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Nutrient Cycling-Tree Species Relationships in Appalachian Forests

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ABSTRACT

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Since the colonization of North America by Europeans, ecosystems in Appalachia and across the continent have been in a prolonged state of flux. Areas particularly rich in natural resources, such as Appalachia, have historically borne the brunt of these swift changes, often with devastating consequences. Downwind of much of the power generation facilities of the Ohio Valley, Appalachian forests have been geographically predisposed to high rates of acidic deposition, a circumstance mitigated by the passage of Clean Air Legislation beginning in the 1970s. Nevertheless, decades of elevated nitrogen (N) and sulfur (S) inputs had a profound impact on the ecology and biogeochemistry of these forests. While inputs of these important plant nutrients can provide fertilization effects on plant life, the acidic N and S forms deposited in precipitation also result in a variety of negative outcomes. Plant nutrition can be influenced by acidic inputs in a variety of ways, including modifications to decomposition processes. Microbially-mediated decomposition results in the liberation of nutrients from organically-bound, often recalcitrant forms. When nutrients are abundant due to acidic deposition or by tree species effects such as N fixation and/or readily decomposable low C/N litter, decomposition processes may be suppressed. Since extracellular soil enzymes (ESEs)–the biomolecules responsible for mediating many of the rate-limiting transformations in terrestrial nutrient cycling–are metabolically expensive, and their synthesis and activities tend to be suppressed under nutrient fertilization. In Chapter 2, I present a literature review of ESE activities in the context of their ecosystem function and responses to disturbance, such as different types of pollution episodes, including acidic deposition. In addition to alterations to forest soil decomposition processes, acidic deposition may have other consequences on forest biogeochemistry. Poorly-buffered forest soils are particularly vulnerable to losses of essential nutrient cations, overriding any potential benefits from plant fertilization effects. In addition, the liberation of phytotoxic aluminum cations in an acidifying soil substrate can be extremely detrimental to plant growth. I hypothesize in Chapter 3 that I will observe declines in soil and foliar nutrient element concentrations as modeled estimates of cumulative historic N deposition in high-elevation red spruce forests increase. Likewise, Chapter 4 considers the effects of acidic deposition in the same ecosystem, testing the hypothesis that ESE activities will decline in concert with a legacy fertilization effect still present in high elevation red spruce forest soils. Lastly, I examine the relationships between plant functional guilds and soil processes using the Stand Initiation and Diversity Experiment (SIDE) at Point Pleasant, West Virginia. I hypothesize that I will observe differences in ESEs in plots dominated by different functional guilds of woody tree species: those bearing arbuscular mycorrhizal symbioses, ectomycorrhizal symbioses, and those capable of fixing N. This research will investigate the interplay between anthropogenic disturbances to ecosystem processes, legacy effects due to historic disturbances, tree species effects on ecosystem processes, and the role of tree species functional guilds on ESE profile and decomposition. Observing the effects of past disturbances will provide insight on the nature of contemporary and future changes to natural systems.

A little boy and his dad were walking in the woods.
“What is this?” asked the boy, pointing at a thicket of shrubs taking advantage of a gap in the canopy.
“Tom says they’re called dogwood,” replied the father.
“*Dog*wood” echoed the boy. “*Whoa...”*

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1 Background and Hypotheses

1.1 Background

Until recent decades, industrialization had caused high rates of nitrogen (N) and sulfur (S) deposition from the atmosphere to Appalachian forests. Atmospheric deposition had a host of direct and indirect effects, such as fertilization, soil acidification, and the liberation of large quantities of previously unavailable and phytotoxic aluminum (Al) to the soil solution. Although Clean Air Act Legislation beginning in the 1970s sharply reduced the effects of atmospheric deposition, there is little doubt that present-day Appalachian forests bear little resemblance to the forest communities observed by the first European settlers.

Although anthropogenic nutrient inputs have declined over the last 3+ decades, legacy effects may still have a significant influence over ecosystem functions, such as nutrient cycling and plant community assemblage. In a pristine ecosystem, it would be reasonable to expect within-site differences in biogeochemical cycling and soil chemistry to be driven by factors intrinsic to the site, such as the spatial distribution of the tree species themselves; conversely, legacy effects of acid deposition could manifest in the form of plant communities dominated by low-pH and Al-tolerant species at sites that have been exposed to the highest amounts of historic deposition.

The first chapter is a literature review on the effects of disturbances on extracellular soil enzyme activities, the biochemical entities responsible for mediating many of the rate-limiting transformations in biogeochemical cycling. The next two chapters are studies that address different facets of potential legacy effects of historic acid deposition, comparing four high-elevation red spruce forests that have experienced a range of historic acid deposition. Investigating the elemental composition of the soil at these sites, as well as analyzing the elemental content of foliage from the dominant species growing upon them, allows

for a comparison of the nutrients present and how the trees are utilizing them. Observing extracellular soil enzymes in the bulk soil beneath these trees provides insight into soil nutrition as well as the health of soil microbial communities, which play major roles in plant nutrition and biogeochemical cycling.

In order to investigate the roles of plant functional traits (PFTs), such as mycorrhizal symbionts, on plant nutrient status, it is necessary to observe these effects on a large sample size in the field. The Stand Initiation and Diversity Experiment (SIDE) provides an opportunity to observe relationships between PFTs and biogeochemistry. SIDE comprises a wide array of native Appalachian tree species with PFTs relating to nutrient cycling, such as litter quality, mycorrhizal association, and N fixation, and is very useful for interpreting the broad results across an array of species and functional combinations.

1.2 Hypotheses

Study 1 *— Four high-elevation red spruce sites were examined for differences in soil and plant nutrient concentrations over a modeled gradient of historic acid deposition.*

1) Legacy soil elemental profiles would exist across this historic N deposition gradient and that these profiles, characterized by low cations and high metals in high deposition sites, would be manifest in foliar elemental concentrations
2) Despite all sites being mixed stands, there will be local, species-specific effects on soil nutrient concentrations resulting from differential nutrient cycling among the trees species in these ecosystems.

Study 2 *— The red spruce sites from the previous study were examined on a seasonal basis for temporal, species, and acid deposition influences on relationships between carbon (C), nitrogen (N), phosphorus (P) and extracellular soil enzyme (ESE) activities.*

1) Soils beneath different tree species will have significantly different ESE activities, corresponding to differences in host-specific soil C:N ratios and/or controls on microbial communities
2) ESE activities will vary with historic estimates of N deposition reflecting shifts in N and P availability
3) Seasonal differences in ESE activities from May, July, and October will reflect changes in C inputs during the growing season in these soils.

Study 3 — *The Stand Initiation and Diversity Experiment (SIDE) in West Columbia, WV was utilized to observe tree species and plant functional trait (PFT) effects on plant growth and nutrient cycling within the SIDE plots.*

1) Tree species diversity will drive mean tree height within plots, and those effects will vary by species.
2) ESE profiles will be vary by tree species within plots
3) The effects of tree species diversity on ESEs will be driven by PFT classification of the species within each plot.

2 REVIEW
Extracellular soil enzymes: Ecosystem function and responses to pollution episodes

2.1 Introduction

Soil microbial communities significantly influence terrestrial ecosystem processes, including biogeochemical cycling and nutrient transformations. The ability of these microbes to perform ecosystem processes is indicative of both the health and functional identities of the microbial community. Extracellular soil enzymes (ESEs) secreted by microbes play important roles and mediate the rate-limiting steps in many nutrient transformations, contributing to the decay of recalcitrant substances such as chitins, celluloses, hemicelluloses, and lignins. In addition, ESEs have been shown to be effective in detoxifying a wide array of artificial organic pollutants in soils, ranging from pharmaceutical compounds, herbicides, pesticides, fungicides, and petrochemicals. The close relationship between ESE profile and microbial community assemblage makes these characteristics uniquely suited to assess remediation efforts and recovery of soil health [1].

The concept of soil health is closely tied to the state of the soil microbial community and the ecosystem services provided by soil organisms. Ferris and Tuomisto (2015) consider soil health to be the abilities of a soil and its associated biota to perform ecosystem functions relative to those in its pristine state; soils with diminished capabilities to provide ecosystem services are considered to have experienced a reduction in functional abilities and, thus, a reduction in health. Advances in analytical techniques have allowed researchers to utilize metagenomic approaches in assessments of soil health by identifying the functional roles of microbes in biogeochemical processes. While the roles of soil microbial communities

in mediating biogeochemical cycling is well known, linkages between community structure and functional roles in these processes are poorly understood.

Numerous studies have observed the impacts of perturbations such as elevated atmospheric CO_2 [3], nitrogen fertilization [4–6], heavy metal contamination [7,8], pharmaceutical compounds [9,10], and polyaromatic hydrocarbons [11,12] on the biogeochemical reactions undertaken by soil microbes. Further, changes in community structure resulting from perturbation have also been reported [13–15]. Although the pathology may vary based upon pollutant identity, any factor that impacts microbial community structure and biomass will therefore impact the ability of the soil to provide ecosystem services and therefore soil health.

While soil metagenomic techniques offer an effective means for characterizing microbial community composition, they are also useful for elucidating roles in terrestrial nutrient cycling [16]. Extracellular soil enzyme profiles are useful for evaluating soil health by observing the enzymatic means by which microbial communities provide ecosystem services. ESEs are secreted primarily by microbes to aid in nutrient acquisition, especially during periods of nutrient limitation [17]. This function of ESEs in nutrient scavenging contributes to their critical roles in decomposition processes, allowing carbon (C), nitrogen (N), and phosphorus (P) to cycle between plant and animal biomass, litter, and microbial biomass pools [18]. Although microbes are the primary source of ESEs in terrestrial soils, plants are also capable of exuding ESEs for nutrient acquisition, most notably acid and alkaline phosphatases in phosphorus-limited systems [19,20], which may also contribute to nutrient cycling in soils. High-throughput techniques for measuring soil enzyme activity have made the use of ESEs for determining soil health increasingly practical

in recent years. Historically, many soil enzyme assays were performed colorimetrically and relied upon the hydrolysis of substrates, such as ρ-nitrophenyl phosphate, to define enzyme activity [21]. In recent years, these colorimetric techniques have been supplanted by the availability of fluorescent methylumbelliferone-linked substrates and the use of microplates for fluorimetric determination. This approach effectively decreases the amount of reagents necessary while increasing the number of samples that can be simultaneously processed, while also reducing spectral interferences associated with nonspecific chromophores in soils [22–24].

The use of ESEs in determining soil health and functional capabilities has a wide array of applications. While the focus of this piece is on the responses of microbial communities and ESEs to soil pollution, ESEs lend themselves well to investigating processes within more pristine systems. The study of ESEs is applicable across a wide array of biomes, whether it is an investigation of nutrient cycling in Antarctic soils [25], boreal tundra [26], or as mediators of greenhouse gas emissions in tidal wetlands [27] and even deserts [28]. Due to their utility in assessing soil functional properties across a wide array of biomes, ESEs and responses to soil contamination are informative proxies for interpreting anthropogenic impacts on biogeochemical cycling. The responses of soil microbiota to changes in climate and the inclusion of ESE-related effects could be important for designing better models predicting climate change outcomes and integrating the influences of climate change factors on biogeochemical processes [29].

While higher plants exhibit the capacity to degrade complex macromolecules and mineralize nutrients in the rhizosphere, such capacity is generally limited. Saprotrophic microbes drive most decomposition of complex carbon sources [30], while nutrient scavenging is largely fulfilled by plant-microbial symbionts

such as ectomycorrhizal fungi and plant growth promoting rhizobacteria [31,32]. Rhizosphere microbial communities are strongly influenced by plant species due to functional characteristics of the plant such as litter and plant residue impacts on pH [33], translocation of nutrients throughout the soil [34,35], and water demand [36]. Successful recruitment of microbial symbionts can provide a significant competitive advantage by enhancing resource acquisition. An ecological strategy observed during plant species invasions is the alteration of rhizosphere characteristics to promote recruitment of symbionts favorable to the invader [37–39]. The ability to influence not only mycorrhizal fungi, but saprotroph composition as well [40], allows plant species to sculpt rhizosphere communities to their benefit.

2.2 Plant controls on rhizosphere microbial communities

Plant species significantly influence microbial community structure directly through the colonization of roots by symbiotic mycorrhizal fungi, plant growth promoting rhizobacteria (PGPR), and additional microbes that in turn form symbiotic or commensal associations with the primary symbionts. Plant functional traits such as organic C inputs via exudates and N fixation can increase bioavailable N levels in the rhizosphere, driving microbial community composition as a result fine-scale nutrient availability. Plant-driven resource heterogeneity within the soil profile shapes microbial community assemblage at fine spatial scales, a trend that is especially evident in the area immediately surrounding plant roots [41]. Indeed, fine roots provide a large surface area around which labile C sources are exuded into soils, creating a rich array of edaphic microhabitats for soil organisms [42].

While belowground factors play important roles within the soil itself, litter inputs to the soil surface and their nutritional content also drive soil development, influencing physical and chemical characteristics

over time. Changes in microbial community structure concomitant with plant species succession has been reported in both forest and grassland ecosystems [43,44]. Microbial community changes have also been observed as a result of land use change from a grassland to pine forest [45]. Consequences of plant functional traits (PFTs), such as litter quality and nitrogen fixation, result in different species of plants having a wide range of effects on soil microbial assemblage due primarily to plant nutrient acquisition strategies. Reports of strong differences in ESE activities in the soil beneath different tree species [46–48] are not uncommon in the literature. As soil microbial community structure is strongly dependent on the heterogeneous spatial distribution of nutrients [49], PFTs such as N fixation are likely to be more significant in influencing these communities than other traits relating to soil nutrition, such as litter quality. Patterns of litter decay are heavily influenced by C:N ratio and more specifically lignin content, the decomposition of which is strongly influenced by the presence of ESEs [50]. Since lignins are compounds that require a great deal of energy to decompose in return for little nutritional benefit, ESEs are critically important for decomposition.

In most terrestrial ecosystems, plant productivity is driven by tripartite relationships between plants, mycorrhizal fungi, and soil bacteria [51]. Were it not for these symbioses, especially the formation of mycorrhizae, acquisition of P would be untenable for higher plants in many terrestrial systems. While fungal diversity in some temperate systems has been shown to be influenced by plant species [52,53], bacterial community composition is more directly influenced by soil organic matter characteristics [49]. In grassland ecosystems, Shannon Diversity of soil microbes has been positively correlated with the breakdown of recalcitrant carbon compounds [54]. This indicates that, as microbial diversity decreases, functional diversity also decreases as there are fewer microbes capable of secreting the compounds necessary for decomposition to proceed.

2.3 Microbial diversity and specialization in nutrient cycling

With the advent of soil metagenomic approaches, investigators have been able to elucidate the genetics behind microbial functional traits, including secretion of ESEs, allowing for characterization of not only microbial community structure, but functional abilities as well. The presence of microbial genes coding for ESEs has been shown to be more dependent on soil nutritional status, especially soil organic C (SOC), than the microbial communities themselves [55]. This suggests that linkages between ESE profile and terrestrial nutrient cycling are not trivial, but are, in fact, intrinsically linked, and that the diverse soil microbial community possesses redundant functional traits that are involved in nutrient cycling [29]. Metagenomics also makes it possible to identify novel genes for enzyme expression; for example, the identification of a novel P-scavenging enzyme that could be useful for improving the P acquisition of transgenic crops [56].

Secretion of ESEs is a functional trait that can vary widely by microbial taxa and community assemblage. Laccase production has been observed only from fungi and some cyanobacteria, and laccase synthesis and secretion varies widely between fungal taxa [57]. However, as laccases belong to the phenol oxidase class of soil enzymes, they are very important for mediating the breakdown of some of the most recalcitrant carbon forms within plant litter but are functionally redundant with phenol oxidases secreted by bacteria. In addition to playing an important role in describing some of the rate-limiting steps in decomposition and C cycling, soil microbial communities play an important role in the N cycle. N fixation, nitrification, and denitrification are important fluxes in the N cycle that are mediated by plant symbionts and/or soil microbial assemblage. Soil metagenomics have allowed investigators to assess soil microbial communities and compare autotrophic and heterotrophic denitrification [58]. Indeed, functional genes

relating to these components of the nitrogen cycle have been identified using forest soil microbial communities [59] and utilized to investigate the impact of fertilization on the N cycle in agricultural systems [60] and more broadly across terrestrial ecosystems [6].

Although N is most frequently the limiting nutrient in terrestrial ecosystems, P can be limiting or co-limiting in some systems. Even as the C and N cycles are tightly coupled, the cycling of N and P are also closely interwoven. Under conditions of N fertilization, the presence of phosphorus can have a large impact on microbial community function and ESE profile [61]. P exists in highly recalcitrant organic forms in the O-horizons of many soils, making enzymes capable of scavenging organic forms of P a sound metabolic investment [18]. P acquisition appears to be the driving need behind the tripartite plant-fungi-bacteria symbioses observed in nutrient-poor Chilean soils [31] and may have played a key role in the early development and present abundance of mycorrhizal symbioses [52].

2.4 Pollution episodes, soil health, and enzyme activities

Any factor that influences abiotic factors of the soil, such as pH, temperature, moisture retention, and nutrient levels, has the potential to negatively affect soil health and cause abrupt changes in soil enzymatic profile. With the passing of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) of 1980 and the attention drawn to the remediation of designated Superfund sites, microbes have been increasingly studied and utilized for bioremediation. In many cases, especially those involving recalcitrant organic compounds, microbes utilized for bioremediation degrade target contaminants using the same suite of ESEs used for nutrient scavenging in pristine systems. This renders metagenomics techniques even more practical for identifying microbial taxa that have genes suited for expressing and

synthesizing enzymes that are effective for degrading toxic, recalcitrant organics. Metagenomics have been useful for identifying novel enzymes capable of operating in colder temperatures [62], under saline conditions [63], and for degradation of toxic organic compounds [64,65].

2.4.1 Acid Deposition

The widespread occurrence of acid deposition in the post-industrial United States has led to many studies on the effects of acid deposition components on biogeochemical processes. Acid deposition from anthropogenic sources typically occurs as a result of the dissolution of gaseous N and S compounds in rain water, although it can also occur as dry deposition on the surface of minute particles, often resulting in soil acidification where the substrate is poorly buffered [66]. As nitrogen and sulfur are macronutrients for both plants and microbes, inputs of these elements can have profound effects on the soil microbial community. In addition, declines in substrate pH may cause shifts in microbial community assemblage. Ectomycorrhizal fungi in particular have been shown to be more diverse in lower-pH soils and exhibit declining diversity with increasing substrate pH [67–70]. N deposition has been shown to reduce microbial biomass over decadal time scales in temperate forest ecosystems [71,72] as well as reducing microbial respiration [73]. Nutrient fertilization can also result in declines in lignin-degrading fungi, as they become less competitive when nutrition is abundant, with potentially large significant implications for decomposition in these systems [74].

Since synthesis and exudation of ESEs is often a stress response to nutrient limitation, nutrient fertilization generally results in declining ESE activities, a trend that has been observed under increasing N [50,75] and sulfur-scavenging arylsulfatases due to S inputs [76]. Interestingly, activities of C- and N-scavenging classes

of enzymes such as β-glucosidases, *N*-acetylglucosaminidases, chitinases, and cellulases have been shown to decline at low levels of N additions but increase at higher levels [77]; a response possibly related to limitation or co-limitation with phosphorus. In a Michigan sugar maple (*Acer saccharum* L.) forest, nitrogen additions resulted in decreases in litter decomposition and phenol oxidase activities concomitant with a decline in fungal Basidiomycete taxa [46].

2.4.2 Metals

Bioavailable metals in terrestrial soils can have many possible origins. Acidic deposition can result not only in fertilization effects, but also in the acidification of the soil substrate itself. As acidification proceeds, metals that are typically insoluble or unavailable due to strong complexation with organic compounds can enter the soil solution as bioavailable species. Heavy metals are often a component of anthropogenically-derived deposition, especially relatively common elements in industry such as Pb, Cd, Zn, and Cu [78]. Since the passing of the Clean Air Act legislation beginning in the 1970s, atmospheric deposition of heavy metals from industrial air pollution has declined considerably. Other sources of heavy metal inputs to terrestrial soils include mining, field application of municipal waste, and locally-abundant ore-bearing parent materials.

