2.53	1.04 \pm 0.37
AM	N	Bulk	11.91\pm 4.31	75.97\pm 8.25	1.51 \pm 0.76
AM	Control	Rhizosphere	7.32 \pm 5.14	85.46 \pm 5.90	1.85 \pm 0.80
AM	N	Rhizosphere	12.21 \pm 7.37	74.67 \pm 11.90	1.74 \pm 0.95
ECM	Control	OH	12.15 \pm 2.49	81.33 \pm 3.25	1.87 \pm 0.62
ECM	N	OH	11.07 \pm 3.76	75.33 \pm 8.90	6.15 \pm 3.75
ECM	Control	Bulk	9.52 \pm 3.85	73.15 \pm 9.42	1.92 \pm 1.05
ECM	N	Bulk	5.26 \pm 2.47	83.90 \pm 5.21	2.59 \pm 0.71
ECM	Control	Rhizosphere	14.46 \pm 5.98	77.69 \pm 8.08	1.62 \pm 0.50
ECM	N	Rhizosphere	10.72 \pm 3.73	80.34 \pm 4.65	2.74 \pm 1.04

Table S3-5. N fertilization resulted in variable, but limited shifts in the top 15 most abundant bacterial phyla. Values presented are relative abundance (Mean \pm SE). Data presented were measured in July 2016 at the Bear Brook Watershed in Maine (n=6 plots per mycorrhizal type, fraction, and treatment). Significant differences in ratios between the reference and fertilized watershed within mycorrhizal type and soil fraction are presented in bold ($p < 0.05$).

