ABSTRACT

Title of Thesis: HARVEST FREQUENCY OF SWITCHGRASS IN A CIRCULAR ECONOMY ALTERS SOIL ECOLOGY

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Modern agricultural management has disrupted the balance of agroecosystems, as reflected in widespread nutrient disequilibrium. Ecosystems have the capacity to restore balance when provided with appropriate ecological conditions that support nutrient cycling, soil structure formation, and plant-microbe interactions.

This thesis begins by proposing the implementation of a circular economy on the Lower Eastern Shore of Maryland to address two pressing local issues: the overabundance of nutrients and increasing rates of marginal land. An important

component of the proposed circular economy is planting and harvesting switchgrass (*Panicum virgatum*). The second and third chapters investigate how different harvest frequencies of switchgrass affect soil ecology. Chapter 2 investigates how harvest frequencies impact arbuscular mycorrhizal fungal (AMF) diversity. The third chapter investigates the relationship among harvest frequency, soil organic carbon, soil aggregation, and AMF. Together, the results show that switchgrass harvest frequency influences AMF community composition, small macroaggregate formation, and soil carbon.

HARVEST FREQUENCY OF SWITCHGRASS IN A CIRCULAR ECONOMY ALTERS SOIL ECOLOGY

by

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Dedication

To the fauna, flora, *and* funga

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Chapter 1: A Circular Economy Approach for Local Biomass and Waste-to-Energy Production

Co-authors: Jonathan Cumming, Stephanie Lansing, Andrew Moss, Eric Burnstein, Priscila B. R. Alves, Marccus D. Hendricks, Gordon F. Custer, Alison Schulenburg, Nate Spicer, Kathleen Brown, Kate Tully

Note: This chapter was prepared as a perspective piece for the Environmental Science & Technology Journal.

Abstract

Biomass waste-to-energy (BWtE) systems provide an opportunity to capture and recycle waste and nutrient streams, converting them into value-added resources. Here, we review BWtE technology options and highlight the potential benefits and barriers to their implementation. Adoption of BWtE systems can close nutrient loops, mitigate greenhouse gas emissions, and foster local economic and climate resilience. Prioritizing local, context-specific solutions can provide environmental and societal benefits, driving the transition to a more sustainable and regenerative energy future. Additionally, we provide a case study on the Lower Eastern Shore of Maryland, USA, to illustrate how local agriculture and waste management practices can be integrated into BWtE systems at a regional level in the face of environmental challenges, such as saltwater intrusion. These practices generate energy and create nutrient by-products that can benefit local stakeholders and economies.

Problem Statement

Current models of economic growth are tightly linked to environmental resource extraction, leading to a decoupling of material cycling between sources and sinks. This disruption has resulted in two major environmental challenges: i) an overabundance of waste, often in the forms of nutrients and carbon, in certain areas, contributing to pollution, runoff,

eutrophication, and greenhouse gas (GHG) emissions; and ii) resource depletion elsewhere, often associated with ecosystem degradation (Harder et al., 2021; Porterfield et al., 2020). Both challenges affect the surrounding ecological communities and the humans living in these environments. Transition pathways away from extractive, linear economies toward more local, circular systems can create resilient and socially-just energy and nutrient-based circular economies.

Circular bioeconomies mimic processes in natural systems, wherein organic wastes and biomass (i.e., food waste, animal waste, and wastewater solids) generated from human-dominated systems can be cycled to capture energy (i.e., biogas, renewable natural gas, syngas, and bio-oil) and nutrients (by-products such as digestate and biochar), using controlled biochemical and thermochemical processes (i.e., waste-to-energy) (Hussain et al., 2020). However, biomass waste-to-energy (BWtE) systems often require additional resources (i.e., co-feedstocks) to optimize and enhance the efficiency of the energy generation processes. Furthermore, there are many technical and practical barriers to implementing BWtE circular economies.

In this paper, we examine potential BWtE systems, highlighting both their potential benefits and key limitations based on a critical review of existing literature. Based on our review, we propose that implementing BWtE circular economies should emphasize localized systems that: i) close nutrient loops, ii) position communities as key decision-makers, and iii) support economic viability. Finally, we explore a case study on the Eastern Shore of Maryland, USA, where rising sea levels and salinization have damaged local agriculture, especially impacting historically marginalized farmers. We discuss a potential BWtE solution to address these local

challenges through a decarbonized, BWtE system that utilizes animal waste and switchgrass to produce bioenergy through anaerobic digestion (AD).

