ABSTRACT

DICK, DAVID LAWRENCE. Forest Floor Manipulation Effects on the Relationship between Aggregate Stability and Ectomycorrhizal Fungi. (Under the direction of Dr. Zakiya Leggett and Dr. Terrence Gardner).

Forest floor and mineral soil manipulations influence the soil biogeochemical properties important for pine tree growth. The impacts of forest floor manipulations on soil aggregate stability and the presence of ectomycorrhizal fungi (ECM), was assessed to elucidate the relationship between ECM abundance and aggregate stability. The study site consists of a 14-year-old loblolly pine (*Pinus taeda* L.) plantation managed by Weyerhaeuser Company in the Lower Coastal Plain, approximately 8 miles east of New Bern, North Carolina. The soil samples were collected from the top 7.62 cm of each soil treatment which includes three levels of forest floor retention: removed, control, and doubled and two levels of forest floor mixing with the mineral soil: mixed and unmixed. ECM abundance was evaluated by fatty acid methyl ester (FAME) analysis and microbial community functionality was assessed by acid-phosphatase activity measurement. Aggregate stability was assessed using the aggregate mean weight diameter (MWD) approach. Results indicate that the forest floor manipulations had an insignificant impact on aggregate stability and ECM abundance. However, a positive relationship between ECM abundance and aggregate stability was identified. Removing the forest floor resulted in a bulk density increase of 0.18 g cm^{-3} compared to doubling the forest floor. Our results demonstrate that some mineral soil properties recover relatively quickly from forest floor manipulations. The study informs forest managers interested in how soil responds to forest floor manipulation and the interaction between ECM and aggregate stability.

Forest Floor Manipulation Effects on the Relationship between Aggregate Stability and Ectomycorrhizal Fungi.

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BIOGRAPHY

David Dick was born in Missouri in the summer of 1993. As a young man, he developed a passion for exploring the great outdoors while in the Boy Scouts of America. He was thrilled by white-water rafting, relaxed by weekend camping, and challenged by lengthy backpacking trips. He was inspired by these experiences to pursue the study of Forestry at the University of Missouri – Columbia, where he received his Bachelor of Science degree in 2017. While there, he enjoyed the hands-on and cross-disciplinary nature of Soil Science in his coursework and during two undergraduate research projects in the forested south of the state. He decided to marry the two fields and took on a research project related to forest soils at North Carolina State University.

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CHAPTER 1 - LITERATURE REVIEW

1.1 Importance of forestry in the South

Southern forests provide important economic, ecosystem, energy, and cultural resources both domestically and internationally (Zalesny et al., 2016; Zhao et al., 2016; Snyder & Butler 2012). The US has approximately a 20 percent share in global wood products production (Prestemon et al., 2015); while Southern forests alone comprise about 16 percent of global and 60 percent of national production (Prestemon & Abt, 2002). The southern region (Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, Oklahoma, Puerto Rico, South Carolina, Tennessee, Texas, the US Virgin Islands, and Virginia) of the USDA Forest Service's Forest Inventory and Analysis research work unit has 215 million acres of forested land, comprising 23 percent of the total forest land in the US (Wicker, 2002). These forests constitute the nation's largest supply of roundwood for the pulp and composites industries. Additionally, the maximum loblolly pine growth response to management practices like competition control, fertilization, and irrigation can be found in intensively-managed forests with low site-index in the South (Zhao et al., 2016). Loblolly-shortleaf pine forests comprise nearly a quarter (50 million acres) of Southern forests (Conner and Hartsell, 2002). Furthermore, approximately 18.7 million acres (54 percent) of North Carolina land is forested (USDA Forest Service, 2020).

Loblolly pine timber production is a major source for widely varying products (e.g., paper products and biofuel). As demand for these products shift (e.g., reduction of paper product use and demand for wood biofuel due to climate policy in the European Union), corresponding shifts in markets occur. The housing crash of 2008 was a testament to the importance of forests in the building materials market. Between 2005 and 2009, three major forestry employment sectors: forestry and logging, wood manufacturing, and paper manufacturing lost a total of 322,805 jobs (Woodall et al., 2011).

Apart from traditional wood products, loblolly pine plantations provide environmental resource benefits as well. For example, increased awareness of the importance of carbon (C) sequestration in mitigating climate change in addition to novel C storage offset programs and markets have added new ecosystem services to Southern forests' list of benefits (Zalensy et al., 2016). Galik et al. (2013) used the National Woodland Owner Survey (NWOS) from the Forest Inventory and Analysis (FIA) conducted by the US Forest Service to assess the C sequestration potential of non-industrial privately owned forests. These forests contribute considerably to C sequestration, but a comparison of C offset program participation

and pricing on private land was needed. Generally, as C prices increase, the sequestration potential for a market region also increases. The survey revealed that medium- to well-stocked stands and large parcel-owners contributed 60.6 percent and 60.2 percent of C stocks respectively for the area. Management practices used in these private forests sought to maintain stocking levels and is therefore important to maximizing their C sequestration potential.

Forests in the South store about 32 percent of total forest C in the conterminous US (Galik et al., 2013). Management of Southern forests can improve productivity and thereby C offset potential. Albaugh et al. (2004) examined long-term growth responses to varying nutrient and water resource levels in loblolly pine (*Pinus taeda*, L.) stands. After 9 years of resource manipulations, standing stem mass increased 119 and 23 percent by optimal levels of nutrients and water availability, respectively. Through careful planning and treatment design, managed forests can reach a harvestable age and contribute biomass to C sequestration more quickly than similar unmanaged forests.

Improvements in loblolly pine genetics have improved growth rates and therefore increased the rate and amount of C sequestration per rotation. Genetic improvement increase productivity, stem straightness, crown compactness, and disease resistance. Nearly 100 percent of loblolly pine plantations in the South are planted with genetically improved stock (Aspinwall et al., 2012). Genetic improvement has resulted in an estimated increase of 3.7 billion m³ (17 percent increase) and 1,100 Tg C (13 percent increase) of volume production and sequestered C respectively (Aspinwall et al., 2012).

Evolving environmental concerns have enabled new forest markets such as renewable bio-energy to be established. Global climate change policy has driven nations to call for greater use of bio-energy sources. Forests are a source of bioenergy in several forms, including residues and manufactured wood pellets. Forests constitute the world's largest single renewable energy source (Scott & Page-Dumroese, 2016). In the Southern US, increasing bioenergy demands from forests have impacted traditional forest product markets (e.g., sawtimber, roundwood, and pulpwood) by encouraging manufacturers to diversify their investing and product lines. Hybrid poplar, eucalypts, and willow have been researched extensively and cultivated for use as a bioenergy crop, prized for their incredibly rapid growth rates (Shmulsky & Jones, 2011, p. 423). However, loblolly pine continues to dominate hardwoods and other softwoods in most wood product categories in the South (Wear and Greis, 2002; Abt et al., 2014) (Figures 1.1 and 1.2).

Southern forests are not only a monetized resource as discussed above, but also provide cultural services to residents and visiting tourists. These services include hunting, fishing, wildlife viewing, hiking, and collection of non-timber resources like mushrooms and other wild edibles (Snyder & Butler, 2012). Some activities appear to be relatively restricted to private rather than public lands. For example, three quarters of hunters in the US operate in private forests, both family-owned and commercial, instead of public land (US Dept. of the Interior, 2001; 2006). If public forests can be utilized for both economic and cultural services, its value as forest land can be increased. Support of recreation in Southern forests does not necessarily come at a cost to forest product output, as advancements in tree genetics and forest management make our silviculture more efficient in production regions and free up lower-production regions for cultural services like outdoor recreation (Bower, 2008). Additionally, recreation can commence in intensively-managed forests, providing both services simultaneously.