Mycotype	Treatment	Fraction	Proteobacteria	Acidobacteria	Verrucomicrobia	Actinobacteria	Planctomycetes	Bacteroidetes	Chlamydiae	Armatimonadetes	Chlorobi	Chloroflexi	Cyanobacteria	PTM7	PTM6	FCPU426	PWPS2
AM	Control	OH	30.60 \pm 6.55	15.64 \pm 3.88	12.50 \pm 3.51	8.68 \pm 2.48	4.18 \pm 1.31	2.87 \pm 0.86	1.09 \pm 0.45	0.64 \pm 0.29	0.06 \pm 0.04	0.00 \pm 0.00	0.51 \pm 0.23	3.26 \pm 1.11	0.15 \pm 0.13	0.31 \pm 0.18	1.10 \pm 0.41
AM	N	OH	37.01 \pm 2.58	12.68 \pm 3.48	7.34 \pm 1.31	7.35 \pm 2.45	8.85 \pm 0.39	4.20 \pm 1.51	1.86 \pm 0.66	0.38 \pm 0.16	0.08 \pm 0.04	0.15 \pm 0.12	0.29 \pm 0.14	5.16 \pm 0.84	0.89 \pm 0.26	0.11 \pm 0.05	3.63 \pm 0.64
AM	Control	Bulk	8.31 \pm 2.38	33.46 \pm 4.21	1.30 \pm 3.12	5.61 \pm 1.94	0.24 \pm 1.40	0.32 \pm 0.53	1.61 \pm 1.30	5.61 \pm 0.15	3.51 \pm 0.03	0.04 \pm 0.07	0.09 \pm 0.26	0.84 \pm 0.52	24.52 \pm 0.52	0.41 \pm 0.17	15.82 \pm 0.45
AM	N	Bulk	30.35 \pm 5.24	23.90 \pm 3.71	9.11 \pm 3.25	8.48 \pm 1.36	8.48 \pm 2.04	2.89 \pm 0.38	1.11 \pm 0.63	0.64 \pm 0.23	0.02 \pm 0.05	1.30 \pm 0.64	0.62 \pm 0.22	2.54 \pm 0.70	0.44 \pm 0.15	0.06 \pm 0.07	8.99 \pm 1.06
AM	Control	Rhizosphere	28.05 \pm 2.11	40.95 \pm 5.32	10.90 \pm 3.31	3.20 \pm 1.16	6.88 \pm 1.53	1.09 \pm 0.42	1.66 \pm 0.85	0.48 \pm 0.18	0.02 \pm 0.02	0.00 \pm 0.00	0.28 \pm 0.18	0.61 \pm 0.43	1.82 \pm 1.62	0.09 \pm 0.09	2.01 \pm 0.81
AM	N	Rhizosphere	28.11 \pm 2.64	30.93 \pm 3.07	10.71 \pm 1.89	5.30 \pm 1.42	10.98 \pm 1.29	1.85 \pm 0.40	1.73 \pm 0.22	0.50 \pm 0.34	0.09 \pm 0.04	1.38 \pm 1.37	0.48 \pm 0.25	1.69 \pm 0.62	0.16 \pm 0.09	0.32 \pm 0.15	4.14 \pm 1.61
ECM	Control	OH	44.79 \pm 4.08	16.59 \pm 3.60	10.57 \pm 0.59	9.18 \pm 1.92	3.85 \pm 0.87	4.99 \pm 0.62	1.24 \pm 0.54	0.44 \pm 0.22	0.08 \pm 0.06	0.00 \pm 0.00	0.21 \pm 0.14	3.04 \pm 0.66	0.34 \pm 0.13	0.47 \pm 0.23	2.32 \pm 0.67
ECM	N	OH	33.65 \pm 3.35	12.39 \pm 2.46	8.55 \pm 1.12	11.64 \pm 1.90	6.72 \pm 1.46	4.28 \pm 1.01	1.81 \pm 0.45	0.20 \pm 0.15	0.06 \pm 0.04	0.00 \pm 0.00	0.75 \pm 0.22	5.81 \pm 0.94	0.62 \pm 0.32	0.45 \pm 0.24	9.54 \pm 4.31
ECM	Control	Bulk	28.50 \pm 2.66	36.42 \pm 8.39	9.80 \pm 1.70	5.60 \pm 2.04	6.54 \pm 2.05	2.27 \pm 0.48	2.79 \pm 1.45	0.32 \pm 0.15	0.04 \pm 0.03	0.07 \pm 0.07	0.25 \pm 0.18	1.51 \pm 0.49	0.62 \pm 0.39	0.25 \pm 0.13	1.50 \pm 0.51
ECM	N	Bulk	30.35 \pm 3.90	23.90 \pm 3.83	9.11 \pm 1.90	8.48 \pm 0.74	8.48 \pm 1.13	2.89 \pm 0.98	1.11 \pm 0.21	0.64 \pm 0.30	0.02 \pm 0.02	0.00 \pm 0.00	0.62 \pm 0.22	2.54 \pm 0.69	0.44 \pm 0.34	0.06 \pm 0.06	8.99 \pm 3.32
ECM	Control	Rhizosphere	28.90 \pm 7.64	19.55 \pm 5.99	11.66 \pm 2.89	4.79 \pm 1.54	5.12 \pm 1.18	1.99 \pm 0.49	1.08 \pm 0.47	0.27 \pm 0.15	0.00 \pm 0.00	0.17 \pm 0.08	0.38 \pm 0.20	0.86 \pm 0.30	0.51 \pm 0.13	0.21 \pm 0.12	1.95 \pm 0.52
ECM	N	Rhizosphere	38.91 \pm 12.42	18.94 \pm 4.81	10.45 \pm 2.72	4.91 \pm 1.77	11.41 \pm 2.42	1.92 \pm 0.74	1.42 \pm 0.43	0.32 \pm 0.11	0.00 \pm 0.00	0.00 \pm 0.00	0.25 \pm 0.16	2.28 \pm 0.73	0.35 \pm 0.16	0.05 \pm 0.05	6.72 \pm 2.45

Table S3-6. Mean enzyme activities across May, July, and September in 2016 at the Bear Brook Watershed in Maine. Each value represents the mean enzyme activity of 6 plots across three sampling dates (n=18) for acid phosphatase (AP), β -glucosidase (BG), and N-acetylglucosaminidase (NAG), phenol oxidase (PO), and peroxidase (PE). Mean values are accompanied by standard error. Significant differences in activities between the reference and fertilized watershed within mycorrhizal type and soil fraction are presented in bold.