Background

Biomass+waste-to-energy technologies

BWtE technologies provide diverse pathways for converting organic wastes and biomass into usable forms of energy while simultaneously generating by-products that can contribute to soil fertility and carbon sequestration in a circular economy. Among these, AD, gasification, and pyrolysis are particularly notable for their potential roles in circular nutrient and carbon management systems. Foundational to closing the 'nutrient loop' in these systems is the use or re-investment of by-products from bioenergy production back into the systems. Here, we outline these technologies and note potential benefits and limitations in terms of 'closing the loop' using their respective by-products.

Biochemical conversion: anaerobic digestion

Anaerobic digestion (AD) is a microbe-mediated biochemical process in which organic substrates are decomposed under anoxic conditions, resulting in the production of biogas, primarily composed of methane (CH_4) and carbon dioxide (CO_2), and a nutrient-rich residual material known as digestate. Co-digesting materials with complementary carbon and nitrogen contents can help stabilize digestion performance and improve biogas yield and substrate flexibility (Rajlakshmi et al., 2023).

The feedstock choice, temperature, and other operating conditions impact the quality and properties of the digestate (Chojnacka et al., 2024). Most AD feedstocks – manure, crop residues, food waste, bioenergy crops – vary in nutrient content, moisture, C:N ratios, and contaminants depending on location (Chojnacka et al., 2024). Due to the fact that most feedstocks are site-specific, it is difficult to create generalized protocols or 'one-size-fits-all' recipes (Tumusiime et al., 2022; Rana et al., 2025). Each new location or combination may require additional testing, creating a technical barrier to widespread, predictable implementation. However, once feedstock optimization is reached, the biogas generated from AD can be used for heating or electricity generation locally or purified to renewable natural gas (RNG) for on-site, local, or regional use.

Thermochemical conversion: gasification & pyrolysis

Gasification is a high-temperature thermochemical process in which biomass undergoes partial oxidation to produce a combustible gas mixture known as syngas (primarily CO and H_2), along with biochar as a residual by-product. Pyrolysis operates similarly to gasification but uses lower oxygen conditions to produce syngas, bio-oil, and biochar, and is particularly suitable for drier materials like poultry litter. The quality and quantity of the products from thermochemical conversion vary depending on feedstock composition and process parameters such as temperature (Bergfeldt et al., 2018). For example, biochar produced from poultry litter would have lower carbon content and a high phosphorus content compared to biochar produced from wood. As with AD, syngas and bio-oil can be utilized to generate heat or electricity, or potentially upgraded to transportation fuels, serving as renewable energy sources in a circular economy (Nusselder et al., 2020).

Value-added by-products from BWtE technologies – potential benefits and limitations

The generation of gases for energy production diverts carbon that would otherwise be kept in waste lagoons, storage, or landfills, where CH_4 may be generated and contribute to climate forcing. Likewise, nutrients in these waste streams can be captured and distributed onto croplands in ways that prevent runoff, loss through landfill leachate, or excessive accumulation which can destabilize environmental systems.

While AD does not alter the total nutrient load of the feedstock, organic forms of nitrogen and phosphorus are converted during the process into inorganic forms that are more immediately available to plants (Porterfield et al., 2020). Digestate can be used as a replacement for synthetic fertilizers, potentially avoiding up to 4 tons of CO_2-equivalent emissions per ton of fertilizer replaced (Akbar et al., 2021). Although anaerobic digestion enhances nutrient availability via mineralization, it does not resolve the low N:P ratios characteristic of many organic amendments. This may cause phosphorus over-application when nutrients are managed to meet crop nitrogen demand, leaving digestate subject to the same phosphorus-driven eutrophication risks as undigested feedstocks (Cordell et al., 2009). Additionally, although AD has been shown to reduce antimicrobials, antimicrobial-resistant genes, and hormones found in most animal manures, further research is still required to determine the best pre-treatment and post-treatment options to fully address these challenges (Congilosi et al., 2021). Additionally, long transportation distances of digestate may offset CO_2-balance benefits, making localized systems for digestate application important. With this, programs that identify where nutrients can be responsibly applied in a locale are essential.