1.2. Soil resistance/susceptibility to change

When managing forests for varying resources, there is a need to not only study how this management affects growing potential and recreational activities, but also how the site quality is affected. One essential component of site quality is soil quality. There is conflicting evidence on the impact of management on soil systems. Some studies point to a resilience of soil to long-term change, while others suggest soil is susceptible to management impacts. The time scale in question is an important consideration. Many studies examine the effects of management immediately after planting, while fewer studies examine management effects later in the life of the stand. Forest managers should be concerned with how site preparation and harvest techniques affect the soil on the long-term, especially soil nutrient supply. Soil type controls nutrient supply to a large extent, with 'buffered' soils able to replenish nutrients lost to leaching and plant uptake more quickly than 'poorly buffered' soils (Richter et al., 1994). Buffered soils have large reserves of nutrient cations retained in primary minerals that can rapidly recharge the mineral soil when depletions occur, maintaining a nutrient equilibrium. As certain nutrients are removed from the soil, the chemical equilibrium is unbalanced and primary minerals and other sources like organic matter (OM) correspondingly release nutrients to restore the equilibrium. Soil pH plays a large role in the maintenance of this equilibrium.

Since soils replenish lost nutrients relatively slowly in some cases, long-term studies are a great resource for tracking loss and replenishment over time and during forest management. Richter et al. (1994, 1995, 1999) published a series of studies detailing the geochemical effects of planting on a loblolly pine

plantation established in 1957 and monitored over the course of the following 28 years (between 1962 and 1990). In these studies, evidence pointed either to resistance or susceptibility to change depending on the layer and type of soil. Over the lifetime of the stands, the forest floor demonstrated a susceptibility to change while the mineral soil was resistant to change. This stands to reason, since the forest floor is more exposed and directly impacted by above-ground management compared to the underlying mineral soil. Furthermore, mineral soils can be well-buffered and may counteract losses over the long-term.

Management techniques can have physical as well as chemical effects on multiple layers of soil. Perhaps the most striking evidence of long-term forest soil change is the physical damage caused by operating heavy machinery on production forest sites. The use of heavy machinery can cause soil compaction, increased erosion rates, and disrupt soil structure (Johnston & Crossley, 2002). One study found that soil compaction significantly increased soil microbial biomass C over the course of 20 years, which may impact C:N ratios and long-term nutrient cycling (Lewandowski et al., 2019). However, the dominant sandy-textured soil on their study site impacted water holding capacity and other factors and is not representative of all forest sites.

Many forest system studies examine the short- and long-term effects of varying levels of biomass removal on the soil with widely variable results. The Long Term Soil Productivity (LTSP) study conducted by the US Forest Service incorporates sites around the country. Some key physical treatments in the study include compaction of the soil, removal of biomass and the forest floor. The LTSP study has demonstrated that after 10 years of forest growth, soil compaction was greatest in low-bulk density (BD) soils and minimal in high-BD soils. BD is a measure of soil mass per unit volume and has important effects on water and gas infiltration and root exploration of the soil (Brady and Weil, 2008). Removal of the forest floor led to reductions in C and other nutrients in the upper 20 cm of mineral soil. Interestingly, the removals had no impact on tree growth after 10 years (Powers et al., 2005). Another study examining forest floor removal effects after 18 years also showed reductions in soil organic C and total N, as well as reduced microbial biomass C and N with biomass removals (Mushinski et al., 2017). Shorter-term observation periods often reveal a greater degree of fluctuation in soil properties following management applications. A two-year study of forest floor removals demonstrated initial decline then recovery of surficial N within the two-year period. Conversely, the same study showed the effects of tillage as a forest management tool to be negligible (Zerpa et al., 2014). Forest management obviously

has mixed effects on long-term soil properties, with upper layers generally being more susceptible to change than lower layers.

1.3. Forest floor and organic matter

Plantation management has direct short-term impacts on the surface O horizon. The O horizon, also called the forest floor in forested systems, is the uppermost soil layer and is dominated by organic material (Buol et al., 2011). The forest floor cover contributes to the prevention of soil erosion insulating the soil in addition to a host of other benefits. The forest floor includes components like litter-fall and harvesting residues and is an important source for nutrient pools in the soil (Piatek & Allen, 2001; Saint-André et al., 2008). The components vary in states of decay, dependent on deposition time and chemical makeup. As materials in the forest floor break apart and decompose, OM is formed and migrates into the mineral soil through percolation of water and movement of microorganisms. A well-drained mineral surface soil will contain 1 to 6 percent OM (Brady and Weil, 2008). Chemically, as OM breaks down, nitrogen (N), phosphorus (P), and other plant-essential nutrients are released into the soil environment and become available for plant uptake and microbial cycling. Successful tree establishment relies partly on adequate nutrient pools during the first several years following establishment. Maximizing nutrient availability for early tree development is one reason managers often limit herbaceous vegetation growth with herbicides after planting.

Through the course of management, and especially in site-preparation techniques, the forest floor can be purposely or inadvertently manipulated to achieve a variety of effects. Forest floor manipulation studies in plantation systems seek to imitate common management practices and/or disturbance regimes. The studies often compare whole-tree harvesting with forest floor removal to stem harvesting with forest floor retention (eg. Busse et al., 2006; Tan et al., 2008; Mushinski et al., 2017). The effects of forest floor removal on C and N pools are well documented, and generally result in a reduction of these pools, although some studies have shown no influence. Similarly, investigations into soil microbe communities reveal mixed results, with forest floor removal reducing microbial activity/abundance (Tan et al., 2008) or showing little to no effect (Bird et al., 2004; Busse et al., 2006). Time after treatment appears to be a factor that impacts the activities of soil microbial communities, with significant results following treatment and a slowly dissipating signal as the stand ages. Recovery of nutrient pools and reestablishment of plant roots in the root zone over time likely explain the dissipation of effects.

Most forest floor manipulation studies compare total removal versus partial retention of the forest floor. However, two studies (Mack et al. 2014; Zerpa et al., 2014) added a forest floor doubling treatment to the conventional study design. The doubled treatment was established by raking the organic material from one plot, creating a removed treatment (Figure 1.3), and spread atop the existing forest floor of the doubled plot (Figure 1.4). A mixing treatment was also applied in the Zerpa et al. (2014) study that imitated disking and bedding in conventional plantation management schemes (Figure 1.5). Both studies confirmed that adding organic matter improves N and P stores, but there was no C increase in the root zone in Mack et al. (2014). The timing of nitrogen release from the forest floor was delayed by organic matter additions prior to planting, potentially better aligning N release with tree requirements (Zerpa et al., 2014). The conflicting results of forest floor manipulations have been attributed to variations in quantifying nutrients such as total C and N versus labile C, N, and P (Zerpa et al., 2014), methods for quantifying soil properties, and the age of stands studied. Finally, one long-term study found that removing the forest floor resulted in an increase of understory cover, diversity, and richness, but negatively affected stand yield compared to doubling the forest floor (Dimov et al., 2015).

1.4. Ectomycorrhizal fungi & soil aggregate stability

Like studying the effects of forest floor manipulations on nutrient supply and C-cycling, measuring the response of one soil property to change in another is important to our understanding of forest ecosystems. One such relationship is the connection between ectomycorrhizal fungi (ECM) and aggregate stability. Aggregation is the binding of soil particles into larger assemblages and is driven by OM, plant root growth, and mycorrhizal fungi like ECM. ECM help to form aggregates through physical and chemical processes. Physically, hyphae can enmesh smaller microaggregates into larger macroaggregates (Figure 1.6). This enmeshment is like a 'net' that binds microaggregates together. The flexibility and tensile strength of hyphae lends the aggregates resistance to break-up (Wu et al., 2014). The second mycorrhizal mechanism involved in soil aggregation is the release of biochemicals such as polysaccharides and proteins that coat particles and 'glue' them together. The most widely recognized exudate is glomalin, a hydrophobic glycoprotein that protects hyphae from decomposition and predation by other soil microbes (Nichols, 2008). Glomalin production occurs in the cell walls of hyphae and is released during hyphae death and decomposition. The glomalin protein is hydrophobic, and therefore serves as a water-resistant 'glue' that works with hyphae during aggregation of particles. Aggregate stability in water can be tied to this hydrophobic property (Nichols, 2008). Similarly, the insoluble glomalin lends more longevity to aggregates compared to other exudates. Aggregation via

hyphal growth is likely the initial process in macroaggregate formation, followed by electrochemical forces between clay particles, OM coatings that 'glue' aggregates together, and precipitation of hard mineral frameworks within the aggregate (Rillig & Mummey, 2006). Consequently, ECM play an important role in aggregate stability, and as soil properties the two play critical roles in forest soil quality. The benefits to the forest ecosystem are discussed below.