Mycotype	Treatment	Fraction	BG	AP	NAG	Phenox	Perox
AM	Control	OH	1.32 \pm 0.16	5.33 \pm 0.33	1.16 \pm 0.17	2.70 \pm 0.49	2.12 \pm 0.34
AM	N	OH	2.64 \pm 0.13	4.64 \pm 0.55	1.67 \pm 0.42	1.72 \pm 0.37	1.50 \pm 0.33
AM	Control	Bulk	0.24 \pm 0.04	0.95 \pm 0.11	0.19 \pm 0.06	0.54 \pm 0.06	2.58 \pm 0.35
AM	N	Bulk	0.42 \pm 0.04	0.69 \pm 0.05	0.08 \pm 0.01	0.99 \pm 0.25	2.57 \pm 0.38
AM	Control	Rhizosphere	0.31 \pm 0.05	1.14 \pm 0.14	0.27 \pm 0.10	0.84 \pm 0.19	2.66 \pm 0.58
AM	N	Rhizosphere	0.51 \pm 0.05	0.88 \pm 0.09	0.10 \pm 0.01	0.65 \pm 0.13	2.70 \pm 0.38
ECM	Control	OH	1.04 \pm 0.09	4.24 \pm 0.34	0.85 \pm 0.14	3.11 \pm 0.76	1.02 \pm 0.19
ECM	N	OH	3.04 \pm 0.17	4.00 \pm 0.34	2.08\pm 0.20	2.19 \pm 0.76	0.85 \pm 0.28
ECM	Control	Bulk	0.28 \pm 0.06	1.26 \pm 0.21	0.14 \pm 0.02	1.06 \pm 0.27	3.14 \pm 0.29
ECM	N	Bulk	0.36 \pm 0.04	0.60\pm 0.04	0.09\pm 0.00	0.76 \pm 0.20	3.38 \pm 0.43
ECM	Control	Rhizosphere	0.27 \pm 0.06	1.31 \pm 0.18	0.19 \pm 0.05	0.78 \pm 0.18	2.67 \pm 0.40
ECM	N	Rhizosphere	0.53 \pm 0.06	0.72\pm 0.04	0.12 \pm 0.02	0.86 \pm 0.27	4.26\pm 0.50

Table S4-1. Mean fine root metrics in AM and ECM dominated soils measured three times over the growing season in 2016 and 2017 at the Bear Brook Watershed in Maine. Each value represents the mean root metric of 6 plots measured in July of the corresponding year (n=6). Mean values are accompanied by standard error. Significant differences in activities between the reference and fertilized watershed within mycorrhizal type and soil fraction are presented in bold.

Year	Mycotype	Treatment	Fine root biomass	Mycorrhizal colonization	Average diameter (mm)		Specific root length		Forks/g	
			(g/m ²)	(%)			(g/cm)			
2016	AM	Reference	335.25 ± 46.18	54.83 ± 0.78	0.81 ± 0.03		870.98 ± 102.32		2985.44	458.08
2016	AM	N	346.51 ± 21.91	33.02 ± 3.17	0.90 ± 0.03		733.75 ± 72.82		2416.52	366.91
2016	ECM	Reference	386.77 ± 36.17	53.80 ± 3.37	0.96 ± 0.03		595.48 ± 45.76		1904.73	157.23
2016	ECM	N	244.16 ± 17.67	33.42 ± 0.73	0.82 ± 0.03		824.99 ± 91.92		2767.26	369.24
2017	AM	Reference	393.86 ± 33.88	66.08 2.62	0.89 0.11		901.40 64.17		3512.09	265.40
2017	AM	N	279.17 ± 26.09	31.56 3.66	0.95 0.08		802.95 45.70		3452.50	368.41
2017	ECM	Reference	381.41 ± 38.68	58.86 4.30	1.11 0.20		791.04 37.93		2963.73	197.17
2017	ECM	N	299.35 ± 22.66	29.75 2.97	0.80 0.07		670.00 67.91		2231.19	418.99