The retention of nutrients during use of thermal conversion technologies, both gasification and pyrolysis, vary based on processing temperatures. Both processes retain phosphorus in the solid fraction and may volatilize nitrogen, contributing to reduced eutrophication potential compared to other waste treatments (Nusselder et al., 2020; Korberg et al., 2021). Biochar from both processes, has a carbon content ranging from 60–85% and can be used as a soil amendment, to increase soil carbon stocks (Panwar et al., 2019). Pyrolysis biochar is typically more carbon-rich, while gasification biochar tends to be more mineral-rich with a higher surface area. Although, both can improve soil nutrient status and sequester carbon, application to soil may lead to nutrient imbalances and concentrated heavy metal residues, depending on feedstock substrate, and thus, biochar should be applied with proper nutrient management plans (Vakalis et al., 2016). In some cases, biochar can sorb contaminants and serve as a remediation agent for soil pollution, further enhancing its value in a circular nutrient economy (Ji et al., 2022).

BWtE for reintegration of nutrient and carbon cycles

BWtE technologies can use waste and biomass from disparate locations and reintegrate them to replicate the nutrient cycling conditions found in natural ecosystems. Two fundamental conditions are consistently met in nutrient cycling in natural ecosystems. First, producers and consumers coexist within the same space (i.e., spatial colocalization). Second, the rates of production and consumption are naturally regulated and balanced (i.e., temporal synchrony) (Liu et al., 2016). Human activities have largely disintegrated the natural equilibrium between nutrient cycling and balanced production and consumption rates. The degree to which nutrients are removed from their original source determines the extent of system disintegration (Harder et

al., 2021). In agriculture, integrated nutrient cycling can exist when livestock consume plants and organisms from their pasture and return nutrients to the soil through manure, maintaining a cycle that supports continued plant production. In contrast, industrialized agricultural systems and concentrated animal feeding operations (known as CAFOs) often rely on imported feed, with manure not being returned at the same rate or location as the feed source (Pagliari et al., 2020). These systems represent two ends of a spectrum, one disintegrated and contributing to nutrient imbalances (Figure 1.1A), and the other integrated and maintaining natural cycling (Figure 1.1C), (Figure 1.1B). BWtE systems represent a functional alternative to disintegrated nutrient systems, by reintegrating disparate waste and biomass sources such as co-feedstocks to produce both renewable energy for communities and value-added by-products that can offer flexibility by replenishing nutrients where they are needed by providing different nutrient product types (Figure 1.1B).

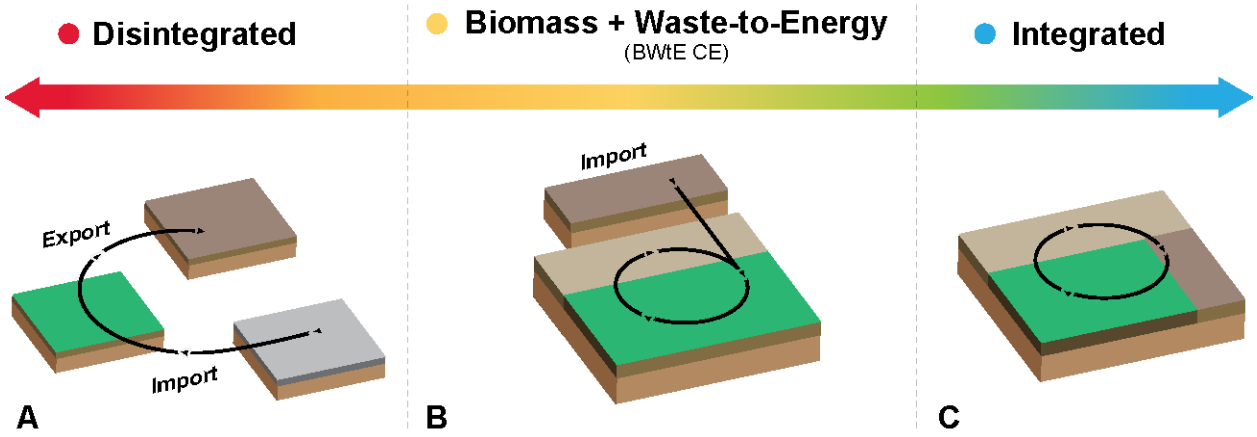

Figure 1.1. *Spectrum of agricultural and waste system integration. Black arrows indicate simplified nutrient flow through systems. In disintegrated systems, nutrients originate from mineral or industrial sources (gray box); in integrated systems, all nutrients originate and cycle within the system; in Biomass+Waste-to-energy systems, nutrients are imported as food from various sources, including on-farm and off-farm sites.*