Exposure of soil microbial communities to heavy metals often occurs as a two-pronged effect: substrate acidification and concurrent increases in bioavailable toxic metal species. Aside from the toxic effects to the microbes themselves, metals tend to suppress ESE activities by deactivating binding sites and altering protein conformation. Li et al. (2009) utilized a stepwise regression to analyze the impacts of Cu, Pb, Zn, Al, and Mn on the activities of eight separate hydrolases in an acidifying tropical forest soil, indicating that metals had negative effects on total enzyme activity, microbial C, N, and P, mineralization, of P, and

especially C-related polysaccharidase and histadase activities. In particular, arylsulfatases have been observed to be particularly sensitive to the presence of heavy metals, and have been proposed as an indicator of heavy metal toxicity in soils [8]. A study of the area surrounding a Cu smelter in China noted depressed acid phosphatase activities as far as 200 meters from the smelter, with evidence for a negative correlation between Cu and Zn availability and ESE activities [80].

Changes in microbial community structure due to heavy metal exposure have also been noted using metagenomics techniques. Gao et al. (2010) developed a dose-response model explaining the reduction in total populations size of the soil microbial community and ESE suppression due to Pb and Cd exposure. Several additional studies have observed the negative correlation between the level of extractable heavy metals and microbial biomass, diversity, and ESE activities [7,81–83]. Soil microbial communities and metabolic processes are sensitive enough to heavy metal toxicity that they are effective as indicators of contamination and have been used to determine damage to soil health and ecosystem function following events such as a mining sludge retention pond dam rupture [84].

2.4.3 Organopollutants

Organic pollutants have become more prevalent in natural systems due to the widespread use of pharmaceuticals and their appearance in wastewater, the use of herbicides, pesticides, and fungicides in intensive agriculture, the widespread use and combustion of petroleum and petrochemicals, and utilization of industrial compounds such as solvents and chemical precursors. Many organopollutants tend to strongly adsorb to soil surfaces; these compounds are considered persistent organic pollutants (POPs) and are extremely challenging to remove once contamination has occurred. This can be especially

damaging in agricultural systems when relatively labile compounds, such as most herbicides, are applied in large quantities, but are retained due to some intrinsic soil property, such as high clay content.

Soil microbial communities exhibit variable responses to organopollutant contamination, due to the wide variety of compounds, retention times, bioavailability, and modes of toxicity. Generally, soil microbial biomass, diversity, and enzyme activities are reduced as exposure to these pollutants proceeds, [9,15,85–87]. However, many microbial communities contain taxa that are capable of detoxifying organopollutants, mineralizing them into bioavailable nutrients. The nutrient-scavenging utility of ESEs can be put to use in this way to aid bioremediation efforts [88], and there are microbial taxa and ESEs that are capable of detoxifying an extremely wide array of organopollutants [89]. Even very toxic compounds, such as bisphenol A, have been shown to be degraded by bacterial isolates from a contaminated soil [90].

2.4.4 Nanoparticles

Nanoparticles (NPs) are classified as objects from 1 to 100-nm in size [91], with the most common and widely studied examples being Ag, zinc oxide (ZnO), copper oxide (CuO), cerium dioxide (CeO_2), titanium dioxide (TiO_2), iron oxides (FeO), fullerenes, and carbon nanotubes [92]. Many of the metal oxide NPs are common components of sludge following wastewater treatment, which is sometimes used a soil amendment. Silver nanoparticles are some of the most common types released into the environment, are widely used in clothing, food packaging, and some cleaning supplies due to their antimicrobial properties [93]. Due to the prevalence of plastic waste in the environment, plastic NPs are extremely common in the environment but are generally inert in terms of biochemical interactions, making them a potential hazard for bioamplification across soil food chains but not directly impacting ESEs [94]. Although plastic NPs are

not as biochemically active as their metallic counterparts, their presence can lead to decreases in ESE activities accompanying declines in microbial biomass, possibly from toxicity associated with amplification across soil trophic levels [95].

NPs can interact with ESEs in many ways, with the physical binding of NPs to the protein potentially resulting in structural and conformational changes, deactivation of active sites, and denaturation of the protein [96]. However, it remains to be seen if studies of NPs on simulated soil microbial communities and their ecosystems functions are truly informative without including organic matter residues such as those found in natural systems [97]. Effects due to sorption to SOM particles are not limited to metallic NPs; following exposure to carbon nanotubes, organic matter content has been shown to play an important role determining differences in soil microbial community structure [98]. Likewise, greater quantities of organic matter have been shown to mitigate negative effects of Ag NPs on ESE activities [99].

Since toxicity effects are related to aggregation of NPs that result in a locally high concentration of the particles [100], the binding of NPs on soil colloids including those comprising SOM can mitigate nanoecotoxicological effects for some time before an exposure threshold is reached. Silver nanoparticles have been shown to reduce ESE activities [101], microbial biomass and respiration[102], nitrification [103], and increase N2O emissions from soils [104]. Lagging negative effects of simulated wastewater Ag NPs on nitrite production and microbial biomass were observed by Schlich et al. (2013), supporting the concept of a toxicity threshold that is reached after sorption capabilities SOM and other particulates are exceeded. Eivazi et al. (2018) observed reductions in acid phosphatase, arylsulfatase, N-Acetyl-β-d-glucosaminidase, and β-glucosidase after exposure to AG NPs; however, after one month, ESE activities

showed signs of recovery. This could be due in part to transformations within the soil, a common fate for Ag NPs [103]. Transformations have been shown to disrupt P and S nutrition for soil organisms and plants by forming Ag_2S and Ag_3PO_4 in soils [107].

2.5 Conclusions

Anthropogenic activities will continue to have widespread impacts on ecosystem processes. The sensitivity of soil microbial communities to widespread forms of industrial pollution may serve as an early warning sign of environmental stress that would otherwise be unapparent, and the fundamental roles played by ESEs in biogeochemical cycling provide direct insight into the disruption of critical ecosystem functions. With the development of high accuracy, high throughput analytical techniques, measuring ESE activities will be an important tool for environmental monitoring.

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3 Soil and tree nutrient status of high elevation red spruce (*Picea rubens* Sarg.) forests

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3.1 Introduction

Nutrient relations of forest trees play a central role in forest ecosystem function and productivity. Nutrient acquisition is influenced by physiological adaptations of tree species, interactions with microbial symbionts in the rhizosphere, and the nature of soils supporting forests [1–4]. The production of enzymes and other compounds by roots and root-associated microbes, differential uptake of nutrients and other elements from the soil, and their return in litter result in tree species acting as "ecosystem engineers" by accumulating and redistributing these elements throughout the soil profiles. The complex interplay between different tree species, mycorrhizal fungi, and the bacterial communities in the rhizosphere determines how tree species respond to nutrient limitation, soil pH, and metal availability and further determines patterns of nutrient cycling that vary across the landscape [5–7].

Since the advent of heavy industry and power generation in the late 19th century, forested ecosystems worldwide have been exposed to high levels of anthropogenically-derived nitrogen (N) and sulfur (S) in acidic deposition. The deposition of acidic compounds varies spatially across the landscape, with the magnitude of inputs strongly influenced by topographic factors such as elevation, slope, and aspect [8]. High elevation ecosystems, such as the red spruce (*Picea rubens* Sarg.) forests in the Appalachian Mountains in the United States, are spatially predisposed to high inputs of acidic deposition [9–12]. With implementation of the Clean Air Act and supporting legislation in the early 1970s in the United States, however, anthropogenic inputs of N and S into the atmosphere have declined, although areas downwind

of the Ohio River Valley, including central Appalachia, still receive some of the highest levels of N and S deposition in the United States [13].

Forest ecosystems are affected by acidic deposition through a variety of pathways, including reductions in plant-extractable nutrient cations in soils, elevated metals in the soil solution, leaching of cations from foliage, and N fertilization effects [14–16]. With continued N inputs into these ecosystems, changes in soils and tree nutrient acquisition continue, including increases in soil and foliar N, decreases in nutrient cations, and decreases in Ca/Al ratio [17–20]. Diminished soil Ca^{2+} has been directly correlated with decreases in winter hardiness of red spruce foliage, leading to region-scale winter dieback events occurring as late as 2003 [21–23] and potentially significant reductions in C storage at the landscape scale [24]. Disruptions in plant-soil nutrient relations and C assimilation due to acid deposition also increase the vulnerability of red spruce to secondary pests and pathogens [25]. These direct and indirect effects of acidic deposition can have wide-reaching implications for ecosystem function and productivity [16,26,27].

There have been numerous studies on biogeochemistry and nutrient relations in Appalachian red spruce stands. Robarge et al. (1989) conducted a survey of red spruce foliar elemental status in southern Appalachia and consolidated the reported literature values for red spruce foliar chemistry available at that time. Foliar Ca in spruce was at the extreme low and Al at the high ranges of values reported for this species in the eastern USA, suggesting these systems were at risk for Ca deficiency and Al toxicity [28]. Johnson et al. (1991) linked the presence of extractable Al to soil solution pulses of NO^{3-} and SO_4^{2-} from acid deposition in southern Appalachia, but failed to detect signs of soil base cation depletion or nutrient deficiency in spruce foliage [29]. More recent studies in the region indicate that soil Ca/Al ratios still

represent a threat to red spruce in the region, although comparison to earlier reported foliar concentrations indicate possible recovery in nutrition since the previous decade [30].

A transect containing high elevation red spruce forests in central Appalachia corresponding to a gradient of historic acidic deposition was established by Smith et al. (2016) using National Atmospheric Deposition Program (NADP) data [31]. These sites experienced cumulative wet N inputs ranging between 117.5 and 206.9 kg ha^{-1} and average annual wet N additions between 4.2 to 7.4 kg ha^{-1} yr^{-1} from 1985 to 2013 [32]. These values are comparable to N deposition ranges observed in studies in other regions in the eastern United States experiencing elevated rates of N deposition, such as the Adirondack Mountains of New York [33] and at sites throughout New England [34,35]. Smith et al. (2016) found that broadleaf deciduous tree relative importance value rather than N deposition rate had the biggest effect on site N status in these red spruce forests [31]. Species-specific differences in nutrient retention have long been considered important drivers of N availability and cycling [36–38].

Building on the Smith et al. (2016) study, we evaluated soils beneath and foliage of three dominant tree species, *Acer rubrum* L. (red maple), *Betula alleghaniensis* Britt. (yellow birch), and *P. rubens* (red spruce), in four central Appalachian forest stands across this historic N deposition gradient to assess soil and plant nutritional status of these mixed spruce-hardwood stands. Although anthropogenic acidic inputs have declined over the last three decades, continued N deposition and legacy effects may still have a significant influence over soil chemistry. While soils provide insight into the status of plant-available nutrients and metals, foliage allows us to gauge element acquisition by the principle tree species at these sites. We hypothesized that: (1) legacy soil elemental profiles would exist across this historic N deposition gradient and that these profiles, characterized by low cations and high metals in high deposition sites, would be

manifest in foliar elemental concentrations; (2) despite all sites being mixed stands, there will be local, species-specific effects on soil nutrient concentrations resulting from differential nutrient cycling among the trees species in these ecosystems.

3.2 Methods

Four high-elevation red spruce stands were selected based upon site characteristics including elevation, tree species composition, and position along a gradient of historic acid deposition in central Appalachia (Figure 3-1). All sites are at high elevation (>1100 m) and have a southwest aspect with slopes from 0—10% within the plot area. Site selection for historic acid deposition relied upon data from the National Atmospheric Deposition Program (NADP) following the procedure of Smith et al. (2016). Sites were chosen from estimated cumulative total inorganic N wet deposition (NO_3^- and NH_4^+) from 1985—2012. Data for dry deposition of N (HNO_3, NO_3^-, NH_4^+) was available for the years 2000—2012, and was incorporated into estimates of total inorganic N deposition by calculating the ratio of wet N deposition to total N deposition for those years and applying it to each pixel in the NADP spatial model for the mid-Atlantic region [39]. These data were then used to estimate total N deposition for the years 1985—1999 that lack available dry deposition data for the highly resolved and well-validated spatial model used by NADP. Although S deposition likely represented a significant component of historic acid deposition, historical data for S deposition were neither robust nor adequately validated for modeling, rendering reliable site estimates unavailable. Elevated levels of N and S deposition in this region are known to spatially coincide [13].

Each site consisted of a single 100-m diameter plot about a center point established in an area with red spruce as the predominant tree species. The Point-Centered Quarter Method [40] was used to analyze

forest tree (>10 cm DBH) community composition in these plots using five points randomly located on three parallel 50 m transects each separated by 25-m. Tree species community composition was similar across all sites, with each site belonging to the red spruce-yellow birch cover type defined by the Society of American Foresters [41]. All four sites have a history of disturbance, including fire and, most recently, timber harvest. Smith et al. (2016) determined year of stand establishment for these sites using tree core cross-dating techniques, with stand age at the time of sampling 75 yr for FLR, 149 yr for LSB, and 156 yr for both CGL and MCG, corresponding to timber harvesting dates. Soils at each site are characterized by generally thick organic horizons, high organic matter content, and relatively low pH. Despite differences in parent material and soil development, pH exhibits little variation across sites or horizons (Table 3-1).

Soil and foliar samples were collected in July 2014. Five individual trees of *A. rubrum*, *B. alleghaniensis*, and *P. rubens* were selected (Table 3-2). Only canopy dominant or co-dominant individuals greater than 45-cm DBH were selected. Soil samples were collected using a 2-cm diameter soil auger to 15-cm. Prior to sampling, coarse litter was brushed away from the sample area. Sampling points were selected on opposite sides of each trunk, midway between bole and edge of canopy. Samples were carefully separated into organic (O) and mineral (B) horizons and then composited by soil horizon for each individual tree. Poorly-developed spodic horizons were occasionally encountered and, being unrepresentative of the bulk soil, were discarded. No C-horizons were apparent at a depth of 15-cm at any of the sample locations, although some samples, most frequently at FLR, consisted of a thick organic horizon and very little mineral soil (approximately 15-cm total depth) before encountering rock. Conversely, there were some red maple individuals at each site that exhibited virtually non-existent organic horizons.

Soil samples (n = 5) were sieved using a 2-mm screen and subsamples dried at 105°C for 48 h for extraction and air-dried for C and N analysis. Dried soil samples were stored long-term at 4°C. Oven-dried samples were extracted with Mehlich-III solution to determine elemental content following the technique outlined by Zhang et al. [42] and adapted from Mehlich [43]. Extracts were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) to determine the concentration of macronutrients, micronutrients, and metals. Air-dried samples were analyzed for C and N concentration using a Thermoquest Elemental Analyzer.

For each species at each site, foliar samples (n = 3) were collected from a subset of the five individual trees sampled for soils at each site. Foliar samples were obtained using a shotgun to collect mid-canopy sun leaves with a southeastern to southwestern exposure [44]. Foliage damaged by shot was discarded. Since different cohorts and stages of development for red spruce needles can vary in foliar chemistry, needles were only utilized from the previous year to ensure uniformity of foliar developmental status for that species [22,45,46].

In the field, foliar samples were placed on ice in sealable plastic bags with a damp paper towel to prevent wilting and desiccation. In the lab, foliar samples were washed in a solution of 0.1% Tween-80 and rinsed repeatedly with Nanopure H2O to remove foliar surface contaminants potentially present on leaves [47]. Approximately 5-g fresh weight samples of foliar tissue were placed into individual paper bags and dried at 70°C for 48 h. Dried foliar tissue was ground using a CyclotecTM 1093 sample mill. Ground tissue was digested in 75-ml Teflon digestion tubes containing 4 ml of trace-metal-grade concentrated HNO3 and 1 ml of 30% H2O2. Microwave-assisted digestion was performed using a MARSXpressTM 5 with settings optimized for foliar tissue [48]. Digests were then subjected to analysis by ICP-OES. National Institute of

Standards and Technology (NIST) standards (Standard Reference Material 1515, apple leaves) were included in this analysis and used to validate elemental concentrations from the ICP-OES data. As with the soils, the C and N content of foliage was determined using a Thermoquest Elemental Analyzer.

We used two-way analysis of variance (ANOVA, species x N deposition), multiple regression, and stepwise regression using site variables to evaluate the impacts of N deposition and species or different site variables, respectively, on soil and foliar elemental profiles using SAS JMP Pro 13 [49]. Data were log- or square root-transformed to meet assumptions of normality. Pearson product-moment correlations for the pairwise comparisons of foliar element values by soil horizon were also calculated, and heat maps representing the correlations between foliar element concentrations and their soil counterparts by horizon were generated in Microsoft Excel.

3.3 Results

3.3.1 Evidence of anthropogenic deposition across the gradient

The sites investigated were selected based on modeled N deposition (Figure 3-1). Sites having received elevated anthropogenic inputs were expected to exhibit characteristic elemental fingerprints of historical deposition, including elevated N and S from atmospherically-borne acids, elevated industrially-released heavy metals, and acid-induced increases in soil Al. Soil pH varied significantly by horizon (O horizon = 3.38, B horizon = 3.50), and increased slightly across the N deposition gradient (Figure 3-S1). Stepwise regression was used to evaluate the importance of modeled N deposition, precipitation volume, and elevation on current soil chemical profiles (Table 3-3, Figure 3-2). Soil N was primarily influenced by precipitation and elevation in the O horizon and by N deposition and elevation in the B horizon (Table 3-3). Parameters were all positive indicating increasing N in soils with increasing N deposition, precipitation,

and elevation. In contrast, S in the O horizons declined with N deposition, but increased in soil B horizons with increasing N deposition (Figure 3-2). The soil metals Zn and Pb are often-used proxies for atmospheric deposition [50]. Zn concentrations in the O horizon were a function of modeled N deposition, precipitation, and elevation and declined in the O horizons but increased in B horizons across the N deposition gradient (Figure 3-2). Pb concentrations were influenced by precipitation and elevation in the O horizon, decreasing with N deposition; Pb was only a function of N deposition in the B horizon and increased with the N gradient (Table 3-3, Figure 3-2). O horizon Al declined with deposition, whereas the change in B horizon with N deposition was not significant (Figure 3-2). Taken together, these patterns suggest that the sites selected have indeed received a gradient in anthropogenically-generated pollutants. While S, Zn, Pb, and Al have moved from the O to the B horizon where reactions with the CEC and amorphous hydroxides may retain them, N is still accumulating throughout the soil profile.

The percent broadleaf tree representation was also initially used in the stepwise regression following Smith et al. (2016), but this variable did not explain any additional variation in the soil properties measured (data not presented). This discrepancy may reflect the species-specific sampling approach used here compared to stand-level sampling employed by Smith et al. (2016). We hypothesized that differences among specific tree species in cycling and remobilization would contribute to variation in soil elemental profiles, and "species" as a variable was included in analyses of soil elemental profiles, below.

3.3.2 Deposition and species effects on soil elemental pools

Sites exhibited differences in soil element concentrations due to N deposition and elevation and these varied by horizon, but typically not by species (Table 3-4). C and N in the O horizons increased beneath the three species with increasing N deposition and elevation, but did not vary between species except for

the higher concentrations in the B horizon beneath *A. rubrum* (6272 mg $^{kg-1}$) versus *B. alleghaniensis* (4732 mg kg^{-1}) and *P. rubens* (4350 mg kg^{-1}) (Table 3-4). C:N ratios did not vary in the O, but declined with N deposition in the B horizon (Table 3-4). Evidence for cation depletion was evident in O horizons of these montane soils, with significant declines in Ca, Mg, and K in organic soils as N deposition increased (Table 3-4); P also declined. In contrast to the O horizon, the concentrations of C and N and all macronutrients in the B horizons increased with N deposition, although elevation had no impact (Table 3-3). Declining Ca/Al, a signal of soil acidification, was not a general characteristic of the sampled soils, but was evident in the O and B horizons only beneath *A. rubrum*; indeed this was the only soil factor for which there was a significant species-by-deposition interaction (Figure 3-S2). Al was greatest beneath *B. alleghaniensis*, intermediate beneath *A. rubrum*, and least below *P. rubens*, reflecting potential differences in uptake and cycling of Al by these species.