Table S4-2. Mean enzyme activities in AM and ECM dominated soils measured three times over the growing season in 2016 and 2017 at the Bear Brook Watershed in Maine. Each value represents the mean enzyme activity of 6 plots across three sampling dates (n=18) for acid phosphatase (AP), β -glucosidase (BG), and N-acetylglucosaminidase (NAG), phenol oxidase (PO), and peroxidase (PE). Mean values are accompanied by standard error. Significant differences in activities between the reference and fertilized watershed within mycorrhizal type and soil fraction are presented in bold.

Year	Mycotype	Treatment	Fraction	AP	BG	NAG
2016	AM	Control	OH	5.33 \pm 0.33	1.32 \pm 0.16	1.16 \pm 0.17
2016	AM	N	OH	4.64 \pm 0.55	2.64 \pm 0.13	1.67 \pm 0.42
2016	AM	Control	Bulk	0.95 \pm 0.11	0.24 \pm 0.04	0.19 \pm 0.06
2016	AM	N	Bulk	0.69 \pm 0.05	0.42 \pm 0.04	0.08 \pm 0.01
2016	AM	Control	Rhizosphere	1.14 \pm 0.14	0.31 \pm 0.05	0.27 \pm 0.10
2016	AM	N	Rhizosphere	0.88 \pm 0.09	0.51 \pm 0.05	0.10 \pm 0.01
2016	ECM	Control	OH	4.24 \pm 0.34	1.04 \pm 0.09	0.85 \pm 0.14
2016	ECM	N	OH	4.00 \pm 0.34	3.04 \pm 0.17	2.08 \pm 0.20
2016	ECM	Control	Bulk	1.26 \pm 0.21	0.28 \pm 0.06	0.14 \pm 0.02
2016	ECM	N	Bulk	0.60 \pm 0.04	0.36 \pm 0.04	0.09 \pm 0.00
2016	ECM	Control	Rhizosphere	1.31 \pm 0.18	0.27 \pm 0.06	0.19 \pm 0.05
2016	ECM	N	Rhizosphere	0.72 \pm 0.04	0.53 \pm 0.06	0.12 \pm 0.02
2017	AM	Control	OH	2.77 \pm 0.43	1.05 \pm 0.11	1.03 \pm 0.18
2017	AM	N	OH	2.44 \pm 0.13	0.89 \pm 0.06	0.62 \pm 0.07
2017	AM	Control	Bulk	1.16 \pm 0.12	0.35 \pm 0.05	0.25 \pm 0.06
2017	AM	N	Bulk	0.62 \pm 0.05	0.30 \pm 0.03	0.10 \pm 0.01
2017	AM	Control	Rhizosphere	1.21 \pm 0.14	0.38 \pm 0.07	0.32 \pm 0.11
2017	AM	N	Rhizosphere	0.67 \pm 0.06	0.28 \pm 0.03	0.07 \pm 0.01
2017	ECM	Control	OH	2.93 \pm 0.39	0.85 \pm 0.12	0.69 \pm 0.14
2017	ECM	N	OH	2.07 \pm 0.20	0.88 \pm 0.10	0.66 \pm 0.07
2017	ECM	Control	Bulk	1.18 \pm 0.11	0.34 \pm 0.05	0.22 \pm 0.03
2017	ECM	N	Bulk	0.54 \pm 0.06	0.27 \pm 0.03	0.12 \pm 0.02
2017	ECM	Control	Rhizosphere	1.02 \pm 0.12	0.39 \pm 0.06	0.30 \pm 0.13
2017	ECM	N	Rhizosphere	0.74 \pm 0.12	0.27 \pm 0.04	0.12 \pm 0.03