ECM constitute perhaps the most important symbiotic relationship with pines in the South. This symbiotic relationship is likely an evolutionary co-adaptation between the tree and fungus. One study demonstrated that root exudates can boost the growth rate of ECM, perhaps a trigger for symbiosis with a developing tree (Futai et al., 2008). Once initial infection of the tree root occurs, hyphae grow between root tissue cells and develop into a complex web of hyphae called the Hartig net. Fungal and cortical cell walls fuse within this zone, facilitating transfer of carbon, nutrients, and water. ECM also form a shroud that envelops short root hairs, adding to root surface area along with the hyphae growing from the shroud (Fisher and Binkley, 2013) (Figure 1.7). ECM benefit trees by increasing the overall root surface area and expanding the volume of soil that can be explored for resources. In turn, ECM gain carbohydrates from the tree while in association with the roots. Fungal hyphae widen the effective rooting area by 5 to 15 cm and may increase uptake efficiency by up to 10 percent (Brady & Weil, 2008). The increased surface area also improves hydraulic conductivity within trees, which may alleviate drought stress. ECM symbiosis is thought to be vital to forest productivity, especially in poor-quality soils. More than 5000 species with non-specific host compatibility levels infect a variety of tree genera including Pinaceae (Futai et al., 2008). The prominence of loblolly pine in SE US forestry reflects the economic importance of ECM symbiosis.

Physical soil processes like aggregation are also vital to healthy forest growth. Aggregation is a soil property where smaller soil particles are joined through chemical, physical, and biological processes to make larger particles. Aggregate stability is a quality that allows an aggregate to resist break-up. Where attractions exist between soil materials like charged clay particles, soil aggregation is facilitated by both chemical and biological mechanisms. However, sandier soils, where attraction among particles is low, aggregation may be entirely biological in nature (Brady & Weil 2010). Aggregate formation has indirect benefits to trees. The most important aspect of aggregation is its improvement of soil physical properties that aid tree growth. First, the formation of aggregates decreases soil bulk density. Tree roots can thereby explore the soil solum more extensively, accessing otherwise unavailable resources.

In addition to improved root exploration, decreasing the bulk density increases pore size and total pore space, which improves aeration and percolation of water. Second, aggregate formation also produces microsites within the aggregates which hold water and OM and harbors microbes, all essential to tree productivity. The microsites may also be anoxic environments that support anaerobic microbes, thereby increasing the heterogeneity of soils. When trapped within aggregates, OM and nutrients are less susceptible to decomposition or leaching within the soil profile and to erosion at the soil surface (Nichols, 2008). Water-stable aggregates (WSA) can resist break-up through wetting and drying cycles and help maintain soil structure within the root zone. However, aggregation and destruction of aggregates occur simultaneously, so the processes driving aggregation are of primary concern. There is some evidence that larger aggregates are key to good soil structure. For example, Minick et al. (2017) found that larger aggregates in the 2000 to 250 μm diameter range may be more sensitive to intercropping management in a plantation setting.

1.5. Justification for study

Forests continue to be an important resource in the South, providing essential pulp and lumber products. Emerging markets such as bioenergy and C-offset monetization reinforce the value of these forests. However, as we require more of our forests and increase the intensity of management practices, it is vital that we consider the sustainability of this management and its impact on forest land. Long-term studies such as LTSP are clearly valuable in assessing the effects of management on the forest soil resource. Since management has varying effects on soil based on soil type, horizon, and region, it is also important to make these assessments in different conditions and locations to broaden the body of knowledge and inform local managers. Many long-term studies have compared such forest floor manipulation strategies as whole-tree harvesting and forest floor removal versus stem-only harvesting. Very few studies in the LTSP system include a doubling treatment (eg. Mack et al. 2014; Zerpa et al., 2014) or a mixing treatment (eg. Zerpa et al., 2014). The impact of forest floor manipulation on aggregate stability and the mycorrhizal fungi that help form aggregates is a question with little study. One such study in aspen forests found that forest floor removal reduced acid phosphatase (an enzyme activity indicator for ECM) by 39 percent (Tan et al., 2008). Due to the close association of aggregate stability and ECM with pine growth, it is important to assess the impact of forest floor manipulation and mixing in the South. As we expect more from our forests, work is needed to ensure our management activities sustain future forest and soil resources.

1.6. Selection of methods

Several techniques are used to quantify aggregate stability including wet sieving, ultra-sonic vibration, and raindrop methods. We elected to use an approach combining wet-sieving with agitation called the modified Yoder method to calculate mean weight diameter (MWD). The Yoder method was selected because it separates the soil sample into five size classes, whereas other lower-resolution methods only identify one class of particles over a certain diameter.

We used acid-phosphatase enzyme activity measurements to biochemically assess potential functional activities of ECM in the soil. We also performed analysis of fatty acid methyl esters (FAMEs) extracted from soil samples to generate community profiles and monitor changes in community structure in the soil. FAME was selected to assess the relative abundance of ECM due to the low cost of analysis compared to other molecular methods, (e.g., DNE sequencing), and to provide estimates of viable biomass.

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Tables and Figures

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Courtesy of Dr. Zakiya Leggett.

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Courtesy of Dr. Zakiya Leggett.

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CHAPTER 2: Forest floor manipulation effects on the relationship between aggregate stability and ectomycorrhizal fungi

2.1. Introduction

Southern forests are important for their economic, ecosystem, energy, and cultural resources (Zalesny et al., 2016; Zhao et al., 2016; Snyder & Butler 2012). From an economic perspective, the US provides about 20 percent of global wood products production, while Southern forests contribute about 16 percent of global production (Prestemon et al., 2015; Prestemon & Abt, 2002). Intensively managed forests in the South, specifically loblolly pine plantations, offer the highest yields and greatest degree of productivity among Southern forests (Zhao et al., 2016). Forests in the South store nearly 32 percent of total forest C in the contiguous US (Galik et al., 2013). Southern forests also provide cultural services, for example, hunting, fishing, wildlife viewing, hiking, and the collection of non-timber resources which includes mushrooms and other wild edibles (Snyder & Butler, 2012).

Although determining short-term effects of management decisions are necessary, the relatively long rotations (20 to 30 years) of commercial forests demand an examination of long-term effects of management techniques. There is conflicting evidence on the impact severity of above-ground management on forest soil systems. Richter et al. (1994) examined the geochemical effects of conventional loblolly pine management in a forest established in 1957. The forest was monitored over the course of 28 years (between 1962 and 1990). The authors reported that cation exchange capacity (CEC) increased as much as $0.28 \text{ cmol}_c \text{ kg}^{-1}$. However, mineral soil exchangeable Ca and Mg decreased markedly while exchangeable K showed little change.

Carbon (C) sequestration is a frequently-studied long-term component of intensively managed forests and has more recently gained importance as a sink to offset CO₂ emissions into the atmosphere. Long term studies confirm the role of plantation forests as a sink for atmospheric C. Over 30-years, one loblolly pine forest accumulated 34.9 Mg ha^{-1} and 96.0 Mg ha^{-1} of C in the forest floor and the upper 0.6 m of mineral soil respectively (Richter et al., 1995). Another study by Aspinwall et al. (2012) compared C-cycling between genetically improved pine stands and unimproved stands and found that genetic improvement increased the amount of ecosystem C by 17 percent relative to the unimproved sites. Litter-fall and post-harvest residues contribute to the composition and establishment of the forest floor and are important sources for soil nutrients (Piatek & Allen, 2001; Saint-André et al., 2008). Trees rely in part on the forest floor for plant-essential nutrients, thus forest managers are concerned with long-term

nutrient supply from the forest floor to maintain the sustainability of forest soils. Forest floor manipulation studies in plantation settings seek to maximize nutrient release and availability by exploring the use of traditional and novel management practices. Such studies often compare whole-tree harvesting with forest floor removal to stem harvesting with forest floor retention (eg. Powers et al., 2005; Busse et al., 2006; Tan et al., 2008; Mushinski et al., 2017). The studies generally show a short-term reduction in nutrient pools following forest floor removal.