3.3.3 Deposition effects on foliar elemental profiles

Foliar elemental concentrations varied strongly by species across the modeled N input gradient (Figure 3-S3), and Ca, K, and Sr were sensitive to deposition (Table 3-5). Ca, K, and Sr exhibited strong evidence for N deposition-induced limitation, declining consistently in *B. alleghaniensis* with N inputs; Ca and Sr declined with N deposition in *A. rubrum* and *P. rubens* as well (Figure 3-3). Foliar Mg and Al were relatively unaffected by N deposition across the modeled N input gradient (Figure 3-3, Figure 3-S3).

3.3.4 Correlations between foliar and soil elemental profiles

Although the tree species were sympatric at all four sites, the foliar-soil nutrient associations differed among them. Pearson product-moment correlations indicated broad differences in foliar element concentrations and their counterparts in each soil horizon (Figure 3-4). There were no obvious diagonal

associations in the matrices in Figure 3-4, indicating that foliar elemental profiles were broadly independent of organic or mineral elemental availability. For example, while Mn concentrations in A. rubrum were positively correlated with both O and B horizon Mn concentrations, Ca, Mg, and Fe were not (Figure 3-4). For some elements such as K, Al, and Ni in yellow birch, and Ca, Zn, and Sr in red spruce, the correlations are significantly positive in one horizon and significantly negative in the other. This latter pattern indicates that the tree species at these sites preferentially uptake selected elements from different depths of the soil profile.

3.4 Discussion

The negative effects of soil acidification, especially the depletion of major nutrient cations and the liberation of Al^{3+}, are major challenges to plant growth and survival [51,52]. The negative impacts and ecological degradation associated with these effects have been the subject of numerous ecological studies in temperate regions, but nutritional data demonstrating evidence of legacy effects or recovery from N deposition are rarer [53]. In the current study, soil N in both the O and B horizons increased with N deposition, precipitation volume, and elevation, reflecting the confounded nature of these variables in affecting the deposition of N into high-elevation ecosystems, but suggesting that N inputs into high elevation forests continues. In the mid-Atlantic region of the United States, Pb, Cu, and Zn from industrial emissions have been shown to occur in elevated concentrations in the organic horizon, although these concentrations have been declining as total deposition from anthropogenic emissions have declined [54]. In the current study, the concentrations of Zn and Pb (Figure 3-2), as well as Cu and Ni, increased with N deposition in B horizons, but, as with other cations, declined in the O. The mobility of metals in soils is pH dependent [55], and this migration from the O to the B may reflect the broad reductions in current rates

of deposition and the movement/retention of historically-deposited heavy metals from the O to subsurface horizons.

The distributions of Mehlich-III extractable soil elements also displayed patterns suggesting that historical acid deposition has depleted cations from O horizons and moved these to B horizons (Table 3-4), where they may be retained on soil CEC when NO_3^- and SO_4^{2-} mobility is reduced by metal sesquioxides [56]. P also declined in the O horizon with increasing N deposition and increased in the B (Table 3-4). Depending on the horizons from which trees obtain nutrients for uptake [57–59], these changes may reflect and/or interfere with tree nutrition and productivity. For example, Dijkstra and Smits (2002) noted that uptake of Ca by deep roots of sugar maple (*Acer saccharum*) was responsible for maintaining high Ca cycling beneath maple compared to hemlock (*Tsuga canadensis*) [60]. Elevated Ca in the litter layer beneath maple was associated with high root density, linking Ca cycling and the deployment of roots by maple to retrieve Ca mineralized from litter (Dijkstra and Smits 2002). In the current study, even though the three tree species exhibited characteristic foliar nutrient profiles (Figure 3-4), we found little evidence that individual tree species were influencing the local distribution of nutrients through the soil profile (Table 3-4), except for Mg in the O horizon, which was less beneath *A. rubrum*, and N in the B horizon, which was elevated beneath *A. rubrum* (Figure 3-S3a). Increases in Al and reductions in the Ca/Al ratio are often indicators of soil acidification. In the current study, Al concentrations declined in the O with increasing N deposition, but increased in the B horizons (Figure 3-2, Table 3-4). Ca/Al ratios remained unchanged overall, but decreased beneath *A. rubrum* (Table 3-4, Figure 3-S2). The divergent responses beneath the species investigated may reflect differential sequestration of Ca in biomass and/or differential mobilization of Ca and Al by each species (Dijkstra and Smits 2002).

Foliar nutrient profiles indicated that the trees along the currently-studied N deposition gradient exhibited some symptoms of cation depletion, although species varied in this response (Table 3-5). Ca and Sr

declined in the foliage of all species across the N gradient, change in K depended on species, but Mg was unaffected (Figure 3-3). A comparison of organic horizon soil solution Al, Ca, and Mg and red spruce foliar concentrations in central and southern Appalachia presented similar results as reported here: trees appeared to be nutrient-sufficient and there was no relationship between nutrient status and estimated site differences in acid deposition [61]. In the Great Smoky Mountains of southern Appalachia, soil solution and species foliar Al, Ca, and Mg were not correlated [30]. These studies are consistent with our results, which demonstrate a consistent lack of foliar response to soil solution nutrient concentrations (Figure 3-4). The paucity of field-based red spruce studies in the literature showing nutrient deficiency except in visibly-stressed trees suggests that red spruce may be most vulnerable when foliar cation-leaching from acid deposition is accompanied by very base-poor, poorly buffered soil substrates, as observed for *Picea abies* (L.) Karst. forests of Europe [62].

Although stand-level soil chemistry, resulting from depositional history interacting with local parent material differences, seemed to override tree species effects on soil elemental profiles (Table 3-4), *A. rubrum*, *B. alleghaniensis*, and *P. rubens* exhibited distinct differences in foliar nutrient concentrations (Figure 3-4, Figure 3-S3a,b), which may contribute to stand-level nutrient cycling. Pearson product-moment correlations indicate large differences in pair-wise comparisons between foliar element concentrations and their counterparts in each soil horizon (Figure 3-4). Leaves of *B. alleghaniensis* consistently exhibited the greatest concentrations of nutrients and metals, followed by *A. rubrum* and *P. rubens* (Figure 3-S3a,b). These differences were typically two- to three-fold greater and as high as eight-fold for Zn in *Betula*. Zn hyperaccumulation is a trait known to exist within *Betula* [63]. For some elements, such as K, Al, and Ni in yellow birch and Ca, Zn, and Sr in red spruce, the correlations are significantly positive in one horizon and significantly negative in the other. This could indicate that the tree species at these sites preferentially uptake selected elements from different horizons of the soil profile, which has

been observed in other systems [58,64–66]. That these differences did not translate to alterations in local soil concentrations may reflect broad redistribution of litter throughout the stands or efficient resorption by Betula before leaf drop. The functional roles of tree species as drivers of biogeochemical differences in high-elevation red spruce stands is supported by the observations of Smith et al. (2016) at these and other sites in central Appalachia [31]. Although there were no effects explained by estimates of historic acid deposition in that study, there were positive effects due to relative importance value of broadleaf deciduous tree species on patterns of N availability: greater N availability from a larger number of broadleaf tree species with lower C:N ratios and higher litter quality diluted any evidence for acid deposition effects on N cycling processes [38]. In the current study, undertaken at a subset of the same sites but sampling beneath specific trees and not at a stand basis [38], soil N levels were sufficiently explained by N deposition, elevation, and/or precipitation volume without evidence of significant species-level effects (Table 3-3).

Ratios of certain elements can be informative as to source, as well as providing an indication for the presence of processes such as chemical weathering. For example, charge and ionic radius result in Ca and Sr displaying similar behaviors in the plant-soil continuum, with the Ca/Sr ratio effective for integrating soil and tree Ca status [67]. Trees preferentially uptake Ca rather than Sr due to its importance as a macronutrient, while Sr is not known to have any necessary biological function [68]. Plant discrimination of Ca over Sr allows scrutiny of plant Ca availability and source, with lower Ca/Sr ratios a potential indication of low plant-available Ca or interference by Al [69]. In the current study, O and B horizon Ca/Sr ratios increased and foliar Sr declined to a greater degree than Ca as modeled estimates of N deposition increased (Figure 3-3), also leading to increases in the foliar Ca/Sr ratio in *B. alleghaniensis* and *P. rubens*, but not *A. rubrum*. It thus appears that N deposition is not/no longer limiting Ca or that the trees are extracting Ca from the B horizon where Ca is accumulating along the N gradient.

Soil and foliar elemental profiles for four central Appalachian high elevation forests suggest that historical and ongoing N deposition increases N concentrations of both the O and B horizons, and induces the migration of cations from the O to the B horizon. Although we sampled soils from beneath the canopies of the dominant *A. rubrum*, *B. alleghaniensis*, and *P. rubens*, species effects on soil element concentrations were strongly overshadowed by the differences between the sites across the N deposition gradient. Foliar elemental profiles suggested that a legacy effect of N deposition still influences tree nutrient acquisition, with foliar Ca, K, and Sr declining with increasing N deposition. In addition, the tree species utilized in this study exhibited distinct foliar element profiles, *with B. alleghaniensis* typically accumulating nutrients and metals in excess of two-fold, and up to eight-fold for Zn, compared to *A. rubrum* and *P. rubens*. Despite these differences, species had minimal influence on soil chemical profiles, suggesting that site N deposition, litter mixing, site parent material, and/or litter nutrient dynamics including resorption may dominate soil chemical profiles.

3.5 Figures and Tables

Site	Code	Location	Total N Deposition (kg N/ha)	Elevation (m)
Flat Ridge	FLR	Slaty Fork, WV	399.6	1320
McGowan Mtn.	MCG	Dry Fork, WV	380.2	1270
Cranberry Glades	CGL	Hillsboro, WV	366.4	1160
Little Spruce Bog	LSB	Pembroke, VA	326.1	1179

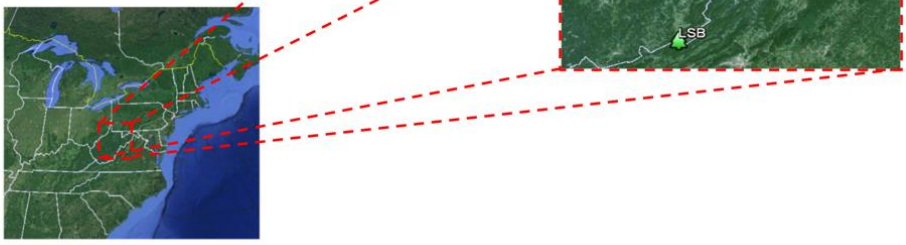

Figure 3-1. Locations and site characteristics of four high elevation sample sites.

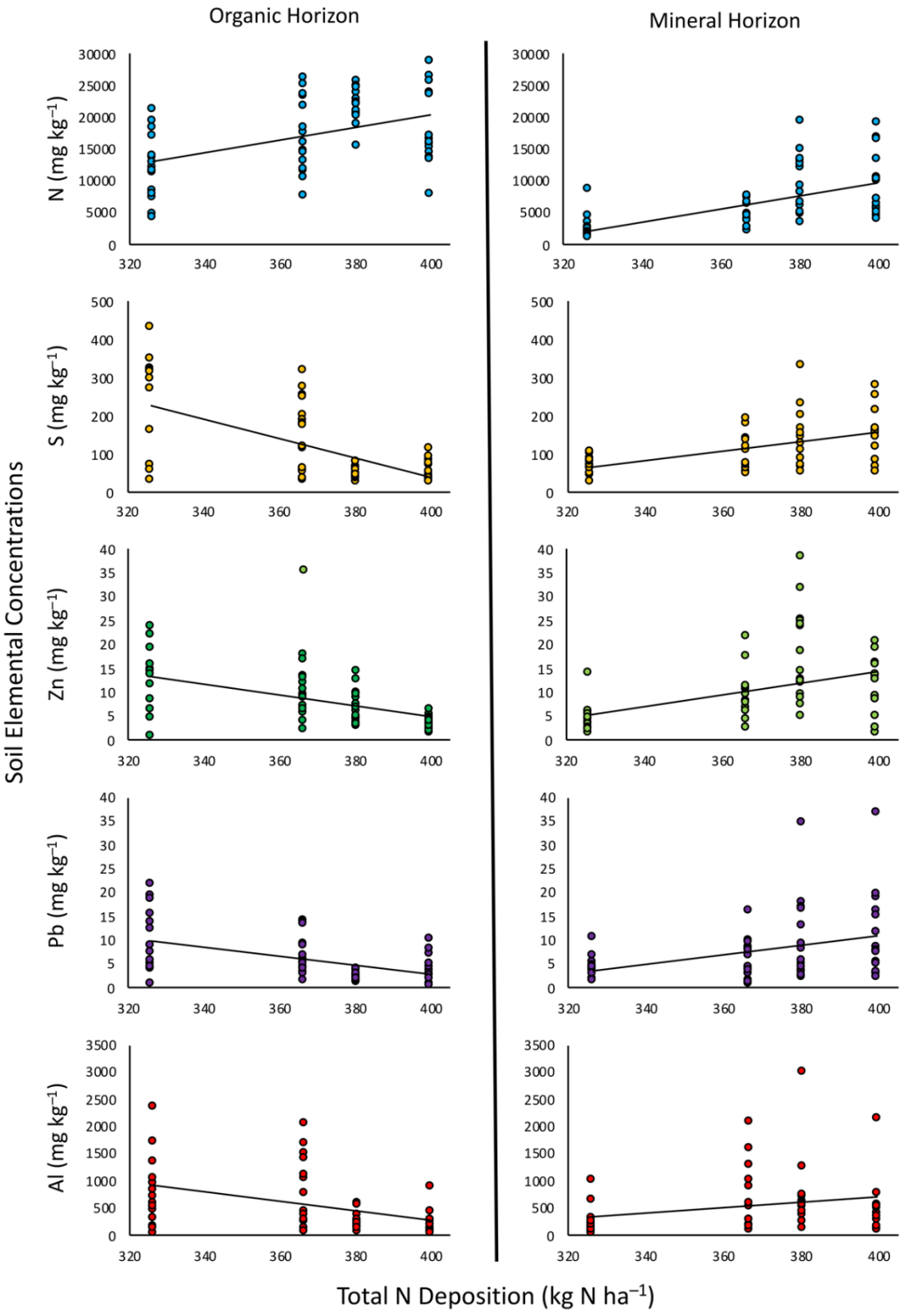

Figure 3-2. Soil elemental concentrations vs. total estimated total N deposition (kg N ha^{-1}) of selected indicators of deposition within the organic and mineral horizons of montane soils.

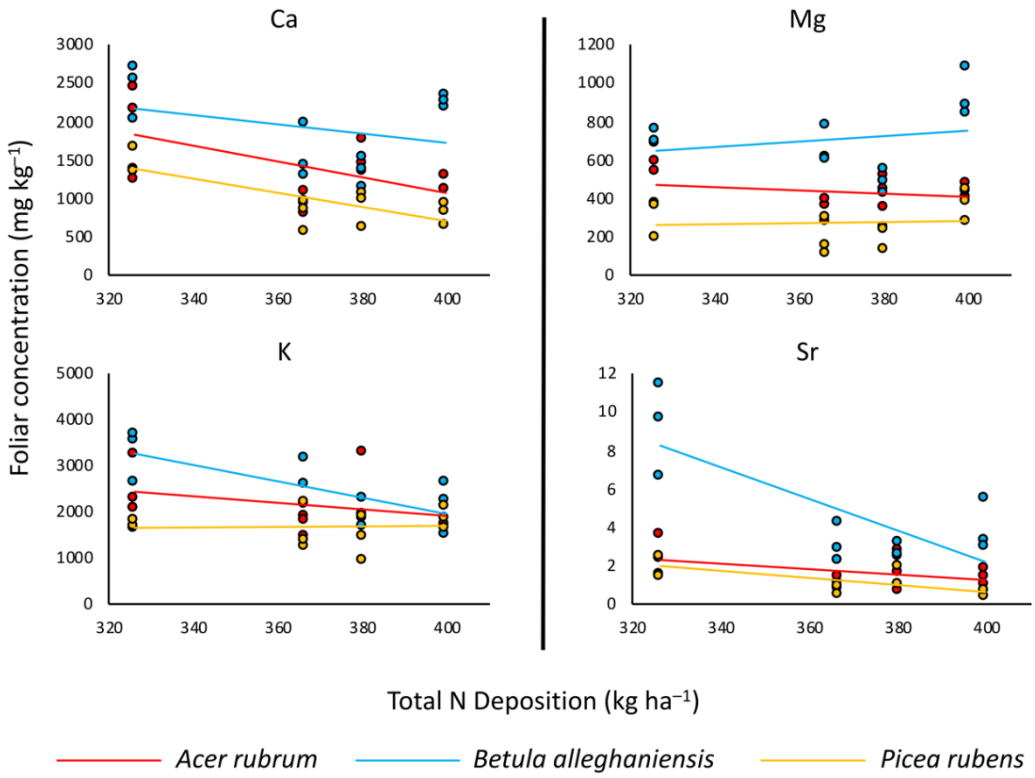

Figure 3-3. Depletion of selected cations in foliage across a gradient of modeled N deposition.

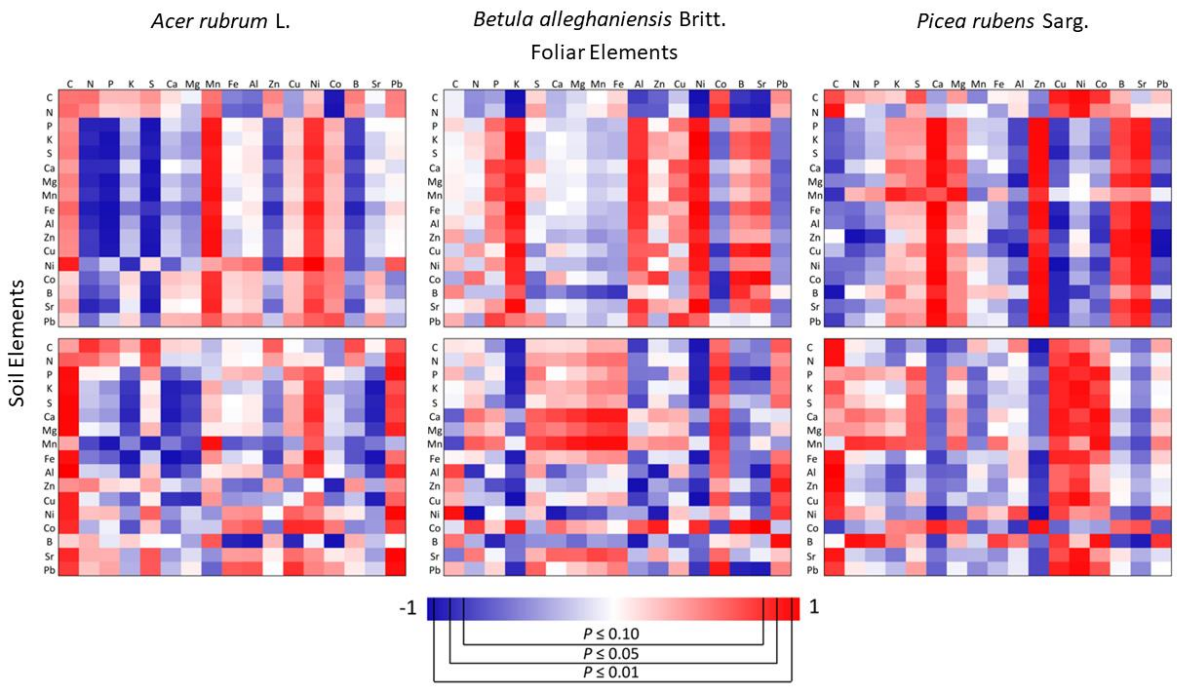

Figure 3-4. Pearson product-moment correlations for the pairwise comparisons of foliar element values by soil horizon (organic fraction above and mineral fraction below, respectively). Color ramp indicates direction of correlation and significance.