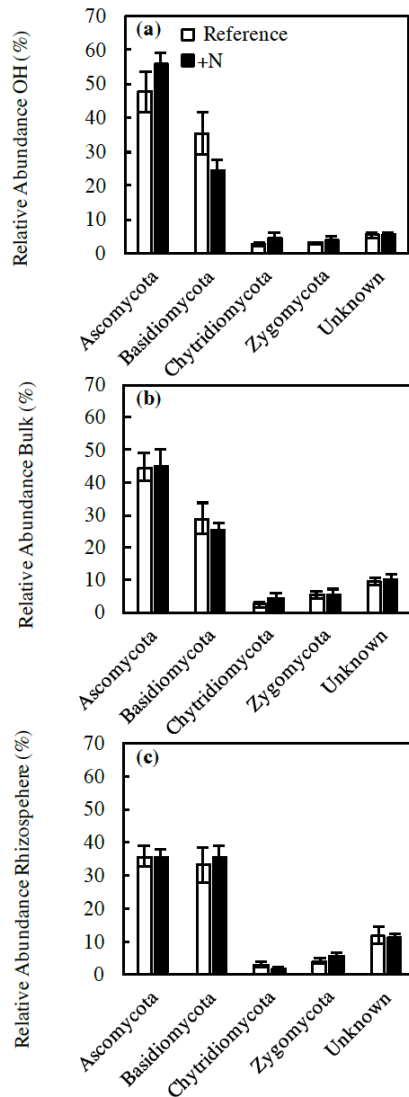


Figure S2-1. N fertilization does not alter the relative abundance of the 4 most common fungal phyla and unclassified phyla in OH, bulk, and rhizosphere soil fractions. Data presented are the watershed-level relative abundances (mean +SE) for each soil fraction (a) organic horizon (b) bulk (c) rhizosphere soil across all plots (n=10 plots per watershed). There were no significant differences in phyla abundance between watersheds in any soil fraction. Data presented were measured in July 2015 at the Fernow Experimental Forest, WV. AM fungi are included in the phylum *Glomeromycota* which for which relative abundance did not vary between watersheds. ECM fungi are represented in the phyla *Basidiomycota*, *Ascomycota*, and *Zygomycota*.

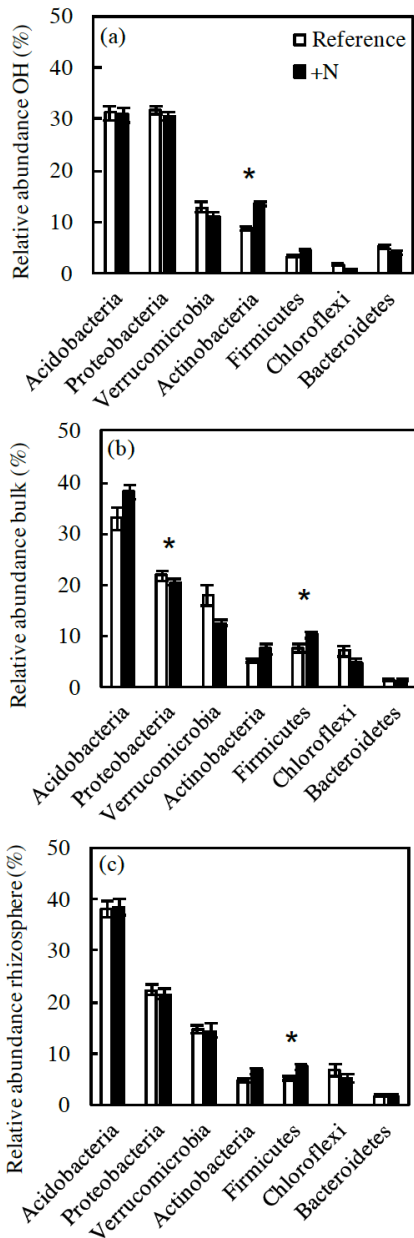


Figure S2-2. Response of dominant bacterial phyla to N fertilization. Data presented are the watershed-level relative abundances (mean +SE) for each soil fraction (a) organic horizon (b) bulk (c) rhizosphere soil across all plots (n=10 plots per watershed). Asterisks indicate significant differences between treatments (p<0.05). Data presented were measured in July 2015 at the Fernow Experimental Forest, WV.