Three studies (Mack et al. 2014; Zerpa et al., 2014; Dimov et al., 2015) added a forest floor doubling treatment to the conventional removal-only study design. To create these treatments, the forest floor was scraped from the 'removed' plots and added atop the existing forest floor of the 'doubled' plots. A mixing treatment was also applied to the Zerpa et al. (2014) study that mimicked disking and bedding in conventional management schemes. Both studies confirmed the addition of organic matter improved N and P stores, without increasing rhizospheric C (Mack et al. 2014). Dimov et al. (2015) found that removal of the forest floor had a negative effect on stand yield relative to doubling the forest floor. Although adding organic material is difficult in forests spanning hundreds of acres, these studies confirm the necessity of forest floor retention when the goal is to maintain the supply of nutrients. The doubling of the forest floor could also simulate the impact of increased litter accumulation on the forest floor due to repeated fertilizations.

OM in the forest floor impacts nutrient pools available to trees. Perhaps equally important as nutrient pools in management decisions are physical and microbial soil properties, which provide suitable rooting conditions and aid in the release, availability, and uptake of plant-available nutrients. Aggregation is a physical process wherein smaller soil particles are joined through chemical, physical, and biological processes to form larger particles. Increasing particle size lowers bulk density (BD), which aids in air and water infiltration to the root zone (Brady and Weil, 2008). Aggregate stability is a measure of quality that is used to describe the resistance of soil to destruction. A more stable aggregate will resist breakdown from water movement in the soil. When trapped within aggregates, OM and nutrients are less susceptible to decomposition or leaching and soil surface erosion (Nichols, 2008).

Fungal associations with plant roots are correlated with increased aggregate stability in the root zone (Wu et al., 2014; Curaqueo et al., 2011). Several classes of soil fungi aid in aggregate formation. ECM are a class of soil fungi that form symbiotic relationships with plant roots. More than 5000 species of

ECM with non-specific host compatibility levels infect a variety of tree genera including Pinaceae (Futai et al., 2008). In this relationship, ECM provide the tree with additional nutrient and water uptake, and the tree provides ECM with carbon. ECM hyphae widen the effective rooting area by 5 to 15 cm and may increase nutrient uptake efficiency by up to 10 percent (Brady & Weil, 2008). ECM contribute to an aggregate size class transformation: the creation of macroaggregates (>3.0mm diameter) from microaggregates (0.3 to 3.0 mm diameter). Zheng et al. (2014) concluded that ECM symbiosis significantly increased either MWD or water-stable aggregation in all aggregate size classes.

Furthermore, a significant albeit weak relationship between hyphal length and MWD was reported. Due to the importance aggregate stability and ECM play in pine growth, it is necessary to assess how these factors are impacted by disturbances imitated by forest floor manipulation and mixing. Studies have compared forest floor removals and whole tree harvesting with forest floor retention and stem-only harvesting, but rarely include a doubling or mixing treatment. Additionally, little research has examined the effects of these treatments on the ECM abundance and aggregate stability. Finally, there is little long-term (greater than 10 years) study of these effects. To address the limitations, the long-term influence of forest floor removals and additions on aggregate stability and ECM abundance was examined on a 14-year-old loblolly pine plantation. Mean weight diameter (MWD) testing was used to assess aggregate stability, (EL) FAME to assess EMC abundance, and acid-phosphatase enzyme activity analyses to describe ECM functionality. The objectives of this study were to determine if forest floor manipulations influenced aggregate stability and ECM abundance and to establish if a relationship exists between ECM abundance and aggregate stability under the environmental conditions of the study site. Our research can provide forest managers with information regarding the application of forest floor site preparation and the tools that affect important soil properties on a long-term basis.

2.2. Methods

2.2.1. Site description

Our study utilizes the plots from a prior study by Zerpa et al. (2014). The original study site was installed in 2006 in the Lower Coastal Plain of Pamlico County, North Carolina (35° 6' 2.00" N, 76° 52' 45.1" W), following the harvesting of a 33-year-old loblolly pine plantation. Site mean annual air temperature is 18.1 °C and mean annual precipitation is 1207 mm. The soil on the site is described as moderately well drained, and is a fine, mixed, subactive, thermic Aquic Hapludult in the Craven soil series (Soil Survey Staff, 2020). The A horizon is a fine sandy loam with an average depth of 10 cm (Zerpa et al., 2014).

Logging of the original stand occurred in the winter of 2005–2006 using a boom-top excavator, and felling equipment was restricted to the previous thinning roads to prevent disturbance of the forest floor on the study plots. In spring of 2006 after harvesting was completed, a complete randomized block study with five replications and six forest floor mixing treatments was established. The treatment design was a 3×2 factorial, with three levels of forest floor manipulation (removed, control, and doubled) and two levels of forest floor mixing with the surface mineral soil (mixed and nonmixed). The forest floor was raked from the removed treatment plots and evenly added to the doubled treatment plots atop its existing forest floor. Control plots retained the original forest floor and harvest debris. Mixing treatments were incorporated by pulling a three-point disk tiller on the first pass and a one-row disk tiller on the second and third passes. The total plot sizes, including buffer zones, were 16.8 m by 9.1 m, and the interior measurement plots were 12.2 m by 4.9 m. Two weeks after the mixing treatments were completed, 96 full-sibling pine seedlings were planted per plot at 1.5 m by 1.2 m spacing for a total of 32 pines seedlings per measurement plot (or 6279 trees per hectare). The pine seedlings were treated with permethrin (Pounce) pesticide prior to planting to prevent damage by pales weevil, *Hylobius pales* (Herbst), and competing weeds were controlled as needed with a post-emergent herbicide (Glyphosate) (Zerpa et al., 2014). Commercial thinning of the plots occurred in 2008, reducing the stocking level to 1350 trees per hectare.

2.2.2. Soil sampling description

Samplings for our study were carried out during June and July of 2019. Soil samples for physical and biological testing were taken from the top 7.62 cm of mineral soil within the A-horizon. The A-horizon is associated with high soil ECM activity where fine roots are concentrated and has been found to contain two-thirds of all ECM species found in a temperate oak forest soil profile (Buée et al., 2007). We selected this depth to capture the area most affected by surface manipulation, to keep within the depth of the mixing treatment, and to represent the depth associated with an abundance of root-associated ECM.

Soil samples for MWD study were sampled using an x-y coordinate system with randomized points on a 30.48 cm by 30.48 cm grid. After removing the forest floor material to the top of the mineral soil, the top 7.62 cm of mineral soil were removed from an approximately 58.06 cm² area excavation using a soil knife with inch-demarcations and placed in Ziploc bags. To preserve the physical structure of the soil, care was taken to not crush the soil during sampling. Two samples were taken per plot (for a total of 60

samples). Upon return to the laboratory, these samples were laid out on paper plates to dry. After the soil was air-dried, it was returned to the bags, which were left open until laboratory analysis could take place.

Soil samples for microbiological study were taken from the mineral soil immediately surrounding loblolly pine trees within the study plots to capture the microbially active area of the root zone. Trees were randomly selected prior to sampling and identified by their tag in the field. If any tree was missing in the plot, soil was sampled from around the next-closest tree. Two trees per plot were selected and three samples per tree were taken and combined to make two composite samples for each plot. Sample locations were 30.48 cm from the root collar of the stem. The forest floor was scraped to the top of the mineral soil and the top 7.62 cm of mineral soil was excavated with an approximately 58.06 cm² area excavation using a soil knife. The soil was placed in Ziploc bags and kept cool in a cooler until transportation to a laboratory refrigerator where they were stored at 4°C until analysis.

Bulk density (BD) samples were taken from approximately plot center, following the protocol of previous measurements at the site. Samples were collected using a soil coring device with a cylindrical interior sleeve measuring 10.08 cm in height and 4.82 cm in diameter for an interior volume of 183.93 cm³. The coring device was pounded into the surface of the mineral soil (0 cm to 10.08 cm). The sleeve containing the soil was removed and stored in Ziploc bags until laboratory analysis.

Finally, a second soil core was taken from approximately plot center in the same method as the BD approach described above to be used for soil chemical analysis.