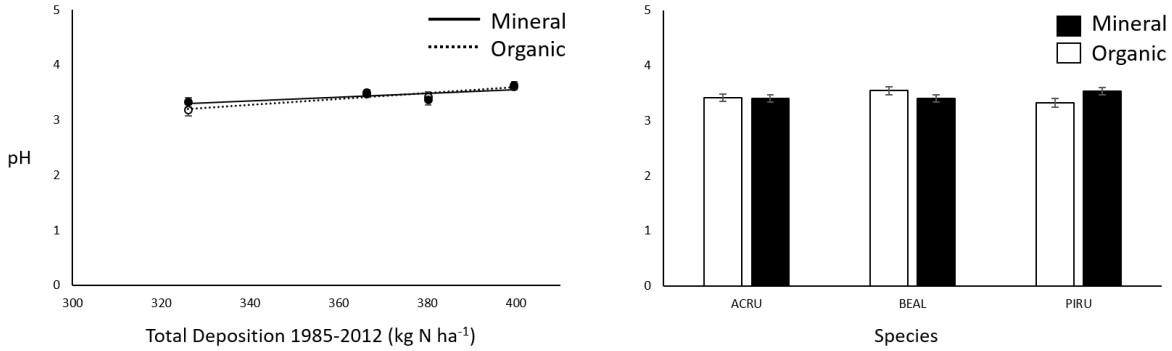

Figure 3-S1. Soil pH as functions of horizon and N deposition (left) and overstory tree species (right).

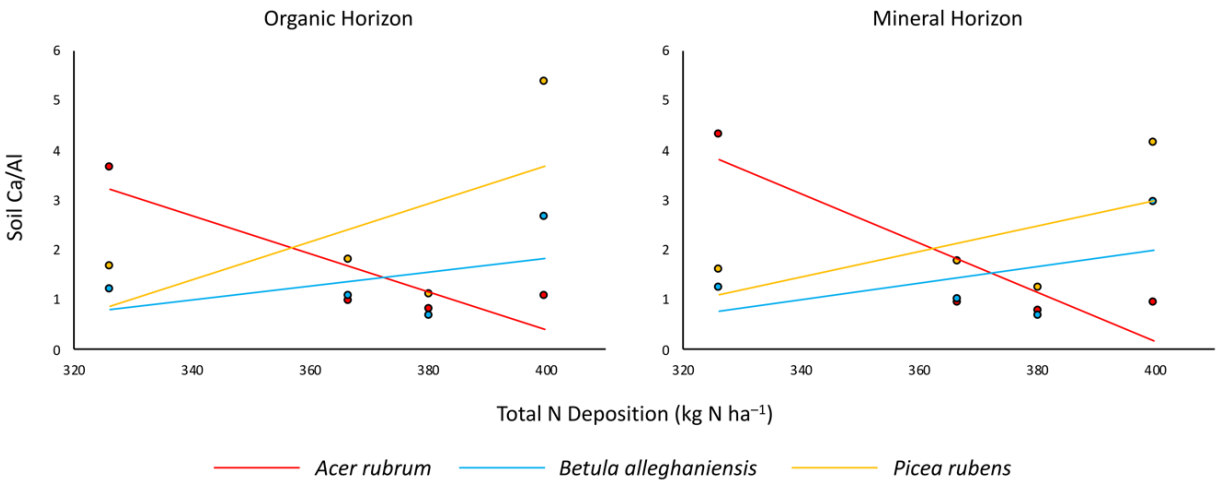

Figure 3-S2. Soil Ca/Al as N deposition and overstory tree species for O (left) and B (right) soil horizons.

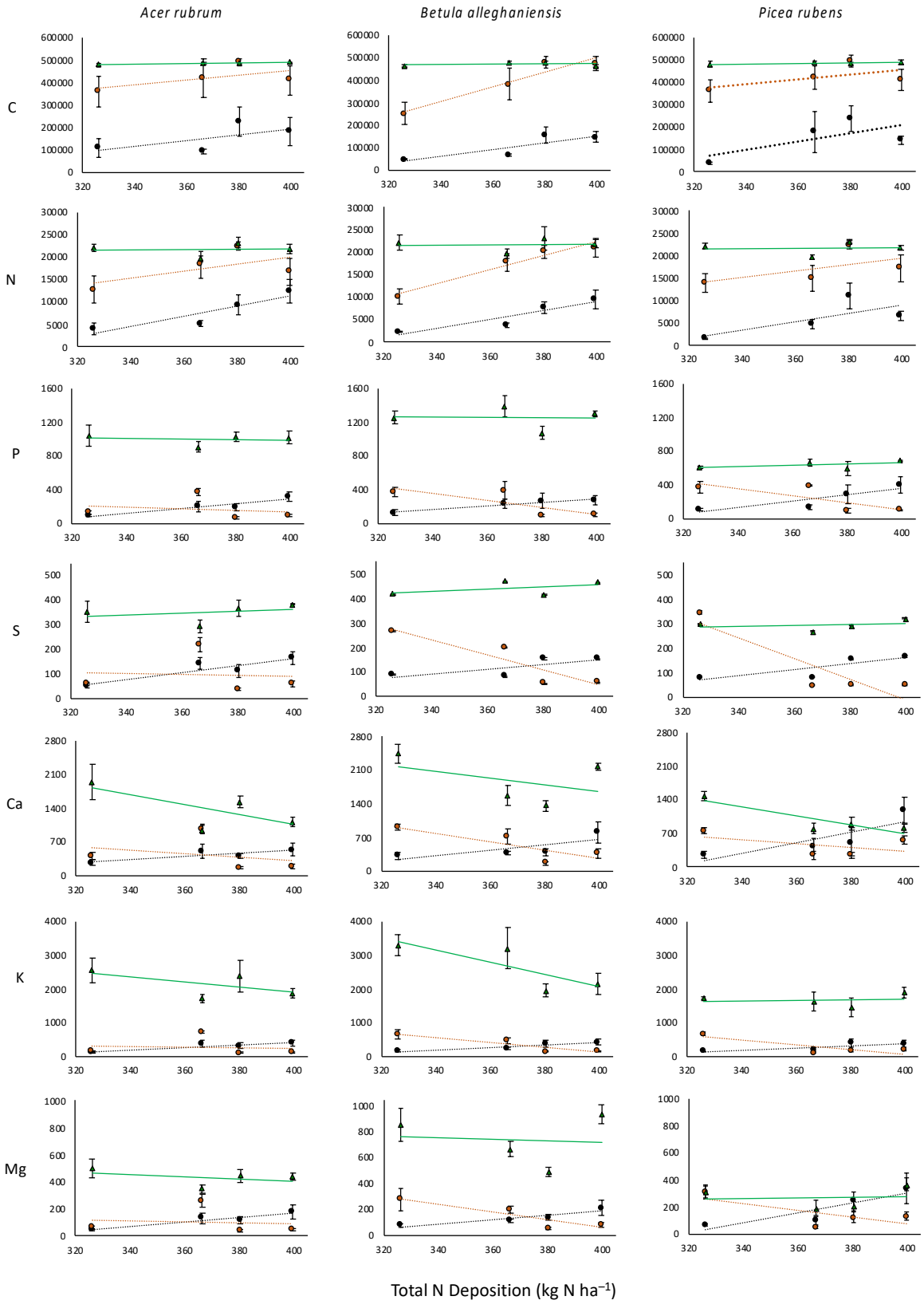

Figure 3-S3a. Soil macro-element concentrations in foliage, and both organic and mineral horizon soils (mg g^{-1}) by forest tree species and modeled estimates of historic N deposition.

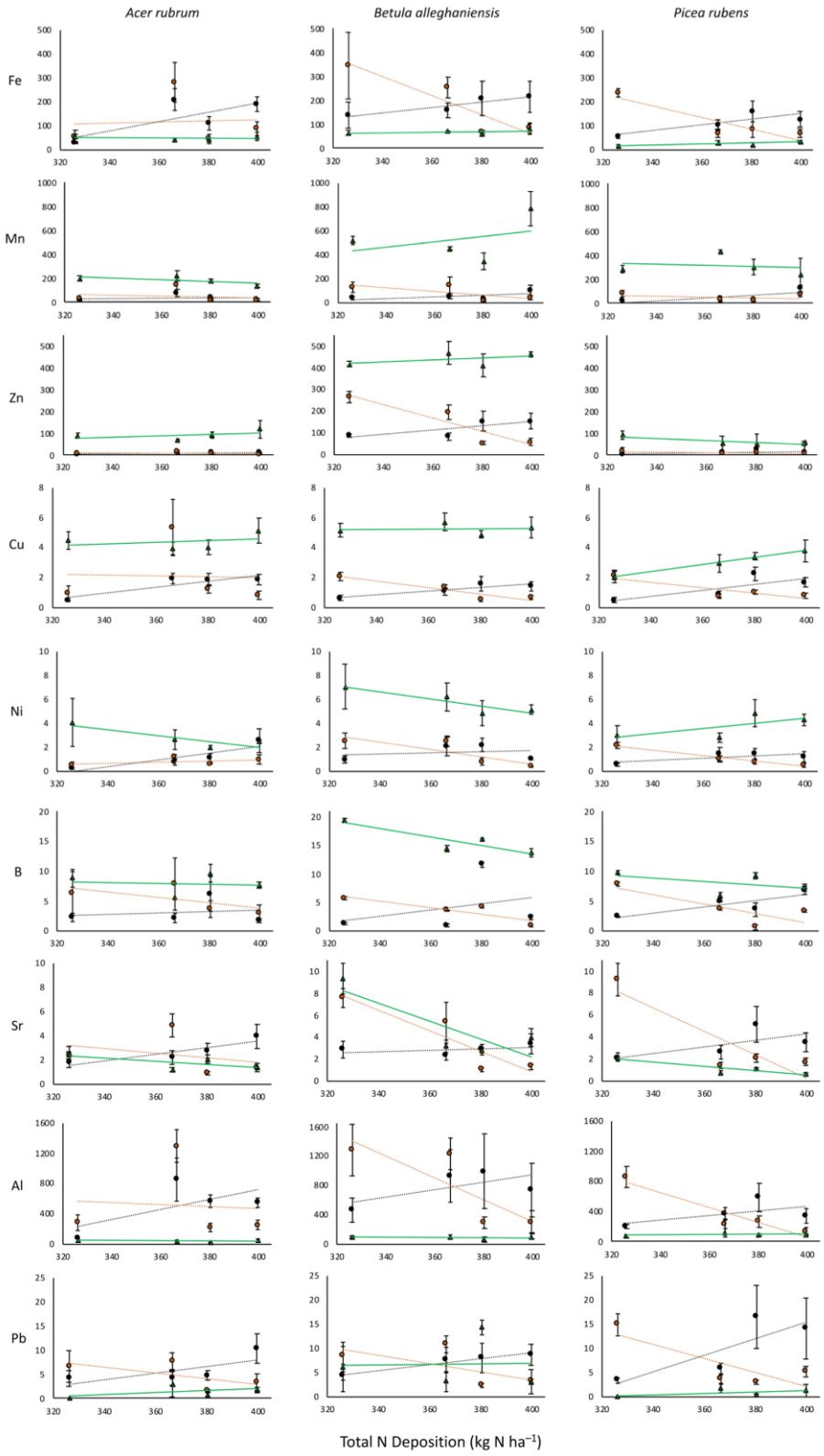

Figure 3-S3b. Soil micro-element concentrations in foliage, and both organic and mineral horizon soils (mg g^{-1}) by forest tree species and modeled estimates of historic N deposition.

Table 3-1. Soil characteristics of the four high-elevation sites. Soil classifications and descriptions were obtained from NRCS Soil Survey Geographic (SSURGO) Database. Mean soil pH values for each site (organic O; mineral M) were measured using 5-g subsamples of homogenized soil from each sample location and each soil horizon.

Study Site	Soil Series	Taxonomic Class	Drainage Class	Description	Soil pH
FLR	Gauley	Frigid Typic Haplorthods	Moderately deep, well drained	Loamy-skeletal, siliceous, superactive	*O:* 3.61 *M*: 3.63
MCG	Ernest	Mesic Aquic Fragiudults	Very deep, moderately well to poorly drained	Fine-loamy, mixed, superactive	*O*: 3.42 *M*: 3.36
CGL	Snowdog	Frigid Typic Fragiudepts	Very deep, moderately well drained	Fine-loamy, siliceous, active	*O*: 3.49 *M*: 3.47
LSB	Lily (LB Complex)	Mesic Type Hapludults	Moderately deep, well drained	Fine-loamy, siliceous, semi-active	*O*: 3.18 *M*: 3.32
	Bailegap (LB Complex)	Mesic Type Hapludults	Deep, well drained		

Table 3-2. Relative importance values for *A. rubrum* (ACRU), *B. alleghaniensis* (BEAL), and *P. rubens* (PIRU) at each high elevation red spruce site; data from Smith et al. (2016).

Site	% ACRU	% BEAL	% PIRU	% Total Broadleaf Deciduous	% Total Needleleaf Evergreen
FLR	15.1	5.0	75.0	26.2	73.8
MCG	6.4	36.8	21.4	49.8	50.2
CGL	18.2	14.0	50.8	43.1	56.9
LSB	13.0	11.0	23.1	32.6	67.4

Table 3-3. Comparison of Corrected Akaike Information Criterion (AICc) values for linear regression models evaluating drivers of N, S, Zn, Pb, and Al concentrations in montane soils. *Indicates most parsimonious model.

	O Horizon					B Horizon				
Model Parameter	**N**	**S**	**Zn**	**Pb**	**Al**	**N**	**S**	**Zn**	**Pb**	**Al**
N deposition	55.0	127.8	132.6	141.8	149.3	92.3	79.2*	129.4	142.9*	157.1
Precipitation	64.5	152.9	146.1	157.3	167.9	127.9	95.4	140.7	152.3	156.9
Elevation	58.2	135.1	143.7	138.9	159.1	120.5	94.3	128.6	149.8	163.9
N deposition, precipitation	57.3	123.9	127.4	139.5	143.2*	93.0	88.2	131.7	144.1	156.6
N deposition, elevation	51.8	120.3*	134.9	133.4*	148.8	88.9*	88.7	121.9	144.6	158.8
Precipitation, elevation	49.9*	132.0	145.7	138.4	160.2	106.3	90.8	118.2*	149.7	152.8*
N deposition, precipitation, elevation	51.6	121.5	127.3*	135.8	145.6	91.2	82.9	120.2	146.4	155.2
R^2 (*best model)	0.30	0.46	0.32	0.36	0.38	0.57	0.29	0.41	0.17	0.25

Table 3-4. Slope coefficients and probabilities for mixed multiple regression for selected elements in montane soils across a modeled N deposition gradient.

Horizon	Nutrient	Deposition Slope (% (% kg N $ha^{-1})^{-1}$)	Elevation Slope (% (% $m^{-1})^{-1}$)	$P_{Species}$
O Horizon	C	1.81*	2.01	0.296
	N	2.00*	2.48*	0.941
	C:N	0.06	–0.42	0.008
	Ca	–3.09**	–6.93***	0.383
	Mg	–3.86**	–4.67*	0.039
	K	–4.59***	–4.98**	0.175
	P	–4.54***	–5.60**	0.182
	Al	–5.73***	–4.03	0.033
	Ca/Al	1.81	–2.93	0.118
B Horizon	C	3.84***	5.19**	0.222
	N	5.98***	3.46*	0.043
	C:N	–1.42***	1.16*	0.173
	Ca	5.01***	–1.57	0.765
	Mg	5.68***	0.785	0.078
	K	4.69***	1.06	0.838
	P	5.14***	-0.41	0.725
	Al	4.66**	1.49	0.155
	Ca/Al	0.45	–3.41	0.181

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Table 3-5. Slope coefficients and probabilities for mixed model regression for elements in foliage of *A. rubrum*, *B. alleghaniensis*, and *P. rubens* across a modeled N deposition gradient.

Nutrient	Deposition Slope (% (% kg N ha^{-1})$^{-1}$	$P_{Species}$
C	0.086	0.009
N	0.083	<0.001
Ca	–2.198***	<0.001
Mg	–0.308	<0.001
K	–1.248*	<0.001
P	0.076	<0.001
S	0.338	<0.001
Fe	0.964	<0.001
Mn	–0.484	<0.001
Cu	1.142*	<0.001
Zn	0.794	<0.001
B	–1.111	<0.001
Ni	–0.279	<0.001
Sr	–4.285***	<0.001
Al	–0.551	<0.001

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

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4 Extracellular soil enzyme activities in high-elevation mixed red spruce forests in central Appalachia, U.S.A.

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4.1 Introduction

Microbial extracellular soil enzymes (ESEs) serve important ecosystem functions by facilitating the biogeochemical cycling of soil organic matter (SOM) and increasing the concentration of plant-available nutrients in the soil solution [1]. Except for acid phosphatases secreted by plant roots, ESEs are generally synthesized and secreted by microbes, and act as drivers for many of the rate-limiting steps in nutrient transformations for resource-scavenging in ecosystems [2]. Both the plant community structure, through the direct influence on microbial communities and indirect effects on litter quality, and soil disturbance, such as acid deposition and accompanying nutrient inputs, will affect the structure and function of soil microbial communities and the production of ESEs [3].

Plant species influence root-zone microbial communities, which influence nutrient turnover and acquisition by the root [4]. Plant nutrient status is especially influenced by the mycorrhizal associations that significantly enhance phosphorus (P) acquisition [5]. Differences in ectomycorrhizal (ECM) versus arbuscular mycorrhizal (AM) fungal communities selected by trees and the quality of detrital inputs from different hosts will influence the microbial community structure and function related to nutrient scavenging. Therefore, it is important to consider tree species effects on the soil microbial community and tree functional characteristics as important drivers of nutrient cycling in forested ecosystems. For example, high-elevation red spruce forests develop an organic soil horizon consisting of large quantities

of nutrient-poor, recalcitrant organic matter. In these ecosystems, microbial ESEs will play important roles in the decomposition of high carbon (C):nitrogen (N) compounds, such as chitin, cellulose, hemicellulose, and lignin [6], and nutrient cycling as a whole.

Nutrient fertilization is another major driver of soil microbial community composition and may promote additional feedbacks by altering the plant communities that influence soil microbial diversity [7]. Although some soil microbial communities exhibit functional resilience despite reductions in microbial diversity [8], ESE profiles often shift as microbial communities change with nutrient enrichment [9–11]. While functional redundancy among soil microbes can minimize the impacts of these community changes on ESE profiles, microbial communities experiencing elevated nutrient availability may lack the microbial taxa and genes to produce certain enzymes needed to mediate nutrient transformations. This includes potential implications for decomposition and related processes [12]. Schimel and Bennett (2004) portrayed the depolymerization of organic nitrogen forms in soil organic matter as the rate-limiting step for soil N cycling [13]. There are also indications that ESE responses to N fertilization are closely intertwined with soil P status in acid forest soils. Chronic N fertilization has been shown to suppress hydrolytic enzyme activities in spruce-fir forests, while nutrient acquisition was governed by inorganic P rather than N [14].

Due to the site preferences of red spruce for high elevations within the Appalachian Mountains, USA, these ecosystems have received, and continue to be influenced by, elevated levels of acidic deposition that contain high levels of N and sulfur (S). Despite reductions in acidic inputs due to the Clean Air Act that curbed industrial emissions in the United States, these N inputs may have impacted soils/soil processes in

these forest ecosystems. Elucidating patterns of soil enzyme activities and the factors influencing their activities could aid in identifying changes in nutrient cycling and, therefore, potential disruptions to ecosystem processes in high elevation red spruce forests.