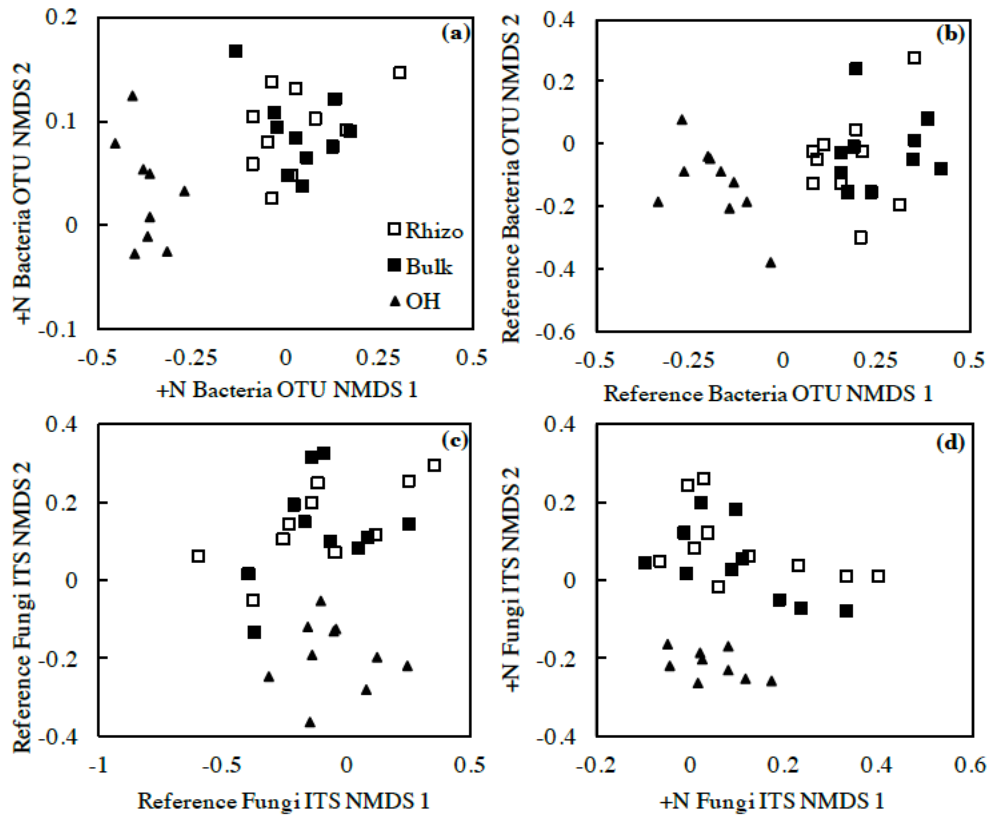


Figure S2-3. Bacterial and fungal community composition did not vary between bulk and rhizosphere soil within either the reference or N fertilized watershed. OH horizon varied from bulk and rhizosphere communities for fungi and bacteria in both watersheds. Data presented were gathered in July 2015 at the Fernow Experimental Forest, WV (n=10 plots per watershed x 3 soil horizons).