2.2.3. Soil mean weight diameter analysis

Soil aggregate stability was assessed using a modified protocol from Kemper and Rosenau (1986) measuring mean weight diameter (MWD) metric and employing the Yoder device. Five grams of air-dried soil were placed at the top of a sieve tower contained in a clear plastic column with descending sieve mesh dimensions of: 2mm, 1mm, 0.5 mm, 0.25 mm, and 53µm. Water was added to the column until the soil was wet and allowed to rest for 5 minutes until the soil was saturated. The entire tower, with the soil resting on top, was then reciprocated vertically within the column with the water level moving above and below the soil level for 5 minutes. The sieves were removed from the column and the soil trapped in each sieve was collected into tins. The soil at the bottom of the column (representing soil passing the 53µm sieve) was also collected. These tins were oven-dried at 70°C for a period of 24

hours, and the soil in each tin weighed. The soil was then returned to their respective sieves, wetted, and then pushed manually through the sieve pores using a rubber policeman then rinsed with water. This process crushed the wetted peds and forced the smaller particles through the sieve but retained the larger sand-sized particles. The sand fraction was then removed from the sieves, oven dried at 70°C for at least 24 hours, then weighed.

To calculate MWD, the sand weight was subtracted from the total soil weight in each size class. This value represented the corrected aggregate weight. This corrected aggregate weight was expressed as a fraction of the total aggregate weight (after removing the sand). This fraction was multiplied by an expansion factor which corrected for the difference in sieve pore diameter. Finally, the weights for each size class were summed to give the MWD in mm for each sample. The calculation is expressed as follows:

$$MWD = \sum_{i=1}^n X_i(W_i)$$

Where *MWD* is mean weight diameter, X_i is the mean diameter of size fraction *i*, and W_i is the proportion of the aggregate weights of size fraction *i* to total aggregate weight (Carrizo et al., 2015).

2.2.4. Soil microbiological analysis

The microbial community composition and structure at the site was characterized using an ester-linked fatty acid methyl ester (EL)-FAME analysis method as described by Schutter and Dick (2000). The lipid extractions from 3 g of field-moist soil, employed mild alkaline methanolysis (15 mL of 0.2 M KOH in methanol), followed by neutralization with 3 mL of 1.0 M acetic acid. FAMEs were then separated into organic phase by adding 10 mL of hexane followed by centrifugation at 480 ×g for 10 min at 37°C. The upper hexane layer was pipetted into a glass test tube and the hexane evaporated under a N₂ stream. FAMEs were dissolved by adding 200 µL of 1:1 methyl tert-butyl ether and hexane containing methyl nonadecanoate (19:0) as an internal standard (0.5 mg·mL⁻¹). Samples were vortexed and transferred into a 250-µL glass insert in a 2-mL GC vial. FAME analysis was conducted using an Agilent 6890 N gas chromatograph with a 25 m × 0.32 mm × 0.25 µm (5 percent phenyl)-methylpolysiloxane Agilent HP-5 fused silica capillary column (Agilent, Santa Clara, CA) and flame ionization detector (Hewlett Packard, Palo Alto, CA) with ultra-high purity hydrogen as the carrier gas. The temperature program ramped from 170°C to 270°C at 5°C min⁻¹ then ramped to 300°C for 2 min (Acosta-Martínez et al., 2010; Gardner et al., 2011).

Fatty acids of key microbial communities were identified and quantified based on comparison of retention times and peak areas to components of MIDI standards using the TSBA6 aerobe program from MIDI (Microbial ID, Inc., Newark, DE). FAMES are described by the number of C atoms, a colon, the number of double bonds, and the position of the first double bond from the methyl (ω) end of the molecule. Other notations used include methyl (Me), cis (c) and trans (t) isomers, and iso (i) and anteiso (a) branched FAMES. Selected FAMES used as microbial markers were Gram-positive (Gram+) bacteria (i15:0, a15:0, i17:0, a17:0), Gram-negative (Gram-) bacteria (cy17:0, cy19:0), and actinomycetes (10Me17:0, 10Me18:0). Fungal markers that included saprophytic fungi (18:1 ω 9c, 18:3 ω 6c), arbuscular mycorrhizal fungi (AMF) (16:1 ω 5c), ectomycorrhizal fungi (18:2 ω 6c), and absolute amounts of FAMES (nmol g⁻¹ soil) were calculated as described by Zelles (1999). Bacterial summation was calculated using the Gram+, Gram-, and actinomycetes markers. In contrast, fungal summation was calculated using both saprophytic and AMF fungal markers. The fungal/bacteria (F:B) ratio calculated by dividing the sum of fungi by the sum of bacteria (Gardner et al., 2011).

The enzyme activities of acid phosphatase, β -glucosidase (β -Gluc), β -glucosaminidase (β -Glm) were determined to characterize the community function of ECM, C-associated microbes, and N-associated microbes respectively. Briefly, 1 g of air-dried soil was assayed under a final concentration of 10 mM of the specific enzyme substrate (p-nitrophenyl-derivate), optimal pH and buffer (without toluene), and incubated for 1 h at 37°C as described in Tabatabai (1994) and Parham and Deng (2000). The reaction product (p-nitrophenol) was determined colorimetrically at 400 nm by spectrophotometer (Thermo Fisher Scientific – Genesys 10S UV-VIS, Waltham, MA). Samples were assayed in duplicate and included a control for each assay, where the substrate was added after the reaction was terminated following incubation. The amount of p-nitrophenol released was calculated by reference to a calibration curve developed with standards containing 0, 100, 200, 300, 400, and 500 nmol of p-nitrophenol (Ntoko et al., 2018).

2.2.5. Other analyses

BD was determined by the volumetric core method. Field samples were oven-dried at 105 °C for approximately 43.5 hours. The mass of the sleeve and dried soil was taken before the soil was removed from the sleeves. The mass of the sleeve was then measured and subtracted from the total mass to give soil mass. This was expressed as a fraction of the sleeve volume (183.93 cm³) to give bulk density of the soil in g cm⁻³.

Soil moisture content was assessed using a loss of moisture approach. A 10 gram field-moist sub-sample was taken from the ECM composite samples. The sub-sample was placed in tins and dried at 105 °C for at least 24 hours. The sub-sample was then weighed and expressed as a fraction of the field-moist weight to give percent soil moisture.

Soil pH of water was measured using a pH meter (Fisher Scientific – accumet, AB200, Waltham, MA). 10 grams of air-dried soil taken from the ECM composite sample were mixed with 25 mL of de-ionized water and allowed to equilibrate for 30 minutes. The pH of the soil slurry was then determined with the pH meter.

Samples for chemical analysis were sent to Brookside Laboratories, Inc. in New Bremen, Ohio for a variety of soil evaluations. Percent OM was measured using weight loss on ignition at 360°C according to Schulte and Hopkins (1996). Cation Exchange Capacity was measured using a cation summation method described in Ross (1995). Percent C and N were determined by combustion according to Nelson and Sommers (1996) and McGeehan and Naylor (1988). Plant-available nutrients were determined with the Mehlich III extractable nutrients test described in Mehlich (1984).

Weyerhaeuser Company contractors conducted an inventory of the loblolly pine trees in the summer of 2019 to add to the historical dataset. Diameter at breast height (DBH) in inches and total height in feet were recorded. Tree volume was calculated using an equation from Amateis and Burkhart (1987) which modeled loblolly pine tree profiles across the South:

$$V_{ib} = -0.09653 + 0.00210D^2H$$

Where V_{ib} is the tree volume-inside-the-bark in cubic feet, D is the tree diameter at breast height in inches, and H is total tree height in feet. Volume in cubic feet was then expanded to cubic feet per hectare for analysis and reporting.

2.2.6. Data analysis

A general model describing the treatment design was used to assess the results:

$$Y_{ijm} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + A_m + E_{ijm}$$

Where Y_{ijkm} is the response to treatments i and j in block m at time k , μ is the overall mean, α_i is the fixed forest floor retention treatment effect ($i=1, 2, 3$), β_j is the fixed mixing treatment effect ($j= 1, 2$), $(\alpha\beta)_{ij}$ is

the fixed interaction effect of the forest floor retention treatment i with the mixing treatment j , A_m is the random block effect ($m=1, 2, 3, 4, 5$), and $E_{ijk m}$ is the random residual error.