The objective of this study was to observe differences in ESE activity profiles from organic and mineral fractions of the bulk soil below the canopies of three of the most abundant tree species in high-elevation Appalachian forests, *Acer rubrum* L., *Betula alleghaniensis* Britt., and *Picea rubens* Sarg.*,* at four sites along a modeled gradient of inorganic N deposition. In this case, we report the activities of acid phosphatase (AP) catalyzing organic P liberation, β-glucosidases (BG) yielding simple C forms from celluloses, chitinolytic *N*-acetyl-glucosaminidases (NAG) that release N, and fungal laccases (LAC), which are a subset of phenol oxidases that are multicopper oxidases expressed by certain taxonomic groups of fungi. We specifically evaluated the following hypotheses: (1) soils beneath different tree species will have significantly different ESE activities, which correspond to differences in host-specific soil C:N ratios and/or controls on microbial communities, (2) ESE activities will increase with N deposition reflecting shifts in N and P availability, and (3) seasonal differences in ESE activities from May, July, and October will reflect changes in C inputs during the growing season in these soils.

4.2 Methods

Four high-elevation red spruce stands were selected based on site elevation (>1100 m), tree species composition (mixed hardwood-red spruce), and position along a gradient of modeled acid deposition, which ranged from 326 to 400 kg N ha^{-1} over the past 27 years in central Appalachia, as outlined by Crim et al. (2019) [15]. Site selection for modeled acid deposition relied upon data from the National

Atmospheric Deposition Program (NADP) from 1985 to 2012. Each site consisted of a single 100-m diameter plot about a center point established in an area with red spruce as the predominant tree species. Three individual trees of *A. rubrum*, *B. alleghaniensis*, and *P. rubens* were selected at each site from the canopy-dominant or co-dominant individuals that were greater than 45-cm diameter at breast height (DBH). Plot tree diversity characteristics are presented in Appendix A. Two soil samples were collected parallel to the slope on opposite sides of each tree midway between the bole and canopy edge and composited. Samples were collected from the same trees once each in May, July, and October using a soil corer with a 2-cm sampling tube to a depth of 15 cm. Samples were carefully separated into organic (O) and mineral (M) fractions and then composited by soil fraction for each individual tree of each species at each site. Following collection, samples were stored in plastic Ziploc® bags and immediately placed on ice. In the lab, samples were sieved using a 2-mm (No. 10) screen and stored at –20 °C [16].

Soil subsamples were dried at 65 °C to calculate soil moisture with subsamples for C, N, and P analysis air-dried and stored in the dark at 4 °C. C and N were measured using a Thermoquest Elemental Analyzer. P was analyzed following Mehlich III extraction by inductively coupled plasma optical emission spectroscopy (ICP-OES). Subsamples for enzyme measurements were thawed and assayed field-moist. Fluorimetric assays were performed for the hydrolytic enzymes acid phosphatase (AP), arylsulfatase (ARS), β-glucosidase (BG), and N-acetyl-glucosaminidase (NAG). Colorimetric assays were completed for the oxidative enzymes polyphenol oxidase (PO) and peroxidase (PER) [1]. The pH of the extracting buffer used in the assays was adjusted to pH 3.5 to approximate the pH of the native soils [17,18]. Enzyme activities were measured for both the organic and mineral fractions of the bulk soil. The reaction of L-3,4-dihydroxyphenylalanine (L-DOPA), the substrate for PER and PO that produces a chromophore as it is oxidized, has a pH optimum approaching 9 [19], which, potentially, results in low measurements given our

assay conditions [20]. While colorimetric assays for LAC were consistent with literature values reported in forest soils [20–22], PER and PO activities were low or undetectable, which indicated that L-DOPA was ineffective as a substrate given the assay conditions simulating the low pH of the native soil at these sites. These data and data for ARS, which were also low, were not analyzed further.

Measurement of extracellular enzyme activities in soils is a challenge since enzymes can be adsorbed to soil constituents such as clays, humic compounds, and other colloidal entities with locally strong charges that can result in strong physical and/or chemical binding of the enzyme [23]. An additional colorimetric assay for oxidative enzyme activity optimized for low pH samples was performed using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate to measure the activity of fungal laccases (LAC) [24]. This method, which is specialized for soil samples, is 3–40 times more sensitive than alternative protocols for soil assays of laccases and provides an effective proxy for soil oxidative enzyme activity [20,25]. Extinction coefficients for chromophores of ABTS in these soils were generated and tested for temporal stability over the course of the soil incubations using lyophilized mushroom tyrosinase (Sigma-Aldrich product #T3824-25KU) [26], and were similar to literature values for forest soils [16,25].

4.3 Results

4.3.1. Soil C, N, and P Responses to N Deposition

Enhanced N deposition led to increases in organic fraction C and N across the N deposition gradient while organic fraction P declined across the sites (Figure 1). Mineral soil C and N increased more substantially than increases in the organic fraction, whereas P increased in the mineral fraction across the N deposition gradient (Figure 1). There was little evidence of species differentiation in soil C, N, or P concentrations among the sites (Figure 1).

4.3.2 Soil Fraction and Species Effects on ESE Activities

Soil enzyme activities, pooled across time points and sites, consistently exhibited higher mean rates ($p < 0.001$ for AP, BG, NAG, and LAC) in the organic fraction than in mineral soils (Figure 2). Rates of AP were the highest of the ESEs measured and, for the species evaluated, organic fractions beneath *P. rubens* exhibited the highest AP (Figure 2). In the mineral fraction, the activity of LAC beneath *B. alleghaniensis* was greater than that of *P. rubens*; LAC activity beneath *A. rubrum* was intermediate between the other two species.

4.3.3 Temporal, Depositional, and Stand Diversity Effects on ESE Activities

Seasonal effects on ESE activity in organic fractions were significant for BG ($p = 0.008$) and NAG ($p = 0.021$) (Table 1) with BG and NAG activity peaking in mid-summer. Soil ESE activities in mineral fractions displayed little temporal change in activity.

ESE activity in the organic fraction was little affected by N deposition (Table 1). In contrast, AP, BG, and NAG were strongly affected by N deposition in mineral fractions (Table 1). In each case, activity increased with increasing N deposition (Figure 3). The significant species $\times$ deposition interactions noted for AP and BG resulted from these ESE responses to N being the greatest in soils beneath *P. rubens*, and less so beneath *A. rubrum*, with little response to N in soils collected beneath *B. alleghaniensis*.

Evaluation of ESE activity as functions of local soil-level and plot-level factors excluding N deposition indicated that ESE activity was not influenced by soil nutrient factors related to N deposition, but rather by plot-level factors that influence the microbial community (Table 2). As noted above, ESE activities were greater in the organic fractions than in mineral fractions (Table 2). In addition, greater plot broadleaf representation depressed AP, BG, and NAG activity, whereas plot tree diversity associated with ectomycorrhizal hosts increased ESE activity as did overall plot tree diversity (Table 2).

Principle component analysis for soil-level and plot-level predictors of ESE activities highlighted separation among sites driven by N deposition, soil nutrient concentrations, and stand species composition (Figure 4). For the organic fraction, the first two principle components (PCs) explained 54.1% of the variation with N deposition, soil C and N, and ECM RIV having positive influences while soil P, tree diversity, and broadleaf RIV had negative weights on PC1 (Table 3). PC2 reflected the strong correlation of activities among the ESEs measured (Figure 4). In the mineral fraction, the first two PCs accounted for 56.4% of the variation. As with the organic fraction, N deposition, soil C and N, and ECM RIV positively loaded PC1 while broadleaf RIV and tree species diversity negatively weighted PC1 (Table 3). In contrast to the PC1 for the mineral horizon, soil P loaded positively to PC1 in the mineral horizon (Figure 4). PC2 for the mineral fraction highlighted the negative correlation between ESE activity and soil N and C. It is evident from Figure 4 that patterns in the data existed with increasing deposition (symbol color intensity) along the first PC in each soil fraction, which supports soil compositional changes highlighted in Figure 1 and ESE activity responses in Figure 3.

4.4 Discussion

The long-term inputs of N-containing acidic precipitation into high-elevation forests in the eastern United States have had numerous impacts on trees and soils in these ecosystems. With the implementation of the Clean Air Act, N and S inputs have declined into these systems, although N deposition is still substantial. Increases in soil N, concomitant reductions in cations, and inputs of metals in these soils [15] have the potential to alter microbial community structure and function. In this study, we evaluated the activity of a suite of microbially-produced soil enzymes involved in SOM and nutrient cycling in soils along a modeled N-deposition gradient in the central Appalachian Mountains.

4.4.1 Soil C, N, and P Responses to N Deposition

Nitrogen inputs into these sites, ranging from 326 to 400 kg N ha^{-1} between 1985 and 2012, stimulated C and N accretion in both the organic and mineral horizons. However, the observed changes were proportionally greater in the mineral than organic fractions (Figure 1). In contrast, P declined in the organic fraction, but increased substantially in the mineral layers, which suggests an acid-induced redistribution of P from the organic to mineral horizons. We also noted substantial redistribution of cations and metals in these soils [15]. Such changes are typical of N-deposition impacted soils [29–32] and have the potential to alter microbial communities and their activity directly or indirectly though changes in tree rhizodeposition. ESEs function to access energy and nutrients stored in recalcitrant organic matter in forest soils [33,34]. Alterations for the processes driving decomposition have important consequences for nutrient cycling and C storage within forests [35].

4.4.2 Soil Fraction and Species Effects on ESE Activities

The predominance of thick, well-developed organic fractions at these sites was typical of the spodic soils that develop under red spruce [36]. Substantially higher enzyme activities in the organic versus the mineral fractions (Figure 2) follow the inverse relationship typically observed between soil microbial biomass and soil depth [37–40]. In the organic fractions, the growth of microbes is stimulated by SOM, and the ramification of mycorrhizal fungal hyphae and their associated microbial communities in the litter enhance degradation of SOM via nutrient scavenging [4,33]. The mineral fractions, which contain lower quantities of organic matter, exhibited lower ESE activities that are related to differential microbial communities established due to lesser influence of host tree litter and roots [41–43].

The microbial community and the activity of ESEs may also respond to changes in the soil environment brought about by variation in the dominant vegetation. The soil microbial community is indirectly influenced by plant diversity through plant traits that influence soil nutrient availability [43]. Quantitative and qualitative differences in litter inputs and differences in root-mycorrhizal associations have large influences on the soil abiotic environment and will influence microbial community structure and function [4,43,44]. In the current study, there were minor differences in ESE activity in soils beneath the three species investigated, such as the higher AP activity beneath red spruce (Figure 2). Multivariate analyses confirmed the importance of function diversity of the forest plots in structuring soil properties and ESE activities (Tables 2 and 3, Figure 4). ESE activities as a whole were not influenced by the mycorrhizal functional status (ECM vs. AM) of the tree of collection, but rather were more responsive to plot-level characteristics, such as broadleaf RIV and ECM RIV (Tables 2 and 3), which may influence plot litter quality. We previously reported [15] that, while foliar nutrient concentrations varied extensively among the tree

species on these sites, soil nutrient pools were relatively homogeneous. This suggests that plot-level redistribution plays an important factor in establishing soil characteristics and the activity of the microbial community [45,46].

4.4.3 Temporal, Depositional, and Stand Diversity Effects on ESE Activities

Temperature and moisture are important factors driving microbial activity and decomposition in soils. Even minor increases in soil temperatures can accelerate microbial activity and litter decay rates under the red spruce [47,48]. Soil amino acid pools, primarily utilized by microbes over plants, have been shown to increase during dormant periods and decrease during the growing season [49]. In the current study, the activity of the hydrolytic enzymes, BG and NAG, in the organic horizon were significantly higher in July than in May or October, which is consistent with Kittredge et al. (2018) who demonstrated sensitivity of these enzymes to organic fraction warming [50]. The mineral fractions, which contain lower quantities of organic matter, are additionally buffered from changes in the surface environment, such as moisture and temperature fluctuations, which may minimize seasonal patterns of microbial population growth and activity.

In the current study, we found the activity of AP, BG, and NAG increased significantly across the modeled N deposition gradient at our sites in the mineral, but not organic fractions (Table 1, Figure 3). This mineral-fraction response may reflect the proportionately greater enrichment of this horizon with C, N, and P as N deposition increases when compared to organic fractions (Figure 1), which may stimulate/alter the growth, metabolic activity, and or diversity of the microbial community. This hypothesis is supported by the relationships between variables in PC1 for the mineral fraction (Table 3), where ESE activities are

positively weighted with soil N, C, and P. In contrast, suppression of ESE activities concurrent with N fertilization has been observed in several studies [3,51,52]. An evaluation of organic fraction soils across a gradient of N deposition in the Adirondack Mountains of Upstate New York showed a trend between N deposition and organic soil N, but stronger relationships with other site factors such as growing season degree days [50].

In addition to the effects of N deposition, we found that activity in the mineral horizon was negatively correlated with broadleaf RIV and tree diversity, which indicates that, across the depositional gradient, differences in stand broadleaf composition (and the quality of leaf and root litter inputs) may also affect ESE activity. Deposition of N and S impacts not only the microbial community structure, but also biotic interactions between fungi, bacteria, and plants. Chronic NH_4NO_3 addition has been shown to increase mycorrhization in black spruce (*Picea mariana* (Mill.) B.S.P.) as well as other species [53,54]. There is evidence that differences in mycorrhizal fungal type, AM or ECM, exert considerable differences in ESE activities. In a similar mixed northern hard-wood/coniferous forest in New England, Brzostek and Finzi (2011) examined the effects of *Acer saccharum* (AM), *Fraxinus americana* (AM), *Tsuga canadensis* (ECM), and *Fagus grandifolia* (ECM) on ESE activities and found that roots of AM tree species exhibit little influence on ESE activities relative to their ECM counterparts [55]. The effects of mycorrhizal type on ESE activities in the current study were difficult to assess, however, as these stands had very similar mycorrhizal compositions (Appendix A) and the effect of plot mycorrhizal status was more prevalent than a tree sample mycorrhizal type (Table 2).

One cannot rule out that specific site factors, such as mineralogy and elevation, may be playing a role in organizing soil microbial communities and affecting ESE activity profiles. These factors will influence tree stand structure and function. While tree species diversity declines across the depositional gradient, broadleaf RIV is lower only in the site receiving the highest N deposition and ECM RIV is lowest only at the site receiving the lowest deposition (Appendix A). Given that these factors also affect ESE activity (Tables 2 and 3), it is difficult to unequivocally ascribe N deposition to the changes in ESE activity observed. Smith et al. (2016) found that broadleaf RIV was the primary driver of nitrogen transformations across a broader modeled N deposition gradient that included our plots [45]. Similarly, Zheng et al. (2018) noted that vegetation change, and the concomitant changes in litter inputs, was the primary controller of soil microbial community structure across an elevational gradient in the eastern Tibetan Plateau [44]. Thus, it may be the quality of N (and other nutrients) cycling in soils across our sites, and not the quantity of N, that is driving patterns of ESE activity observed in this case.

4.4.4 Conclusions

Nitrogen deposition into high elevation spruce forests in the Appalachian Mountains has increased soil organic fraction C and N and mineral fraction C, N, and P, whereas P concentrations in organic fractions have declined. These changes are also associated with site differences related to tree species composition, including broadleaf deciduous RIV, tree diversity, and ECM RIV. Soil ESE activities increased with modeled deposition across the four sites sampled, and ESE activities for most enzymes were negatively associated with broadleaf RIV and tree diversity while positively correlated to ECM RIV. Sampling time had little effect on the activities of most enzymes, and the paucity of differences observed between seasons may indicate that enzyme activities mediating many of the rate-limiting steps in nutrient transformations are relatively robust against differences in ambient temperatures and precipitation across small temporal scales at

these sites. The enzymes most influenced by changes in soil quality relate to P availability and decomposition of complex carbon compounds, which, potentially, results in greater rates of litter decomposition, lower C storage, and a transition towards P limitation as N deposition increases in these forest soils.

4.5 Figures and Tables

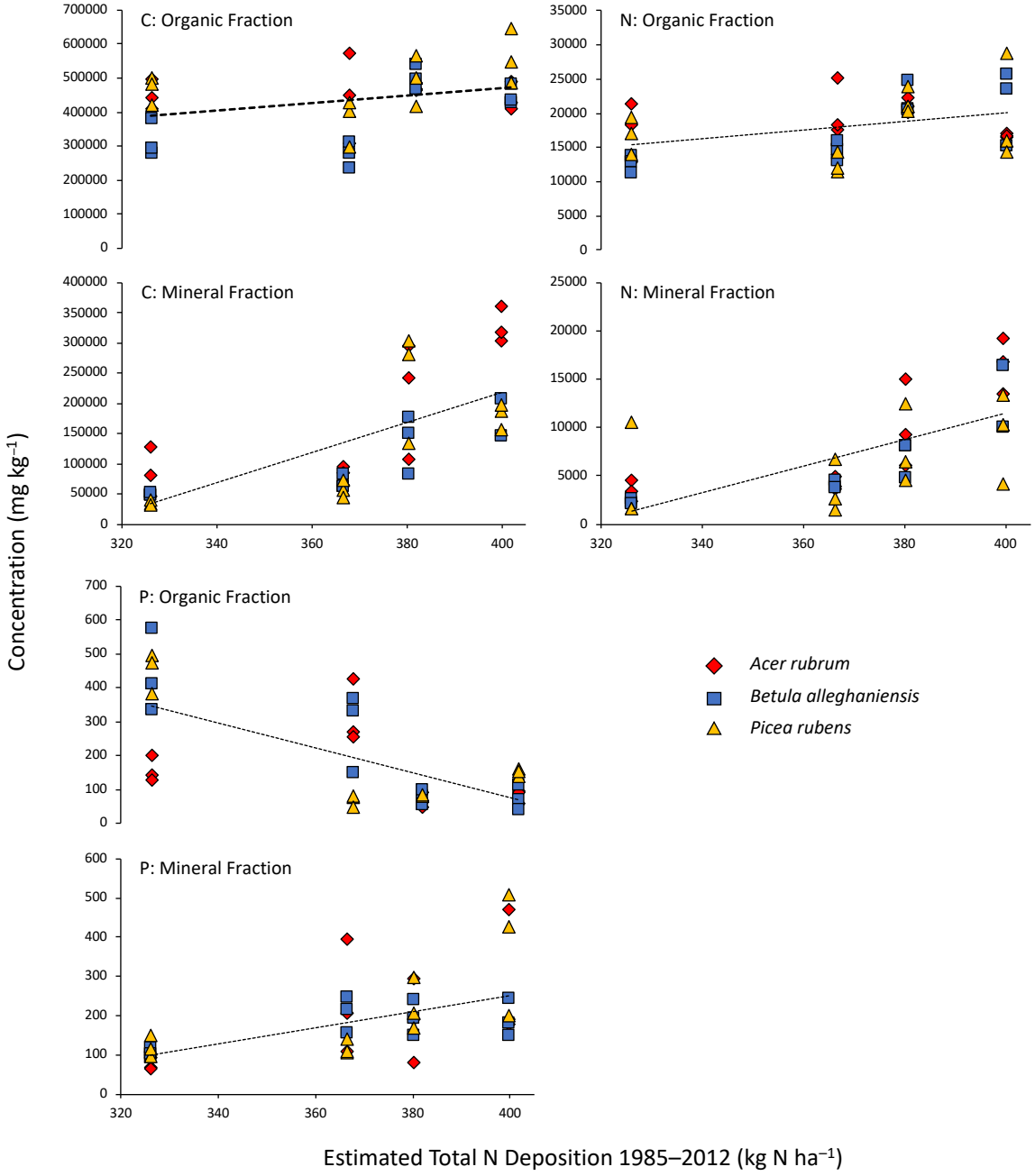

Figure 4-1. Mean soil C, N, and P concentrations for organic and mineral fractions as functions of estimated total N deposition into high elevation spruce forests. Each symbol represents the mean beneath three replicate trees of each species. Regression lines are best fits across all species. Slopes ± SE [(mg kg^{-1}) (kg N ha^{-1})$^{-1}$] for the regressions are: C_{org}, 1133 ± 574, C_{min}, 2421 ± 435, N_{org}, 64.7 ± 25.9, N_{min}, 131.0 ± 20.9, P_{org}, –3.76 ± 0.70, and P_{min}, 2.36 ± 0.56. All slopes are significant ($p < 0.05$).