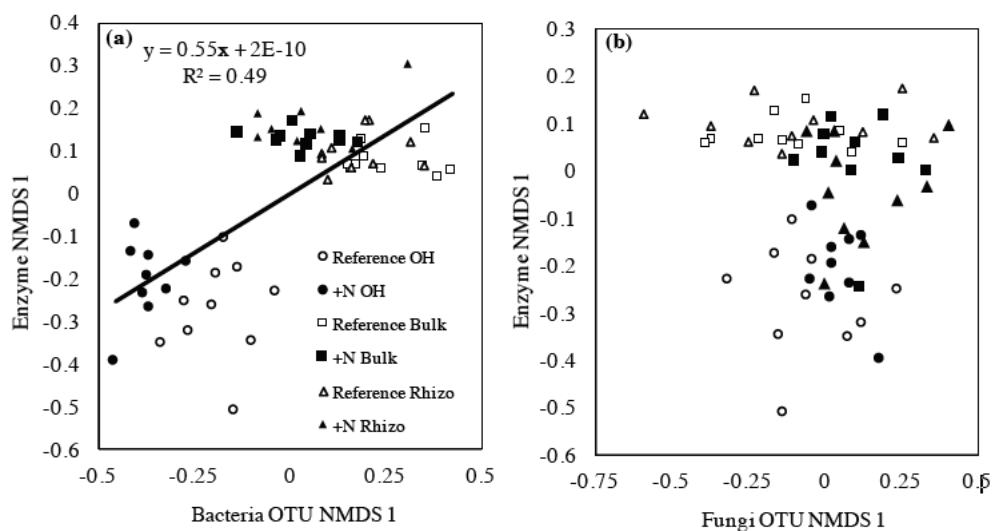


Figure S2-4. Bacterial community composition is significantly correlated with the first NMDS axis of enzyme activity across OH, bulk, and rhizosphere soil fractions (a). There is no relationship between fungal community composition and enzyme activity. Data presented are from microbial community analysis of each soil horizon between both watersheds collected in July 2015 at the Fernow Experimental Forest, WV (n=10 plots per watershed x 3 soil horizons).

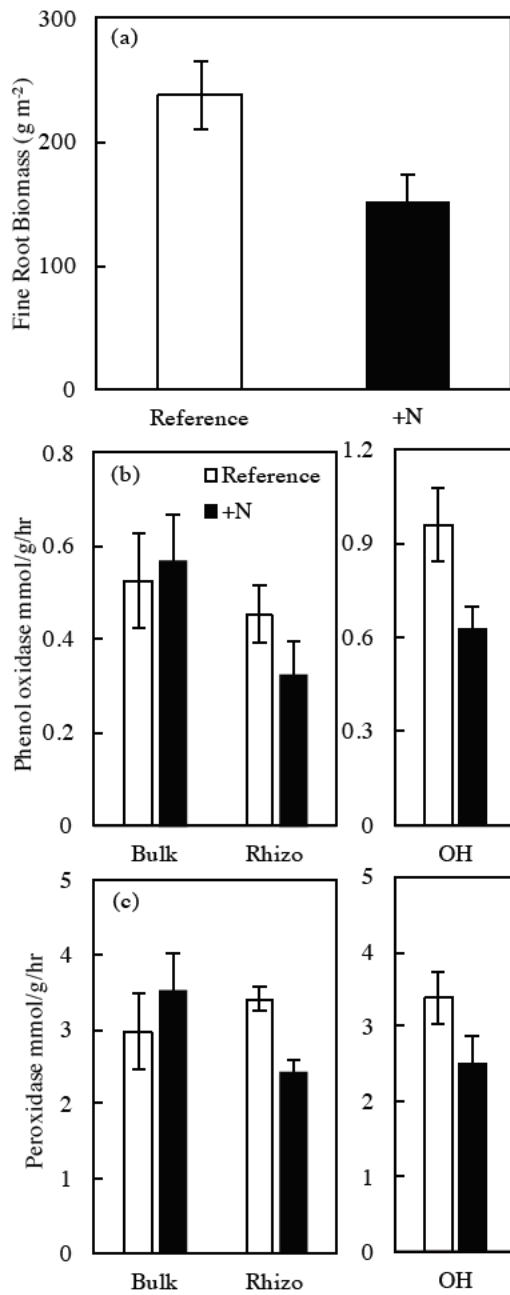


Figure S2-5. Figure S5. Fine root biomass (a), phenol oxidase (b), and peroxidase (c) responses to N-fertilization at the replicated Long-Term Soil Productivity experiment at the Fernow are similar to the watershed scale pseudo-replicated study. Data presented were gathered in June 2015 at the Fernow Experimental Forest, WV (n=6 replicate plots per treatment).