Data were initially digitized and tabulated using Microsoft Excel (Microsoft Corp., Redmond, WA, 2020). Data was analyzed using R Studio (RStudio, PBC, Boston, MA, 2020) and various packages therein. Descriptive statistics were calculated using the *describeBy* function. Analysis of variance (ANOVA)s for individual soil properties were calculated using the *fit <- aov* function. Where significant differences in factors were found, we performed the Tukey's post-hoc test to compare the means between the soil properties using the *tukey.test* function. To assess the relationship between MWD and ECM, we employed a linear regression and compared P-values using the *fit <- lm* function. Principal component analysis (PCA) of FAME indicators was carried out with the *(factor).pca* function.

Data visualization was done in both Excel and R Studio, with tables constructed in Excel and figures constructed in both Excel and R Studio.

2.3. Results

2.3.1. Soil Physiochemical Properties

The effect of the treatments on soil aggregate stability was assessed with mean weight diameter (MWD) testing. MWD values appeared to trend higher in the mixing treatment relative to the non-mixed treatment. However, the difference ($P=0.96$) between the treatments was insignificant (Figure 2.1). ANOVA tests were run on the experimental data with full and partial models to find factor levels with significant impact on the independent soil properties (Table 2.1). Tukey's post-hoc test was used to identify differences among treatment levels when a factor significantly impacted a variable. Table 2.1a shows a significant impact ($P < 0.05$) of forest floor retention on BD. Table 2.1b shows a significant impact ($P < 0.05$) of mixing on percent N.

Differences in physiochemistry were insignificant except for percent nitrogen (N) and BD, which were significant (Table 2.2). Percent N was on average higher in the mixed treatment (0.13 percent) than in the non-mixed treatment (0.12 percent) independent from the forest floor treatment ($P < 0.05$). However, when percent N is converted to a kg ha^{-1} basis using BD and soil depth, there is no significant difference between the doubled and removed treatments ($P = 0.97$), (Table 2.2b.). Additionally, BD averages were higher in the removed treatment (1.35 g cm^{-3}) than in the doubled treatment (1.17 g cm^{-3}).

³) independent from the mixing treatment ($P < 0.05$). Percent organic matter (OM), percent carbon (C), percent moisture, cation exchange capacity (CEC), and pH of water (pHw) all showed no significant difference.

2.3.2. Soil ectomycorrhizal fungi and other microbiology

Soil microbial community composition and structure was assessed using fatty acid methyl ester (FAME) testing (Table 2.3). The FAME biomarker/indicator, 18:2 ω 6c is used to represent the ECM group within the microbial community structure. The ECM FAME indicator was not significantly different among any treatment factors but did show a difference in the blocking level factor.

Enzyme activities were measured to determine the functioning and dynamics of soil microbes related to nutrient cycling in the soil (Table 2.4). The acid phosphatase enzyme is used here as an indicator of ECM activities (Ntoko et al., 2018). There appeared to be a trend in the acid-phosphatase data pointing to increasing activities with increasing forest floor retention as well as in the mixing treatment. However, there was no significant difference found among treatments for any enzyme activity measured.

A principal component analysis (PCA) was conducted using the detected FAME indicators: 18:2 ω 6c; bacteria total, fungi total, fungi:bacteria ratio, gram + sum; gram – sum, and total FAME (Figure 2.2). PCA aids in the visualization of large data sets by simplifying a large set of variables to a smaller one while retaining key information. The principal component will be the variable that best explains the variance in the dataset. The overlap of the ellipses indicates no significant difference among the treatment distributions, supporting the initial assessment of lack of statistical difference among the variables.

2.3.3. Relationships among soil property variables

The ECM FAME indicator 18:2 ω 6c was found to have a significant relationship ($P = 0.023$) with the aggregate stability metric MWD within the root zone. A linear regression between the two variables yielded a positive relationship with a weak adjusted $R^2 = 0.17$ (Figure 2.3). This indicates that 17 percent of the variance of MWD is explained by the predicted ECM FAME indicator curve. In other words, there is a significant but weak relationship between the two variables. With increasing ECM abundance there is an increase in aggregation in the root zone. The relationship between MWD and acid-phosphatase activity was not significant ($P = 0.24$).

2.3.4 Tree volume

Tree volume was assessed by an equation that used DBH in inches and total height in feet. The volume of the double non-mixed treatment appeared to trend higher than the other treatment volumes.

However, volume had no significant difference ($P = 0.52$) among the treatments when ANOVAs were tested (Figure 2.4).

2.4. Discussion

2.4.1. Soil physical properties

There was no significant difference in mean weight diameter among treatments. Literature suggests that mixing significantly reduces aggregate stability due to losses in OM and soil organic carbon (SOC), especially in agricultural soils, where tillage is a common soil treatment (Eynard et al., 2005).

Investigations of tillage effects are less common in forest systems, but it was expected that our study would yield similar results. It was surprising that there were no significant differences in aggregate stability, especially between the mixed and non-mixed treatments. Some of the studies reporting reductions in aggregate stability after mixing represent a relatively short measurement interval after treatments when compared to our 14-year interval (eg. Eynard et al., 2005; Kibet et al., 2016).

Additionally, some of these studies cover sites with repeated mixing treatments, often several times during the year (eg. Kibet et al., 2016). However, one study confirmed reductions in MWD with two- and four-year repeating intervals between mixing in an agricultural setting (Shu et al., 2015). Our longer measurement interval may have allowed a recovery of aggregation to occur in the soil compared to short-term and repeated mixing effects of the other studies.

Another potential explanation for our lack of difference in aggregate stability may be explained by soil type. The A horizon is a fine sandy loam and may lack the charged clay particle attraction present in more clayey soils. When the charge attraction is absent or reduced, aggregation is driven by other mechanisms like OM 'gluing' or binding by ECM hyphal growth and exudates like glomalin (Nichols, 2008). The relatively long interval between the treatments and our sampling may have normalized the effects of forest floor manipulation and mixing through OM and ECM recovery, both of which show no significant difference among the treatments. Furthermore, 91 percent on average of the non-sand weight fraction of aggregates was present in diameters above 53 μm and 65 percent on average present in the 2mm and above size class. This suggests that there was minimal small-diameter material available

for further aggregation, supporting the contention that aggregates have largely recovered since the manipulation and mixing treatments were imposed.

There was a significant difference in bulk density (BD) between the forest floor doubling and removal treatments. Removing the forest floor increased BD by 0.18 g cm^{-3} relative to doubling. This may be due to the initial reduction of OM that likely resulted from the forest floor being removed. Additions of OM decrease BD in the soil partly through its involvement in macroaggregate formation and the addition of the lower density material (Shu et al., 2015). Interestingly, after 14 years, OM did not show any difference in the soil, so the effects of manipulation on BD may have occurred early in stand establishment. The resulting BD was not an area of concern as far as having an impact on tree growth since our results were equal or less than the typical Hapludult profile A horizon (1.42 g cm^{-1}) (Brady and Weil, 2008).

The apparent recovery of aggregates is useful information for forest managers interested in the impacts of forest floor manipulation or mixing on the soil when establishing loblolly pine plantations. Since plantation forestry is conducted on rotation intervals of 20 years or greater, our study suggests that aggregate stability can recover after 14 years or less in sites with similar conditions and similar soil types. This finding supports the idea of soil's overall ability to recover from manipulation. Increases in BD with forest floor removal suggests that retaining debris and litter after a harvest is preferred over whole-tree harvesting and forest floor removals where soil physical properties are concerned.