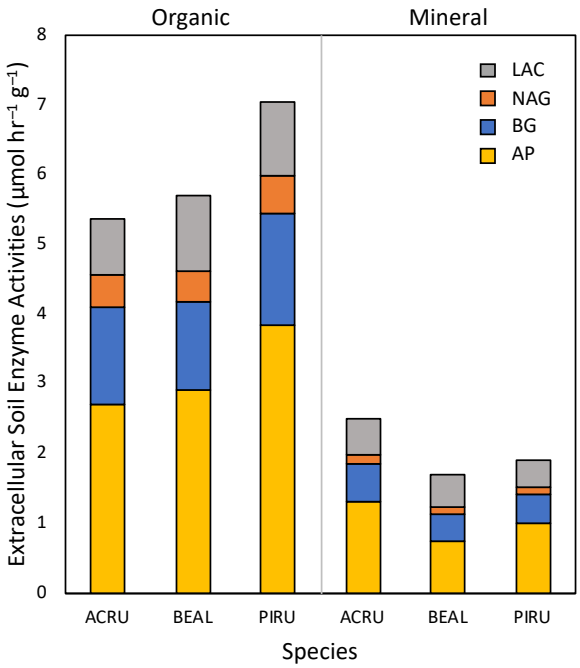

Figure 4-2. Extracellular soil enzymes (ESE) activities pooled across sites and sampling times by tree species for organic (left) and mineral (right) soil fractions. Stacked bars represent the mean activity for each enzyme for three replicate trees of each species assayed at four sites and at three times (*n* = 36).

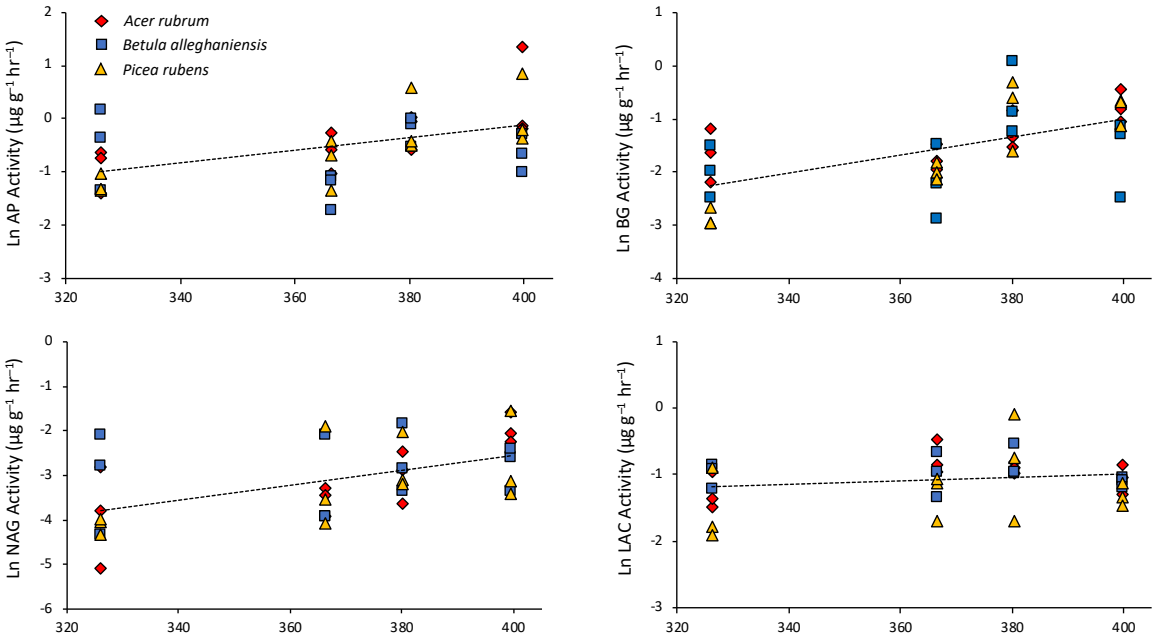

Figure 4-3. Activity of AP, BG, NAG, and LAC in mineral soil fraction as functions of estimated total N deposition into high elevation spruce forests. Each symbol represents the activity in samples beneath three replicate trees of each species. Regression lines are best fits across all species. Slopes ± SE [ln(µg g^{-1} h^{-1}) (kg N ha^{-1})$^{-1}$] for the regressions are: AP, 0.0115 ± 0.0027 ($p < 0.001$), BG, 0.0173 ± 0.0036 ($p < 0.001$), and NAG, 0.0161 ± 0.0041 ($p < 0.001$), LAC, 0.0026 ± 0.0018 ($p = 0.161$).

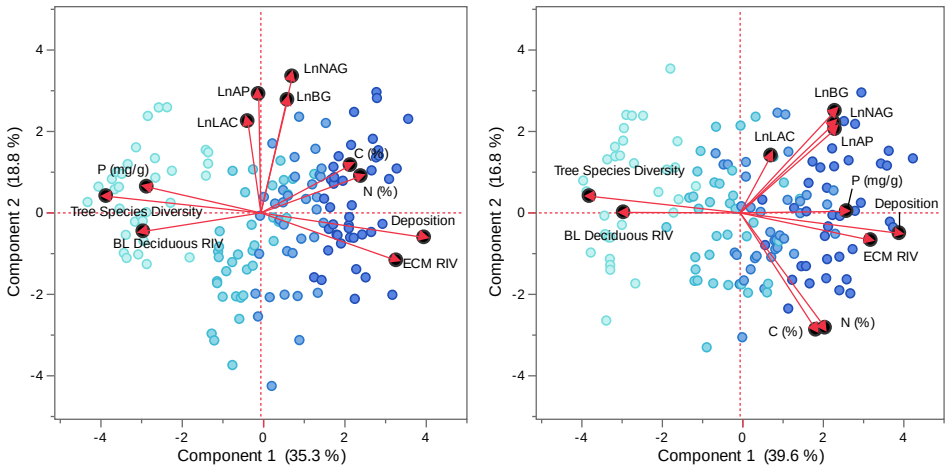

Figure 4-4. Principle component analysis for soil extracellular enzyme activities and soil-level and plot-level predictors of ESE activities in the organic and mineral horizons, respectively. Symbol color intensity reflects N deposition at each site (light blue, low, to dark blue, high, N deposition).

Table 4-1 F-statistics for three-way ANOVA tests for soil extracellular soil enzymes (ESE) activities.

Factor	DF	AP	BG	NAG	LAC
Organic Fraction ESE Activity					
Month	2	0.666	3.149*	4.640*	1.385
Total Deposition	1	0.004	1.684	0.710	0.718
Species	2	7.317**	0.060	1.643	1.113
Month × Deposition	2	0.741	0.325	0.120	1.049
Month × Species	4	0.145	0.602	1.180	0.706
Deposition × Species	2	0.134	0.576	0.877	1.635
Month × Deposition × Species	4	0.582	2.110	0.448	0.786
Mineral Fraction ESE Activity					
Month	2	0.271	2.627	1.480	0.360
Total Deposition	1	17.883***	25.011***	17.741***	1.914
Species	2	1.874	0.782	0.286	3.637*
Month × Deposition	2	2.346	1.183	2.254	0.669
Month × Species	4	2.891*	1.196	1.055	1.171
Deposition × Species	2	4.949*	3.634*	2.320	0.793
Month × Deposition × Species	4	1.987	0.467	3.377*	0.921

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4-2. Parameter estimates for multiple linear regression of soil-level and plot-level predictors of ESE activities.

Factor	AP	BG	NAG	LAC
Soil C (%)	0.0073	0.0127	–0.0098	–0.0056
Soil N (%)	–0.1606	–0.3901	0.3888	0.1173
Soil P (mg kg^{-1})	–0.0001	–0.0001	–0.0001	0.0001
Mycorrhizal Type [ECM–AM]†	0.0012	–0.0561	0.0103	0.0169
Soil Fraction [O–M]†	0.7489***	0.8020***	1.076***	0.3232***
Plot Broadleaf RIV	–0.0629***	–0.0762***	–0.0624**	–0.0285
Plot Shannon Diversity	1.827**	2.429***	1.602*	1.158*
Plot ECM RIV	0.0747**	0.1272***	0.0627*	0.0473*
R^2	0.4748	0.4431	0.5245	0.1534
P_{model}	<0.0001	<0.0001	<0.0001	<0.001

†Parameter estimate for the difference in response between ECM and AM hosts and between mineral (M) and organic (O) fractions, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4-3. Principle component loadings for soil-level and plot-level factors, ESE activities, and N deposition in high elevation red spruce forests. ESE activities were natural log transformed. Values marked with * contribute disproportionately to the PC loadings.

Organic Fraction				
Factor	PC1	PC2	PC3	PC4
% Variation	35.3	18.8	15.4	8.2
Ln AP	–0.0123	0.6876*	–0.3729*	–0.0655
Ln BG	0.1550	0.6539*	–0.0518	0.2456
Ln NAG	0.1823	0.7893*	–0.0361	–0.1083
Ln LAC	–0.0755	0.5304*	–0.1304	0.4710*
Soil C (%)	0.5201*	0.2760	0.7677*	–0.1472
Soil N (%)	0.5802*	0.2127	0.7500*	–0.1279
Soil P (mg kg^{-1})	–0.6600*	0.1510	0.0101	–0.3866*
Plot Broadleaf RIV	–0.6803*	–0.1070	0.4515*	0.4662*
Shannon Index	–0.8951*	0.0968	0.3541*	0.1685
Plot ECM RIV	0.7857*	–0.2749	0.0089	0.4019*
N Deposition	0.9467*	–0.1418	–0.2222	0.0678
Mineral Fraction				
% Variation	39.6	16.8	14.4	9.0
Ln AP	0.5479*	0.4771*	0.4186*	–0.2655
Ln BG	0.5474*	0.5809*	0.2683	–0.0050
Ln NAG	0.5443*	0.5108*	0.3081	–0.2963
Ln LAC	0.1788	0.3263	0.3637*	0.6830*
Soil C (%)	0.4375*	–0.6663*	0.5397*	–0.1597
Soil N (%)	0.4908*	–0.6549*	0.5378*	–0.0700
Soil P (mg kg^{-1})	0.6149*	0.0096	–0.1796	–0.0634
Plot Broadleaf RIV	–0.6711*	0.0030	0.4961*	0.3031
Shannon Index	–0.8681*	0.0951	0.4336*	–0.0292
Plot ECM RIV	0.7540*	–0.1556	–0.0425	0.4547*
N Deposition	0.9187*	–0.1164	–0.2800	0.1805

Table 4-S1. Tree stand composition of the four field sites. Data from Smith et al. (2016).

Site	AM RIV[1]	ECM RIV[2]	Broadleaf Deciduous RIV[3]	Shannon Index
FLR	15.78	84.22	20.76	0.403
MCG	11.97	88.03	50.15	0.287
CGL	20.83	79.17	49.22	0.500
LSB	28.80	71.20	53.16	0.574

[1]Arbuscular mycorrhizal host tree relative importance value

[2]Ecctomycorrhizal host tree relative importance value

[3]Broadleaf tree relative importance value

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5 Tree species effects on extracellular soil enzyme activities at the Stand Initiation and Diversity Experiment, WV

5.1 Introduction

Relationships between tree species diversity and forest ecosystem productivity and function have been studied extensively in recent years. Positive tree-species diversity–productivity relationships have been observed across ecosystems at the global scale [1], as well as in central Europe [2], across North America [3], and in Appalachia and the mid-Atlantic [4]. One primary tenet to controlling these broad trends in diversity–productivity is that plant functional traits (PFTs), which can used to categorize species by the way they function in ecosystems, underlie the acquisition and utilization of resources within the soil environment [5]. Indeed, the broad positive relationships between diversity and productivity across a broad array of ecosystems [6] has also been observed for systems with increasing variation of PFTs [7].

Disentangling PFTs contributing to ecosystem function can be aided by distinguishing between traits responsible for direct biological function, such as photosynthetic capacity, and those relevant to broader ecosystem function, such as nitrogen (N) fixation [8]. In a biogeochemical context, PFTs such as N-fixing symbioses, vesicular arbuscular mycorrhizal (AM), and ectomycorrhizal (EC) fungal associations have major implications for ecosystem function and productivity. While tree species diversity, richness, and evenness have been shown to positively impact ecosystem aboveground productivity, there is a growing body of evidence that plant PFTs are also an important driver of microbial community composition: trees with divergent PFTs may influence microbial community structure and function, subsequently altering soil chemical and physical conditions and ecosystem productivity [9–13]. For example, in temperate

deciduous forests, tree species have been shown to directly affect microbial community composition through their mycorrhizal symbionts and indirectly through functional traits that influence soil pH and nutritional status through root exudates and litter quality [14]. While fungal diversity in some temperate systems has been shown to be influenced by plant species [15,16], bacterial community composition is more complexly derived by interactions among plant roots, plant-specific mycorrhizal fungi, and soil characteristics [17].

Central to belowground PFTs influencing forest productivity is the function of root-mediated microbial communities in cycling soil nutrients by extracellular soil enzymes (ESEs). Although plants have the ability to synthesize extracellular soil enzymes (ESEs), such as acid phosphatases, to access organically-bound nutrient sources in the soil, the majority of ESEs are secreted by microbes [18]. ESEs are excreted by microbes to aid in resource-scavenging during periods of limitation, mediating some of the rate-limiting steps in nutrient mineralization, such as during cellulose and protein degradation [28–31, 37]. These ESEs are highly beneficial to plants, as their activity increases the concentration of plant-available nutrients in the soil solution [20].. Thus, the availability of nutrients in forest soils is facilitated by the complex microbial community interacting with soil bound/sequestered nutrient pools. Although changes in soil microbial community diversity have been examined because of their potential impacts on ecosystem function [21], research assessing the relationships between tree species diversity, PFTs such as mycorrhizal associations, and ESE profiles is scarce [22].

The Stand Initiation and Diversity Experiment (SIDE) in Point Pleasant, WV consists of 182 plots designed to evaluate tree species diversity effects on forest stand productivity. Installed in March 2012, SIDE

incorporates a pool of 14 potential tree species in various combinations, providing a unique opportunity to observe differences in belowground processes and measure responses of ESE activities over a gradient of tree species diversity, evenness. and functional types and to assess the relationships between plant community structure and ESE activities. Tree species PFTs relating to plant-microbe interactions for nutrient acquisition (AM and EC fungal associations as well as N-fixation) were central to this analysis of ESE activities of the SIDE experiment, allowing us to ask the following: 1) Are there discernable effects of tree species diversity on height? (2) Is there evidence for species diversity as a driver of enzyme activities? (3) Do the PFTs drive differences in ESE activities at SIDE?

5.2 Methods

The Stand Initiation and Diversity Experiment (SIDE) was established in 2012 on property managed by the Clements State Tree Nursery in West Columbia, outside of Point Pleasant in Mason County, WV (38°57'27"N, 82°05'03"W). SIDE occupies approximately 3.6-ha of agricultural land selected for its homogeneous distribution of soil and physiographic features. The site is almost completely flat, consisting of soils of the Connotton Series (Loamy-skeletal, mixed, active, mesic Typic Hapludalfs) and the Lakin Series (Mixed, mesic Lamellic Udipsamments), with Connotton gravelly sandy loam and Lakin loamy fine sand present [23]. SIDE is at 183-m elevation with mean annual temperature 13°C and receives approximately 107 cm of precipitation annually [24]. In the past, the site had been used to raise tree seedlings of various species sold by Clements State Tree Nursery, and had been unoccupied and unvegetated for approximately ten years preceding the installation of the SIDE plots. Before that, the site was in cover crops, such as sorghum-sudangrass (J. Huffman, personal communication, February 10, 2020).

A simple completely randomized design (CRD) was utilized at SIDE to test the effects of species richness and species evenness on ecological function. Seedlings (2-0 bare root stock) of 14 species selected to simulate components of central Appalachian forest communities were planted in March 2012 without supplemental irrigation. These species included *Acer saccharinum* L. (silver maple), *Alnus glutinosa* (L.) Gaertn. (black alder), *Carya ovata* (Mill.) K. Koch (shagbark hickory), *Castanea mollissima* Blume (Chinese chestnut), *Cercis canadensis* L. (eastern redbud), *Cornus racemosa* Lam. (gray dogwood), *Nyssa sylvatica* Marshall (black tupelo), *Picea abies* (L.) H. Karst. (Norway spruce), *Pinus strobus* L. (eastern white pine), *Prunus serotina* Ehrh. (black cherry), *Quercus alba* L. (white oak), *Quercus montana* Willd. (chestnut oak), *Quercus rubra* L. (red oak), *Robinia pseudoacacia* L. (black locust). Species richness was incorporated into the experimental design at four levels (3, 5, 7, and 9) along with two levels of species evenness (1:1, 3:1) combined into eight treatments. Each of the eight treatments are replicated 21 times. Tree species were selected at random to satisfy the richness and evenness criteria of each individual plot, with one monoculture control plot present for each species in the study for a total of 182 plots. The plots are arranged in a 14 $\times$ 13 grid with 3-m spacing between each respective plot. Within plots, seedlings were planted in an 11 $\times$ 7 grid with individual seedlings planted on 1-m centers. In addition to the species selection for each plot being randomized, the plots comprising the monoculture controls and the replicates for each treatment level are randomized throughout the 14 $\times$ 13 grid (Figure 5-1). To minimize deer herbivory, a dual-layer electric fence was installed around the perimeter of the site.

For this analysis, plots were re-classified into three levels of species richness ($3 \leq 5$, $6 \leq 9$, > 9) to represent species composition after nearly four growing seasons (Figure 5-1). This resulted in 14 monoculture and 168 mixed-species plots, with the mixed-species plots consisting of 53 low diversity ($3 \leq 5$ species), 90 medium diversity ($6 \leq 9$ species), and 25 high diversity (> 9 species) plots. Soil samples were collected from

each plot, and height (cm) of every tree in each plot was recorded using measuring poles in July 2015. Importance value index (IVI) was calculated for each species by coupling height and relative abundance within plots [25]. Dead individuals at the time of sampling were removed from the data set.

For soil collection, litter was removed from sampling points and discarded. Soils were sampled to a depth of 10-cm using a 2-cm soil corer from four locations within each plot; samples within plots were composited within a plastic Ziploc bag. Following collection, samples were immediately put in a cooler on ice. Immediately on returning to the lab, soil samples were sieved using a 2-mm (No. 10) screen and stored at –20°C. Soils were subsampled to measure pH and dried at 65°C to measure soil moisture. Additional subsamples for C and N were air-dried and analyzed using a Thermoquest Elemental Analyzer. In addition to within-plot samples, soils were also collected from 13 locations in the grassy medians between plots for comparison.

Fluorimetric assays were performed on field-moist soils for the hydrolytic enzymes acid phosphatase (AP), arylsulfatase (ARS), β-glucosidase (BG), and N-acetyl-glucosaminidase (NAG), and colorimetric assays for the oxidative enzymes polyphenol oxidase (PO) and peroxidase (PER) [26]. Colorimetric assays for fungal laccases (LAC)—polyphenol oxidases utilized by certain taxonomic groups of fungi often associated with ectomycorrhizal fungi—were also performed [27]. Enzyme extraction buffer pH was adjusted to 5.5 to match assay conditions with those of the native soils at SIDE [28–30].

Field recording and management of the SIDE growth data set was performed in Microsoft Excel, exploratory data analysis in SAS JMP 14.0 [31], and multivariate analyses were completed in JMP and R

version 3.5.2 [32] to characterize relationships between height, diversity, and soil variables. Soil measurements, including ESE activities, C:N molar ratio, % soil C, and % soil N were square-root transformed to meet assumptions of normality.

5.3 Results

5.3.1 Tree growth and soil characteristics

Since installation of the SIDE plots in early 2012, seedlings had experienced approximately 12% mortality by the time of sampling in July 2015. Over the three-year period, the species exhibited a wide range in height growth response. Black locust, silver maple, and black alder accounted for approximately 41% of total tree height for the SIDE plots across all plots (Table 5-1). Black alder and black locust are both classified here as N-Fixers, with black alder bearing EC fungal associations and black locust associating with AM fungi. The third species, silver maple, is an AM species. All three are early successional species exhibiting rapid juvenile growth, and this behavior is strongly represented in the SIDE growth data at the time of measurement.

Compared to the species monocultures, mixed-plot individuals of grey dogwood (GD), black tupelo (BG), and shagbark hickory (SH) exhibited the greatest increases in total height in mixed-culture plots, while Chinese chestnut (CC), silver maple (SM), and eastern redbud (RB) exhibited the greatest reductions in growth in mixed culture plots (Figure 5-2). Across all-plots and all species, tree height was negatively correlated with plot %EC host stems (r = –0.2985, P < 0.001), positively correlated with %N-Fixer host stems (r = 0.5202, P < 0.001), and positively correlated with functional richness (r = 0.1535, P = 0.0385); there was no correlation between tree height and species richness or Shannon Diversity.

Evaluation of soil nutrient levels taken with a set of random samples across the SIDE site indicated that soil pH was uniform at approximately 5.5. Soil concentrations of macronutrients exhibited little variation across the study plots and were within healthy ranges for plant growth (Figure 5-S1). Across all of the SIDE plots, patterns in the diversity measures and soil chemical characteristics highlighted the relative independence of soil chemistry and plot diversity (Figure 5-3, plot colors as in Figure 1). PC1 accounted for host species tradeoffs, with EC and N-Fixing host species positively loading PC1 and AM hosts negatively loading PC1 (Table 5-S1). Soil C, N, and C:N also loaded negatively on PC1 while soil moisture contributed positively. PC2 positively separated data based on soil C, N, and C:N as well as EC and N-Fixing importance values and negatively based on AM host importance (Figure 5-3). Across variables, soil C, N, and C:N were positively correlated, but were uncorrelated with any PFT variables (Table 5-S2). Soil moisture was negatively correlated with AM IVI, but positively corelated with N-Fixer IVI and the Fxn Shannon Index. Plot total tree height was negatively correlated with increasing EC host prevalence in the plots (Figure 5-4, Table 5-S1).

5.3.2 Extracellular soil enzyme activities

Across all plots, soil ESE activities were generally positively correlated, with the greatest correlations being between AP, BG, NAG, and PER (Table 5-2). Soil C and N variables had little effect on ESE activities, with only a positive association existing between C:N and BG and total normalized (ESE_T) activity across all plots. Additionally, AP, PG, and ESE_T were negatively correlated with stand tree height across the plots (Table 5-2). Species monocultures exhibited distinct ESE activity profiles, although, without replication, these patterns are not statistically separable in any way (Figure 5-4). Notable is the different pattern

exhibited by the grassy paths separating plots, which exhibited high PO activities in comparison to the other plots (Figure 5-5).

Across all SIDE plots, soil ESE activities were influenced by plot diversity and overall stand growth (Figure 5-6). PC1 was positively influenced by soil ESEs, except LAC, and negatively weighted by height and species and functional diversity (Table 5-S2). LAC, PO, and NAG, as well as Functional and Species Shannon Indices positively contributed to PC2 while PC2 was negatively influenced by stand height. Interestingly, importance indices for EC, AM, and N-Fixing hosts exhibited different influences on the PCs, although their loadings were relatively small (Figure 5-6). The divergent loadings of several ESEs and stand diversity metrics in the PCA led us to evaluate specific relationships between ESEs and diversity measures. When specifically evaluating ESE responses, ESE activities broadly declined as plot diversity increased, with negative slopes for activity as species richness, functional richness, and the Shannon Index increased (Table 5-3).

5.4 Discussion

The Stand Initiation and Diversity Experient (SIDE) plots were established to evaluate the connections between forest diversity and stand establishment for forests in the mid-Appalachian region. Here, we specifically evaluated relationships between tree stand diversity and soil characteristics and soil extracellular enzyme (ESE) activities. Combinations of trees with different plant functional traits (PFTs) allowed us to further investigate the importance of AM, EC, and N-fixing host trees in influencing soils. Three early-successional pioneer species dominated height measurements at SIDE with two of them being

N-Fixers (BA and BL) and the third (SM) an AM-forming species. In their responses to the presence of other tree species, BA and SM exhibited decreases in mean plot-level heights compared to their respective monocultures, while BL was ambivalent (Figure 5-2).

The predominance of some of these species may have had other effects on the growth of other species in the plots; trade-offs between soil moisture and % arbuscular mycorrhizal host abundance (Table 5-S1) suggest the greater growth and leaf surface area of silver maple and black locust (Table 5-1) may have depleted soil water. The predominance of BA, BL, and SM explain the positive correlations between plot-level %N-Fixers, functional richness, and the negative correlation with %EC individuals across SIDE. These effects likely overshadowed other potential relationships between mean tree height and productivity at this early stage in development when these early successional species exhibit such predominant growth relative to other species. Notable increases in mean height of GD, BT, and SH reflect their mid-successional species roles as being well-adapted for establishing themselves after the initial wave of pioneer species following disturbance [33]. The lack of relationships between species richness or Shannon Diversity, and height may be an artifact of plot-level height being driven so strongly by the presence of BA, BL, and SM.

Despite their disproportionate contribution to overall growth across the SIDE plots and to species IVI within any plots, the two N-fixing tree species, BA and BL, contributed few statistically significant effects on the soil variables measured here (Table 5-2, Figure 5-7). N-Fixer relative abundance was associated with NAG and PER activities plot-wide, with a strong influence detected on N-scavenging NAG within low-diversity plots that, however, disappeared as species diversity increased (Table 5-3). Paradoxically, BL IVI was negatively associated with soil % total N, although this pattern was not statistically significant (Figure

5-7). Soils under BL have been shown to cycle N relatively rapidly [34], with rates of net nitrification as high as 30-150+ times the rate of nearby soils in BL-free areas in the Albany Pine Bush of Upstate New York [35]. Seasonal differences in total soil N have been observed in soils under BL [36], and it is possible that sampling in July during a period of high inorganic N-demand under this AM N-fixing species could explain measurements of low soil N. While BL has been show to increase total soil N, observable differences are dependent upon stand age and tree size [37–39]. The young age of the SIDE stands may have precluded detection of N accretion.

The short time period between establishment and the measurements in this study is also reflected in the general absence of strong species and PFT impacts on soil chemistry metrics. Agricultural soils often have vastly different microbial communities compared to neighboring forest soils that have escaped land use change and/or disturbances associated with human settlement [40–42]. Since soil microbial communities in agricultural settings are strongly driven by cropping and management practices, changes in soil biological and chemical characteristics may lag relative to aboveground changes, as it takes time for the effects of vegetation change and belowground processes relating to associated microbial community shifts to accrue [43–46]. There is evidence that mycorrhization can occur in the early years of a seedling's development for AM [47–49] and EC [50,51] species, as well as infection of bacteria involved in symbiotic N-Fixation in BA [52–54] and BL [55,56]; indeed, assumptions of widespread mycorrhization and infection with symbiosis-promoting microbes are key in our interpretation of these SIDE data through the prism of the PFTs chosen for this study.

Species diversity can be negatively influenced by the presence of a single taxon possessing adaptations granting it a superior ability to acquire resources in a given environment [57]. Early colonization on a

disturbed or post-agricultural site can promote legacy effects on plant community assembly as succession proceeds [58]. Different plant species, themselves recruiting different fungal and bacterial symbionts, produce a large array of niche spaces for microbes to occupy, allowing microbial communities in more diverse stands to be more resilient to environmental changes, such as alterations in precipitation and temperature, as well as nutrient inputs through functional redundancy [59]. The relatively few differences in AP and BG activities across these plots do not match well with activities measured in nearby Ohio in stands dominated by old-growth deciduous broadleaf EC taxa, possibly indicating that the young age of these plots indeed results in a much different suite of soil biochemical processes [60]. In addition, the complexity of these relationships can make comparisons of soil chemical and biological metrics difficult to interpret; significant correlations are often observed within sites, but a loss of significance when data from similar sites is pooled [61].

As soil microbial communities experience nutrient limitation due to plant growth and nutrient uptake in high productivity plots, they were expected to respond by secreting additional ESEs for nutrient scavenging. This effect was hypothesized to be particularly strong in high-diversity plots with a larger number of tree species. However, there was little evidence for coupling between ESE activities and aboveground growth. ESE activities displayed few relationships with diversity measures, including Shannon-Wiener species diversity. Zechmeister-Boltenstern et al. (2011) observed stronger effects on microbial community composition due to plant species diversity than to N enrichment, and we expected to see a similar pattern expressed in ESE profiles, since the plant functional traits observed in this study are closely tied to nutrient acquisition [62]. While there were plot-level relationships between functional composition and ESE activities, this was only the case for NAG and BG across all plots, and AP across low diversity plots. Indeed, intraspecific variation within species also plays an important role in ecosystem

function, but it can be difficult to detect in biodiversity-ecosystem function (BEF) experimental designs [63]. In addition, effects of tree species complementarity on growth may be more obvious at small spatial scales than at the stand level [64]. However, species interactions are not always as straightforward as species-species complementarity, such as the effect of surrounding individuals to attract or repulse herbivores and pathogens [65].

Soil microbial community structure is strongly dependent on the heterogeneous spatial distribution of nutrients [17], and ESE activities are sensitive to a number of factors, such as tannins and other organic compounds released by plants [66]. Belowground plant-microbial processes can vary according to the predominant development stages of trees present within a community. ESE activities of Norway spruce in Europe varied considerably for seedlings planted in established forests versus areas that had been clear-cut, with soil in proximity to seedlings in clear-cut areas exhibiting distinct EC fungi community structures and higher enzyme activities in association with more labile organic residues [67]. In forest ecosystems, the patchy nature of established trees of different species interwoven with areas of disturbance can lead to belowground microbial community patchiness as well, with localized implications for nutrient cycling [68,69].

These strong localized species effects and classification of PFTs can be very important for disentangling complex plant-microbe-soil relationships. Phillips et al. (2013) have formalized some of these trait-dependent biogeochemical functional differences into a novel concept referred to as the Mycorrhizal-Associated Nutrient Economy (MANE) [70]. Within this paradigm, stands dominated by AM species are assumed to follow a nutrient economy more dependent on rapid cycling of inorganic nutrients, while EC

stands will exhibit slower cycling characterized by greater coupling between roots, microbes, and ESE activities recycling nutrients from organic sources, providing a straightforward framework for hypothesis testing. While we do see some differences that align with the expectations of MANE, it appears that the SIDE plots are too recently-established to consistently demonstrate strong differences by functional type, as well as indications that some long-held assumptions about species PFTs require amendment. For example, *Carya* is often listed as forming AM associations, although recent work has detected evidence that members of the genus can form associations with both AM and EC fungi [71–73]. Indeed, the *Carya ovata* (SH) monoculture, classified as an AM species for the purposes of the current analyses, occupies a distinct position in our principal components analysis that fits well with the main cluster of EC species (Figure 5-6). SH displayed a relationship with AP; since this is the only ESE measured here that is secreted by plant roots to any large extent, this could reflect the disproportionate belowground biomass displayed by SH relative to its aboveground state, making it appear to outperform its IVI relative to observed AP activities.

Laccase was one of the few enzymes to display differences due to species diversity. Unlike other phenol oxidases, laccases possess redox potentials too low to oxidize the non-phenolic structural components of lignins, but are nevertheless effective drivers of lignin decomposition through the creation of organic radicals that can oxidize these linkages [74]. As a result, soil laccase measurements using the substrate ABTS are likely to detect a finer range of soil oxidative enzyme activity than the phenol oxidase and peroxidase activities calculated from measurements of gross oxidation using L-DOPA [30]. While this could lead to low estimations of overall oxidative enzyme activities, this greater specificity makes laccase measurements relatively sensitive to detecting differences in the suite of oxidative enzymes released by microbes, allowing fungal taxonomic groups to display distinct signatures [75–78]. In this study,

differences in laccase activities were driven most strongly by EC species with high foliar C:N ratios (Figure 5-7).

Globally, positive relationships between tree species diversity and productivity are common [1], however, elucidating the nutritional and biogeochemical linkages between PFTs, diversity, and productivity across ecosystems remains a challenge. There are some indications that relationships between PFTs and nutrient cycling are currently stronger than effects due to environmental changes such as warming, at least with respect to processes such as decomposition [46,79]. Although the SIDE plots were early in their stage of establishment, if these relationships can be validated and generalized across terrestrial ecosystems, this knowledge could inform management practices to take advantage of plant-microbe interactions driving these poorly understood ecosystem services provided by systems with high species and functional diversity.

5.5 Figures and Tables

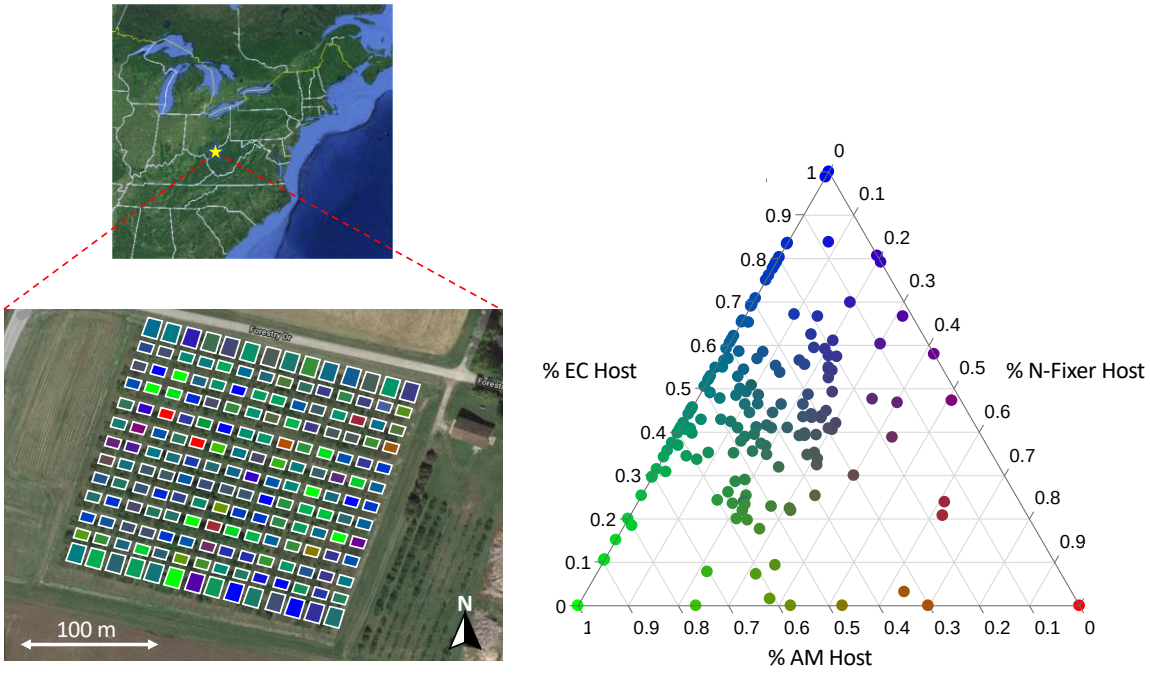

Figure 5-1. SIDE experimental site in West Virginia, with recolored plot layout diagram showing the spatial distribution of plots by functional composition (left). Plots were colored using RBG color ramp for EC, AM, and N-Fixer host species. Ternary plot of plot diversity composition (right).

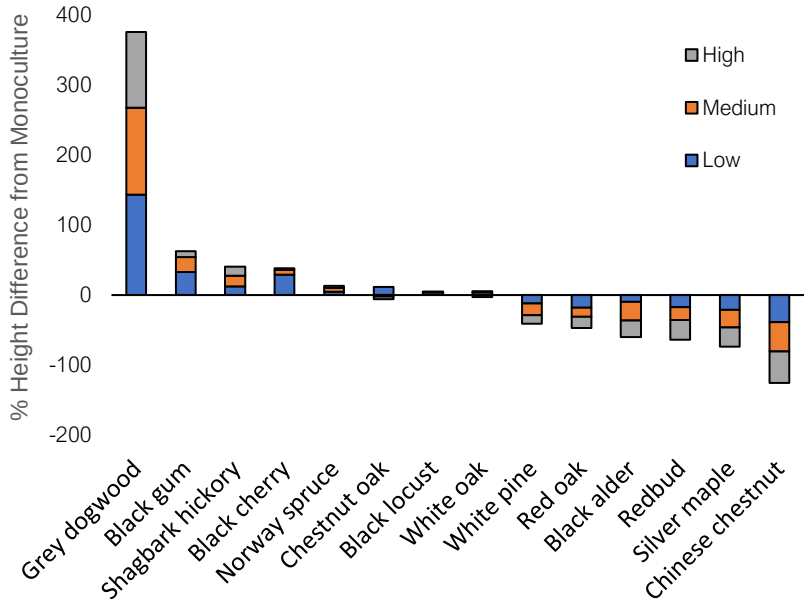

Figure 5-2. Total height for each species in low, medium, and high diversity plots as a percent difference from the total height of each respective species monoculture.

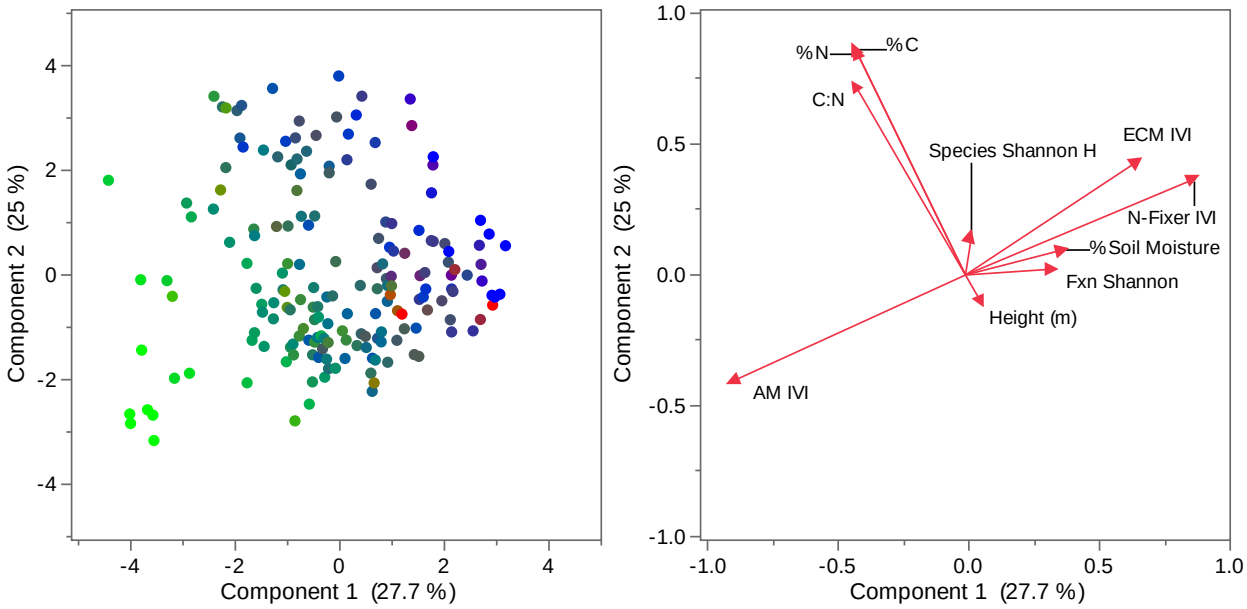

Figure 5-3. PCA for tree growth, soil variables, and plot diversity measures at the SIDE plots. Left: Individual plots with color notation as in Figure 1. Right: Eigenvectors for effects. AM IVI = arbuscular mycorrhizal host importance value index; EM IVI = ectomycorrhizal host importance value index; N-Fixer IVI = nitrogen-fixing host importance value index; Fxn Shannon = Shannon Index based on functional attributes; Species Shannon = Shannon Index based on number of species. PC loadings are presented in Supplemental Table 1.