2.4.2. ECM and soil microbiology

The assessment of microbial community composition and structure performed using FAME analysis indicated that the groups were insignificantly different among the treatments. Similarities in microbial community composition and structure suggests soil microbes are resistant to forest floor manipulation and mixing or that the community can recover from treatment impacts after 14 years or perhaps both. Some studies have found that conventional mixing practices in agricultural systems reduces fungal hyphae extensions (Curaqueo et al., 2011; Hu et al., 2015). Hyphae may be mechanically disrupted during mixing, however this does not necessarily kill the fungal assemblages. Although the differences are insignificant, the removed-non-mixed treatment had the highest concentrations of FAME indicators for each group. Regardless of mixing, doubling the forest floor resulted in the lowest FAME concentrations for most indicator groups. Therefore, there may be a trend in forest floor retention and

FAMEs, with a negative relationship between additions of forest floor material and a reduction of FAMEs. A negative relationship between forest floor mass and FAMEs is unexpected however, since decreasing OM has been shown to decrease microbial biomass and fungal phospholipid fatty acids (Busse et al., 2006). The fungi:bacteria ratio was highest in the removed treatments. It is possible that removing the forest floor creates inordinately optimal conditions for fungal growth, making fungal abundance the driving factor in its representation among the treatments.

Differences among the treatments on any enzyme activity value were insignificant. However, acid-phosphatase activity (used to detect ECM function) did not display the same trend as the FAME indicator for ECM, where additions of forest floor material reduced FAMEs. Conversely, increasing forest floor retention and mixing appeared to increase acid-phosphatase enzyme activity. However, the metrics describing the microbial community involved in these two tests are different enough from each other to make comparison difficult and it is better to evaluate them separately.

2.4.3. Relationships between soil property variables

The relationships between aggregate stability and the variables associated with ECM (acid-phosphatase enzyme activity and 18:2 ω 6c FAME concentration) were examined. A significant association was detected between aggregate stability and the 18:2 ω 6c FAME indicator for ECM. The positive association suggest that as ECM abundance increases, aggregate stability is improved. The relationship between aggregate stability and the ECM FAME indicator supports the hypothesis that increasing ECM abundance will improve soil aggregation. Testing for aggregate stability and ECM FAMEs was conducted using different field sampling approaches; aggregate stability was sampled using a randomized grid point approach while ECM samples were taken around loblolly pine tree stems. Aggregate stability sampling was located away from the immediate vicinity of the tree stems to avoid the interference of large roots and thereby minimize destruction of aggregates during sampling. However, both sampling approaches captured the same depth (0 cm to 7.62 cm) from the biologically active root zone. The relationship's low explanation of variability (adjusted $R^2 = 0.17$) may be a result of this difference in sampling approaches. We did not find a significant relationship between aggregate stability and acid-phosphatase enzyme activity. This is surprising considering the positive relationship with ECM FAMEs, but may be explained by the difference in metrics between FAME and enzyme activity. FAME analysis

shows indicator group abundance while enzyme activity testing shows activities related to nutrient cycling in the soil.

2.4.4. Impacts on management decisions

Our results lend useful insight into making management decisions in loblolly pine plantations with similar conditions to our study. Generally, forest floor manipulation and mixing were not detrimental to aggregate stability and the soil microbial community after 14 years. Mixing and forest floor manipulations are not common site preparation techniques. However, they may mimic disturbance events like heavy machinery on the landscape or the result of other techniques, such as fertilization increasing forest floor mass over time. Managers concerned with maintaining site quality across many forest rotations may utilize our results when considering how disturbance affects certain soil properties. Retaining the forest floor resulted in lower BD values, which improves water infiltration and aeration in the root zone. This result supports other studies that applaud the benefits of retaining debris after a harvest. However, although bulk density differed significantly among treatments, tree volume was not different. Therefore, the benefits of forest floor retention are more in terms of soil physical structure rather than tree growth. Furthermore, all BD values reported here were within the accepted range found in Ultisols, the soil order present on the study site. Finally, maintaining suitable conditions for ECM growth and symbiosis was positively related to increased aggregation, with presumably positive benefits to site quality and tree growth.

2.5. Conclusion

Southern forests are vital for their economic, ecosystem, and cultural services. Managers implement techniques like genetic improvement and site preparation to maximize tree growth and site quality. Managers can devise best management practices through evaluation of the effects of these techniques over time. Our study provides useful information for managers interested in the impacts of forest floor manipulation and may be concerned about the subsequent impacts on soil properties. The forest floor manipulations had little to no effect on aggregate stability or ECM abundance in the mineral soil. A weak but positive relationship was found between ECM abundance and aggregate stability, pointing to site quality benefits in maintaining a healthy soil microbiome. Our study can inform managers of the impact of certain forest floor manipulation techniques on aggregate stability and ECM abundance. Furthermore, our results indicate that promoting ECM abundance improves aggregate stability, a key soil physical property. Our study supports the notion of soil recovery from forest floor manipulation and

reveals a potential relationship between aggregate stability and ECM abundance in Coastal Plain loblolly pine forests.

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Tables and Figures

Figure 2.1: Results of mean weight diameter testing for study treatments. Soil mean weight diameters in the top 7.62 cm of mineral soil for different forest floor and mixing treatments. Different colors denote the three forest floor treatment factors (control, double, and removed). Error bars are standard error of the means.

Table 2.1: Results of ANOVA tests on study factors. a. P value results of ANOVA testing on full model including Block, forest floor retention (Floor), mixing (Mixed), and the interaction of Floor and Mixed. b. P value results of ANOVA testing on partial model including Block and mixing (Mixed) while excluding forest floor retention. Factors with significant impact on the soil property variables are in bold.

2.1a. Soil Property	Factor			
	Block	Floor	Mixed	Floor:Mixed
	Pr (> T)			
18:2 ω6c FAME indicator	<0.0001	0.43	0.26	0.26
N (%)	<0.05	0.93	0.06	0.87
N (kg ha ⁻¹)	<0.05	0.43	0.25	0.97
C (%)	0.52	0.87	0.55	0.73
C (kg ha ⁻¹)	0.42	0.35	0.74	0.87
BD (g cm ⁻³)	0.61	<0.05	0.21	0.38
pHw	<0.01	0.55	0.25	0.90
moisture (%)	<0.0001	0.11	0.21	0.84

Table 2.1 continued: **Results of ANOVA tests on study factors.** a. P value results of ANOVA testing on full model including Block, forest floor retention (Floor), mixing (Mixed), and the interaction of Floor and Mixed. b. P value results of ANOVA testing on partial model including Block and mixing (Mixed) while excluding forest floor retention. Factors with significant impact on the soil property variables are in bold.

Soil Property	Factor	
	Block	Mixed
	Pr (> T)	
18:2 ω6c FAME indicator	<0.0001	0.27
N (%)	<0.05	<0.05
N (kg ha ⁻¹)	<0.05	0.23
C (%)	0.49	0.52
C (kg ha ⁻¹)	0.40	0.74
BD (g cm ⁻³)	0.64	0.26
pHw	<0.01	0.23
moisture (%)	<0.0001	0.22

Table 2.2: **Results of ANOVA tests on physiochemical properties.** a. Means and results of ANOVA and Tukey's post-hoc tests on soil physiochemical properties in the top 10 cm of mineral soil. Significant differences are bolded and denoted by different letters within a given soil property (row). For example (a) is significantly different from (b). b. Results of ANOVA and Tukey's post-hoc tests on soil chemical properties in the top 10 cm of mineral soil after expanding from percent to kg ha⁻¹.

Soil Property	Treatment					
	Control - Mixed	Control - Non-mixed	Double - Mixed	Double - Non-mixed	Removed - Mixed	Removed - Non-mixed
OM (%)	3.5	2.8	3.8	3.3	3.3	3.4
C (%)	2.0	1.8	2.1	2.1	2.0	2.5
N (%)	0.11(a)	0.13(b)	0.12(a)	0.13(b)	0.11(a)	0.13(b)
moisture (%)	28	24	30	32	28	30
CEC (meq 100 g ⁻¹)	6.1	4.8	8.1	5.8	6.5	5.5
pHw	5.0	4.1	5.0	5.0	5.1	5.0
BD (g cm ⁻³)	1.3	1.2	1.2(a)	1.2(a)	1.4(b)	1.3(b)

Soil Property	Treatment					
	Control - Mixed	Control - Non-mixed	Double - Mixed	Double - Non-mixed	Removed - Mixed	Removed - Non-mixed
OM (kg ha ⁻¹)	43000	39000	45000	38000	46000	44000
C (kg ha ⁻¹)	25000	25000	25000	25000	28000	32000
N (kg ha ⁻¹)	1400	1500	1400	1600	1600	1700

Table 2.3: Results of FAME tests on soil microbial functional groups for study treatments. Fatty acid methyl ester (FAME) indicators of bacterial and fungal functional groups. The 18:2 w6c fungal biomarker/indicator is associated with ectomycorrhizal fungi.