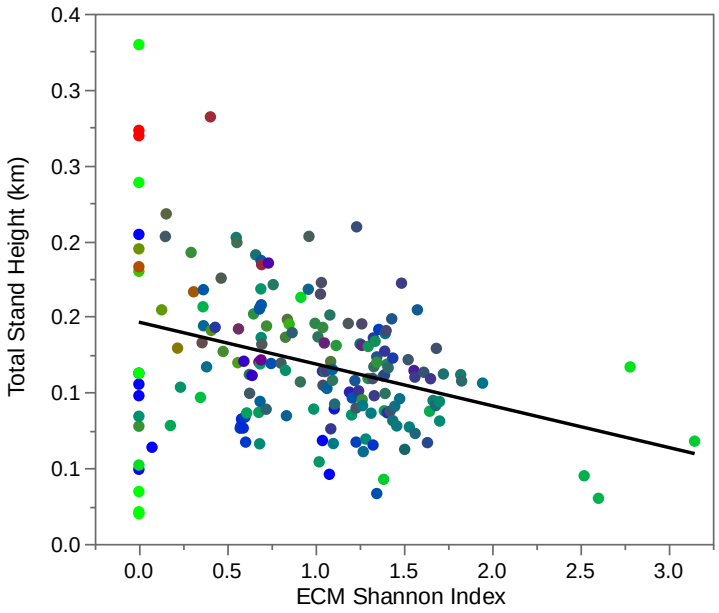

Figure 5-4. Total tree height in SIDE stands as a function EC Shannon Index. Individual points (plots) with color notation as in Figure 1.

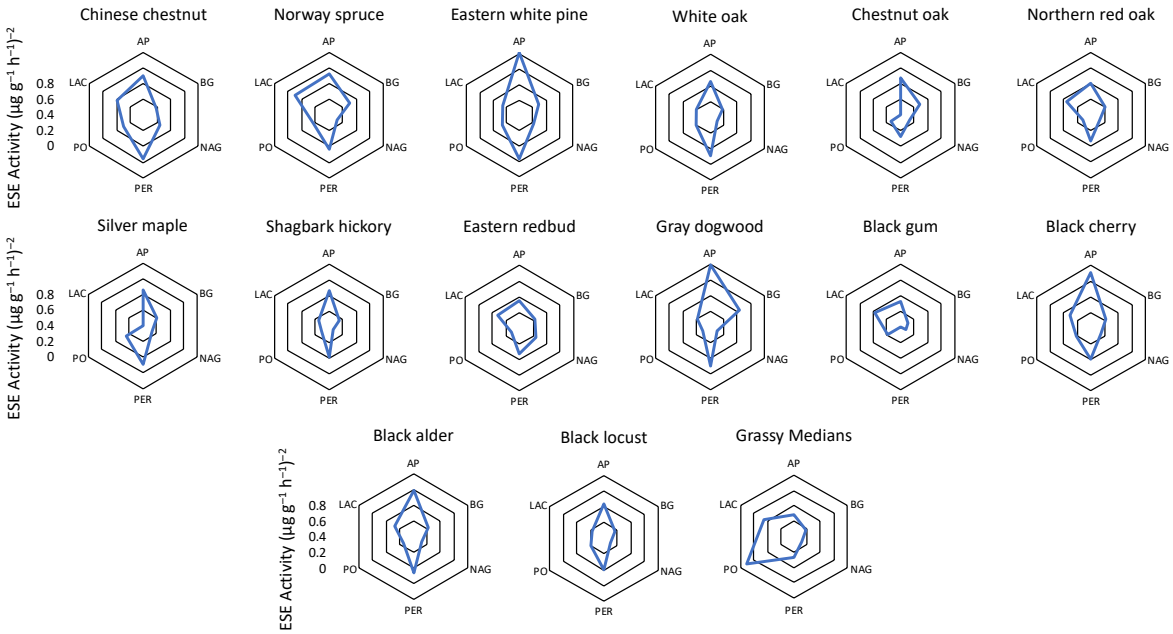

Figure 5-5. Radar plots of square-root transformed ESE activities for each species monoculture and the grassy medians (N = 10). Top row exhibits EC-host species, middle row AM-host species, and bottom row contains N-Fixing hosts and the open grassy median samples.

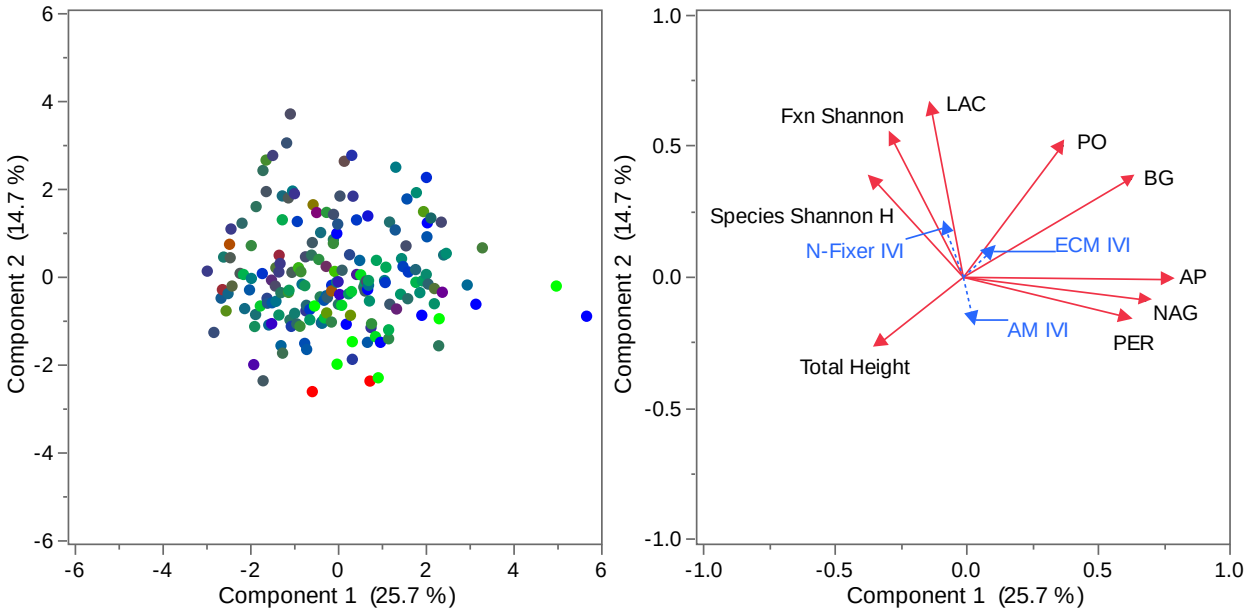

Figure 5-6. PCA for soil ESE activity and plot diversity measures at the SIDE plots. Left: Individual plots with color notation as in Figure 1. Right: Eigenvectors for effects. AM IVI = AM host importance value index; EM IVI = EC host importance value index; N-Fixer IVI = N-fixing host importance value index; Fxn Shannon = Shannon Index based on PFTs; Species Shannon = Shannon Index based on number of species. IVI values in blue reflect eigenvectors for importance value indices for EC, AM, and N-Fixing hosts. PC loadings are presented in Supplemental Table 2.

Figure 5-7. Loading plots of partial least squares regression models for species IVI effects on soil variables. Proportion of variance explained by species IVI for each individual variable in parentheses. Significant relationships identified via multiple regression with significance thresholds †P<0.1, *P<0.05, **P<0.01, ***P<0.001.

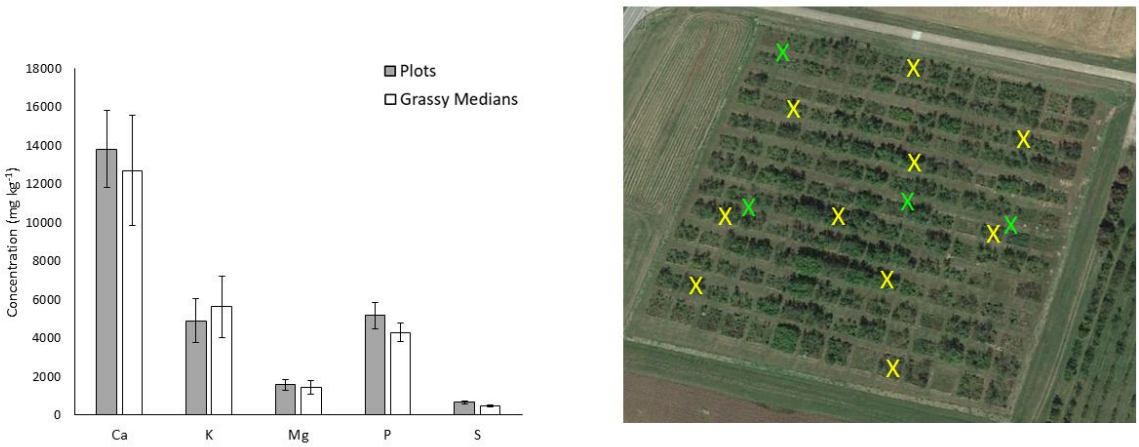

Figure 5-S1. Soil macronutrient concentrations taken from randomized locations across the SIDE study area (*N=14*). Yellow markers indicate sample taken from plot center, and green from the center of a grassy median between plots. Data was collected from Mehlich-III soil extracts via ICP-OES at the Agricultural Analytical Services Laboratory at The Pennsylvania State University.

Table 5-1. SIDE study species descriptions and relative height contribution across all plots.

Species Code	Common Name	Scientific Name	Family	Functional Trait	% Height Contribution
BL	Black locust	*Robinia pseudoacacia*	Fabaceae	N-Fixer (AM)	0.1570
SM	Silver maple	*Acer saccharinum*	Sapindaceae	AM	0.1445
BA	Black alder	*Alnus glutinosa*†	Betulaceae	N-Fixer (EC)	0.1070
RB	Eastern redbud	*Cercis canadensis*	Fabaceae	AM	0.0808
CC	Chinese chestnut	*Castanea mollissima*†	Fagaceae	EC	0.0750
CO	Chestnut oak	*Quercus montana*	Fagaceae	EC	0.0716
RO	Red oak	*Quercus rubra*	Fagaceae	EC	0.0710
BC	Black cherry	*Prunus serotina*	Rosaceae	AM	0.0651
WP	Eastern white pine	*Pinus strobus*	Pinaceae	EC	0.0601
WO	White oak	*Quercus alba*	Fagaceae	EC	0.0575
GD	Grey dogwood	*Cornus racemosa*	Cornaceae	AM	0.0359
BG	Black tupelo	*Nyssa sylvatica*	Nyssaceae	AM	0.0268
SH	Shagbark hickory	*Carya ovata*	Juglandaceae	AM	0.0251
NS	Norway spruce	*Picea abies*†	Pinaceae	EC	0.0212

† Species non-native to Appalachia.

Table 5-2. Correlations between soil ESE activities and soil chemical parameters across all SIDE plots.

		Probability (*P*)										
	Variate	[AP]	[BG]	[NAG]	[PER]	[PO]	[LAC]	ESE_T	C:N	%C	%N	Height
Correlation Coefficient (*r*)	[AP]		**<0.001**	**<0.001**	**<0.001**	**0.014**	**0.007**	**<0.001**	0.984	0.662	0.865	**0.001**
	[BG]	0.446		**<0.001**	**0.001**	**0.001**	0.113	**<0.001**	**0.010**	0.162	0.114	**0.002**
	[NAG]	0.490	0.368		**<0.000**	**0.019**	0.349	**<0.001**	0.500	0.598	0.930	0.053
	[PER]	0.414	0.250	0.343		**0.041**	0.077	**<0.001**	0.075	0.480	0.271	0.623
	[PO]	0.181	0.240	0.173	0.151		**0.007**	**<0.001**	0.501	0.246	0.069	0.998
	[LAC]	–0.201	0.118	–0.070	–0.131	0.201		**<0.001**	0.145	0.496	0.542	0.969
	ESE_T	0.633	0.696	0.681	0.584	0.574	0.281		**0.031**	0.366	0.125	**0.015**
	C:N	0.001	0.192	0.050	0.132	0.050	0.108	0.160		**<0.001**	**<0.001**	0.855
	%C	–0.033	0.104	–0.039	0.053	0.086	0.051	0.067	0.698		**<0.001**	0.553
	%N	0.013	0.118	–0.007	0.082	0.135	0.045	0.114	0.634	0.923		0.131
	Height	–0.236	–0.231	–0.143	–0.037	0.000	–0.003	–0.179	0.014	–0.044	–0.112	

Table 5-3. ESE activity responses to SIDE stand diversity measures analyzed by MANOVA.

ESE	Parameter	Species Richness	Fnx Richness	Shannon Index
AP	Intercept	0.505	0.515	0.496
	Slope	–0.00477	–0.0164	–0.0172
BG	Intercept	0.218	0.229	0.217
	Slope	–0.00260	–0.0108	–0.0122
NAG	Intercept	0.145	0.153	0.141
	Slope	–0.00325	–0.0114	–0.0133
PER	Intercept	0.401	0.440	0.399
	Slope	–0.00453	–0.0259	–0.0205
PO	Intercept	0.202	0.186	0.193
	Slope	–0.00316	–0.0027	–0.0095
LAC	Intercept	0.336	0.328	0.327
	Slope	–0.00174	–0.0020	–0.0030
MANOVA *P*		0.0114	0.0049	0.0338

Table 5-S1. PCA loading matrix for SIDE soil properties and stand diversity. Values in red contribute disproportionately to the PC loadings.

Variable	PC1	PC2	PC3	PC4
% Variation	27.62	25.00	13.58	11.14
Bartlett *P*	< 0.001	< 0.001	< 0.001	< 0.001
C:N	–0.4173	0.7180	0.0348	0.1641
%C	–0.4205	0.8612	0.0419	0.0623
%N	–0.4113	0.8439	0.0101	0.0111
%Soil Moisture	0.3705	0.0957	0.2164	0.2671
ECM IVI	0.6597	0.4352	–0.3939	–0.2062
AM IVI	–0.8891	–0.4049	0.0823	–0.0289
N-Fixer IVI	0.8763	0.3671	0.0099	0.0645
Fxn Shannon	0.3347	0.0226	0.7606	0.0266
Species Shannon	0.0231	0.1471	0.7394	–0.4003
Total Stand Height	0.0607	–0.1046	0.1451	0.8963

AM IVI = arbuscular mycorrhizal host importance value index; EM IVI = ectomycorrhizal host importance value index; N-Fixer IVI = nitrogen-fixing host importance value index; Fxn Shannon = Shannon Index based on PFTs; Species Shannon = Shannon Index based on number of species.

Table 5-S2. PCA loading matrix for SIDE ESE activities and stand diversity. Values in red contribute disproportionately to the PC loadings.

Variable	PC1	PC2	PC3	PC4
% Variation	25.72	14.71	14.02	11.53
Bartlett *P*	< 0.001	< 0.001	< 0.001	0.004
AP	0.7776	–0.0088	0.2792	0.0775
BG	0.6355	0.3772	0.0099	–0.1932
NAG	0.6915	–0.0869	–0.0231	0.1141
PER	0.6191	–0.1514	0.1180	0.3520
PO	0.3751	0.5024	–0.3740	0.3194
LAC	–0.1224	0.6464	–0.5397	–0.1846
Total Height	–0.3182	–0.2528	–0.3880	0.7060
Fxn Shannon	–0.2699	0.5278	0.3953	0.4639
Species Shannon H	–0.3394	0.3723	0.6570	0.0788
AM IVI	0.0373	–0.1633	0.0041	–0.1662
ECM IVI	0.1043	0.0984	–0.0855	–0.0983
N-Fixer IVI	–0.0687	0.1901	0.0264	0.1981

Abbreviations as in Supplemental Table 2.

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6 Summary and Conclusions

Anthropogenic emissions stemming from industry have resulted in historically high levels of acidic deposition into central Appalachian forests. Despite the reduction in acidic inputs due to legislation curbing industrial emissions in the United States, continued N deposition may contribute to legacy effects in these forest ecosystems. Soil and foliar samples were collected from four high elevation red spruce sites along a modeled gradient of historic N deposition. The three most abundant tree species at all sites, *Acer rubrum* L., *Betula alleghaniensis* Britt., and *Picea rubens* Sarg., were sampled. Mehlich-III soil extracts of both organic and mineral horizons and foliar digests from these trees were subjected to elemental analysis.

Soil N concentrations supported the presence of a N deposition gradient: in the O horizon, N concentrations were driven by precipitation volume and elevation; in the B horizon, N concentration was explained by modeled N deposition rate and elevation. Cation depletion was evident in O horizons, with significant declines in Ca, Mg, and K as N deposition increased. Foliar Ca, K, and Sr declined in foliage with increasing N deposition. Although the three species were sympatric in mixed stands at all four sites, the foliar-soil nutrient associations differed among them across the gradient, indicating differential uptake and cycling of nutrients/metals by these forest tree species.

Anthropogenic emissions have impacted terrestrial forest ecosystem processes in North America since the industrial revolution. With the passage of the Clean Air Act in 1970 in the United States, atmospheric inputs of nitrogen (N) and sulfur (S) into forests in the Appalachian Mountains have declined, which have,

potentially, mitigated their effects on processes such as decomposition and nutrient cycling. Activities of microbial extracellular soil enzymes (ESEs) mediate many rate-limiting nutrient transformations in forest soils and play important roles in the decomposition of complex organic compounds. Soils in high-elevation red spruce forests are characterized by low pH and high carbon (C):N ratios and, having historically received extremely high levels of N deposition, may exhibit legacy impacts of deposition on nutrient availability and decomposition. We utilized four sites along a modeled gradient of N deposition in central Appalachia to assess contemporary ESEs in bulk soil under *Acer rubrum* L.*, Betula alleghaniensis* Britt., and *Picea rubens* Sarg. in May, June, and July 2016. Increasing N deposition led to increases in organic fraction C and N and decreases in phosphorus (P). Sites receiving higher N also exhibited greater mineral fraction C, N, and P. ESEs were highest in organic fractions with acid phosphatases (AP) exhibiting the highest activity.

There was little influence of N deposition on organic fraction ESEs, but strong evidence for a positive relationship between N deposition and activities of AP, β-glucosidases (BG), and chitinase (NAG) in mineral fractions. Species effects on ESEs were present with high AP in organic fractions under spruce and high mineral fraction fungal laccase (LAC) under birch. The sampling season demonstrated little effect on ESEs. ESEs were more strongly influenced by plot-level factors, such as tree species diversity and abundance of ectomycorrhizal (ECM) tree species, than temporal or soil factors or nutrient status related to modeled cumulative N deposition across these sites. Decreases in AP, BG, and NAG activities with greater abundance of broadleaf deciduous species and increases in activities with ECM host abundance indicate that microbial communities driven by these plant functional groups are responsible for the differences in ESEs observed in these high-elevation mixed red spruce stands.

Relationships between tree species diversity, plant functional groups, and nutrient cycling in terrestrial ecosystems have been difficult to quantify. The SIDE study examined the effects of species richness on productivity, utilizing 14 tree species encompassing six forming EC fungal associations, six forming AM fungal associations, and two species forming associations with N-fixing organisms. Species-specific effects on plot-level mean plant heights were present, especially as compared to species monoculture mean heights.

ESE activities were more responsive to plot diversity, species composition, and plant functional composition than tree growth. These relationships generally declined with diversity level, as increases in species richness appeared to have muted any detectable individual effects characteristic of some species. Species monocultures exhibited unique ESE profiles. Across all plots, acid phosphatases and β-glucosidases showed some of the greatest differences, with a few species-specific effects. Likely due to the early stage of establishment, less than four full growing seasons, there was little observable evidence for differences in soil %C, %N, and C:N ratio, even in plots dominated by N-fixers. ESE activities were generally not strongly associated with these soil variables, which follows from the few observable differences in measurements of those variables across plots. The oxidative enzyme laccase was the only enzyme that was sensitive to differences in species richness. There was some evidence that species importance values were correlated with some ESE measurements, especially black alder, black locust, and silver maple; the three species exhibiting the most growth. To assess the generalizability of these early results at SIDE, other diversity studies should be utilized with similar species compositions, and these studies should be allowed to proceed at the decadal scale to be most relevant for forest management and modeling.