FAME Indicators (nmols g ⁻¹ soil)	Treatment					
	Control - Mixed	Control - Non-mixed	Double - Mixed	Double - Non-mixed	Removed - Mixed	Removed - Non-mixed
Bacteria						
Gram + sum	37	32	31	29	30	39
Gram - sum	26	24	22	21	22	28
Bacteria total	87	76	72	69	71	93
Fungi						
18:2 w6c	20	19	17	18	17	24
Fungi total	92	84	77	76	81	110
Fungi:bacteria ratio	1.1	1.1	1.1	1.1	1.1	1.2
FAME total	1300	1200	1100	1100	1100	1400

Table 2.4: Results of ANOVA tests on enzyme activities for study treatments. Means and results of ANOVA and Tukey's post-hoc tests on enzyme activities in the top 7.62 cm of mineral soil for different forest floor and mixing treatments. Significant differences are denoted by different letters within an enzyme row. Acid phosphatase, β -Glucosidase, and β -Glucosaminidase are indicators for ECM, C-cycling, and N-cycling microbes respectively (Ntoko et al., 2018).

Enzyme Activities (mg PNP kg ⁻¹ soil h ⁻¹)	Treatment					
	Control - Mixed	Control - Non-mixed	Double - Mixed	Double - Non-mixed	Removed - Mixed	Removed - Non-mixed
Acid Phosphatase	180	160	220	190	140	130
β -Glucosidase	33	32	31	34	30	35
β -Glucosaminidase	35	36	35	47	38	39

Figure 2.2: Principal component analysis of summary FAME indicators. Treatment groups are delineated by the colored ellipses and overlaps indicate similarity in variance (not significantly different). The arrows and variables in red are the key FAME indicator sums and the ECM indicator. Overlapping variables are: 18:2 ω 6c, bacteria total, gram + sum, and gram – sum.

Figure 2.3: Relationship between FAME ECM indicator and mean weight diameter. Results of a linear regression between 18:2 ω 6c FAME and MWD, which relate to ECM community composition and aggregate stability, respectively. There is a positive relationship ($P = 0.023$) with an adjusted R-squared value of 0.1702.

Figure 2.4: Results of tree volume measurements for study treatments. Tree volume for different forest floor treatment levels and mixing. Different colors denote the three forest floor treatment factors (control, double, and removed). Error bars are standard error of the means.

CHAPTER 3 – REFLECTIONS AND FUTURE STUDY

3.1. Study objectives and takeaways

The objectives of this study were (1) to determine if forest floor manipulation and mixing had an effect on ectomycorrhizal fungi (ECM) community structure, soil aggregate stability, and a variety of other soil properties and (2) to determine if there was a relationship between ECM and aggregate stability. The results show that after 14 years, there were no residual effects from manipulation or mixing on aggregate stability or ECM community structure. It was speculated that the time between treatment and this study's measurements allowed aggregation and the soil microbiome to recover after disruption. These results support the contention of long-term studies that soils recover well from forest floor manipulation in forest systems (Richter et al., 1994, 1995, 1999). There was a weak positive relationship between ECM and aggregate stability. As ECM community structure increased, there was an increase in aggregate stability. This positive relationship suggests that improving conditions for ECM symbiosis may improve conditions for aggregation in the soil. Bulk density (BD) was greater in the removed treatment (1.35 g cm^{-3}) than in the doubled treatment (1.17 g cm^{-3}). It was speculated that organic matter (OM) additions in the doubled treatment contributed to a decrease in BD through flocculation and the addition of a lighter material.

3.2. Significance

This study contributes to the body of knowledge where forestry and soil science intersect, especially where forest floor disturbance is concerned. Few studies have examined the effects of forest floor additions and mixing on aggregate stability and ECM community structure or the relationship between them in a plantation forest setting. The initial study (Zerpa et al., 2014) used forest floor manipulation to mimic litter increase due to repeated fertilization in site preparation and mixing as a tool to improve soil physical properties and nutrient availability. The results of this study demonstrate that manipulation and mixing effects are nominal after 14 years. Although BD was significantly higher in the removed plots relative to the doubled plots, none of the BD values were outside of the range reported for the soil type represented by the study site. Furthermore, differences in tree volume were insignificant, suggesting that the treatments provide no long-term advantage to tree growth. Soil aggregates and ECM display an ability to recover from intermittent physical disturbance. Compared to agricultural studies where mixing is conducted annually or more frequently, this study suggests that some soil properties can recover from a single disturbance in less than 14 years. Furthermore, this study reveals a positive relationship between ECM and aggregate stability. Current site preparation

techniques to maintain soil physical quality such as slash redistribution and mixing are time-consuming and costly. The positive relationship between ECM and aggregate stability reveals a potential need for future inquiry into improving soil physical properties through encouragement of the native ECM community.

3.3. Study limitations and further inquiry

There were several limitations present in this study. First, the study site represented only one soil type in the Lower Coastal Plain of North Carolina, making broad claims on ECM and soil physical properties in plantation forests in the South impossible. Similarly, field sampling and laboratory measurements were made over the course of the summer growing season, limiting the capture of seasonal effects on the several variables, especially ECM community structure, which varies over the course of the year. Second, the study captures the relationship between aggregate stability and ECM at one point in time. Both properties are known to change with time, so the results reflect what may be a transient relationship. Finally, the study design implements a significant but relatively small sample number due to time and workforce limitations. A larger sample size, especially in the aggregate stability sample set, could have provided a stronger signal in the relationship between ECM and aggregate stability. The strongest case for future study lies in the positive relationship between ECM and aggregate stability.

Our study design only confirms the relationship but does not explore the mechanisms involved in aggregation by ECM. Several mechanisms associated with ECM are thought to be involved in aggregation, including fungal hyphae forming “nets” around smaller soil particles, fungal exudates “gluing” particles together, and OM inputs by senesced fungal bodies increasing aggregation. Hyphal length, glomalin-related soil protein, and microbial biomass carbon (MBC) relatively could be assessed to parse out these effects. Further study could quantify the relative impact of these mechanisms on aggregation to clarify the relationship between ECM and aggregate stability in plantation forest systems. The study only assesses the ECM-aggregation relationship at one point in time. Further study could examine how that relationship shifts over the course of the year or longer. Repeated measures could shed light on which seasons show the greatest amount of aggregation via ECM activity and which seasons other factors predominate in that role. Finally, considering this study’s treatments, future long-term studies could elucidate the interval after forest floor manipulation and mixing where ECM and aggregate stability return to pre-treatment levels. Since this study found insignificant results in these

two variables while other studies demonstrated a difference, a repeated measures study following treatment could shed light on ECM and aggregate stability recovery following forest floor disturbance.

Additional study may include a more detailed examination of the microbial community structure and diversity. The ECM community was assessed using FAME concentration testing, which reports the relative abundance of groups of microbes like gram-negative and -positive bacteria, actinomycetes, or ECM. However, FAME testing does not indicate which species of microbes are present. Future study may utilize molecular DNA sequence analysis, which provides an individual species-level breakdown of the community. DNA analysis would have greater resolution than the FAME approach and could add ECM diversity to the understanding of the relationship between aggregate stability and ECM.

3.4. Conclusion

This study adds to the understanding of the interaction of ECM and aggregate stability in Southern plantation forest systems with a commonly planted species. The results support the contention that forest soils recover well from forest floor manipulation and provide valuable information to managers seeking to understand how manipulation affects physical and microbial soil properties after 14 years. The positive relationship between ECM and aggregate stability reveal opportunities for further inquiry, including a more detailed examination of the mechanisms involved in ECM-aided aggregation and repeat measures studies of that relationship, perhaps following site preparation. The results of such studies will broaden the understanding of the role of ECM in aggregation and the temporal scales important to the relationship.

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