Local is context-specific and often challenging

While co-locating BWtE facilities near waste sources is ideal, it may not always be feasible due to zoning, population density, or infrastructure constraints. Transportation costs and emissions can diminish the benefits of using distant feedstocks, but in some cases, integrating diverse waste streams from a broader region may offer greater net value for energy yield. There is potential for broader biomass streams to generate greater net value for the environment and communities, particularly when those materials would otherwise be mismanaged. Additionally, some biomass sources, such as bioenergy crops grown on marginal land, could provide environmental co-benefits through carbon sequestration in local soils (Liebig et al., 2008). Defining the boundaries of biomass and waste inputs must be context-specific and guided by holistic decision-making that weighs the trade-offs among material types, distances, energy yield, environmental impacts, and community outcomes. To support this, tools such as techno-economic analyses (TEA) and life cycle assessments (LCA) should be employed with a focus on maximizing benefits and minimizing harms within the defined BWtE circular ecosystem.

Economics, Policy, and BWtE Development

Circular economies for BWtE systems require deliberate economic and policy frameworks to support their development. Within the current and dominant economic framework operating today, market forces can force businesses to primarily act in pursuit of profit maximization. There is little inherent incentive to adopt practices that prioritize environmental sustainability or community well-being without a positive return on investment. Through mechanisms such as subsidies, tax incentives, grants, and regulatory frameworks, governments

can create conditions where circular economic practices are viable within the current system that is otherwise driven by extractive profit.

Alternative economic systems to support a circular economy

While government and state interventions show promise, the fundamental structures of capitalism do not naturally align with the regenerative, localized, and cooperative principles at the heart of many circular economy models (Velenturf et al., 2021). This tension suggests that while state-led efforts can create temporary remedies to support circular economies, a durable transition may require more comprehensively supported approaches for the integration of new practices within the existing economic frameworks. Scholars, practitioners, and grass-roots movements are recognizing that the dominant economic system is incompatible with ecological sustainability and equitable development (Brand-Correa et al., 2022). A few emerging economic theories that are notable and potentially ideal frameworks for implementing socially and environmentally sound economies are ecological economics and social and solidarity economics (Bruel et al., 2019; Villalba-Eguiluz et al., 2023). However, for transitory circular economies to emerge now, it is essential to focus on generating policies and programs that can support the circular economy within the current economic system.

Engage stakeholders in BWtE development

Beyond environmental goals, circular economies must consider the social dimensions of sustainability by embedding community needs and knowledge into their design and governance. The people who live in the communities where these systems operate should not only benefit from them but should also be key decision-makers shaping how they function; those that have

been marginalized and excluded from power and decision making should have influence over the new economy's planning and operation (Wheeler et al., 2020).

Each process for engaging communities in planning and public decision making must be tailored to the social, economic, and environmental contexts. Outlined below are several steps that can help ensure that affected communities are engaged in a just manner.

Step 1: Identify justice and engagement goals. These goals may include centering and empowering groups that have been otherwise marginalized, ensuring that the potential benefits and harms from the economic transition are distributed fairly, or that future generations will have access to quality services and resources (Schlosberg et al., 2003).

Step 2: Gather data on who is affected by the proposed changes. This data includes both who lives, works, or otherwise spends time in areas geographically impacted and who belongs to social groups who are likely to be more impacted (Mohai & Saha, 2007). This step may involve collecting demographic, economic, and environmental data which can be analyzed spatially. Determining the scale of analysis is critical (Mohai et al., 2009). For example, scales may vary from whole counties to specific neighborhoods.

Step 3: Identify and engage relevant, trusted local institutions representing affected communities. Community engagement and outreach may include local or regional governments, churches, advocacy groups, unions, charitable groups, schools, or others that represent particular communities otherwise marginalized in decision-making (Davidoff et al., 2016; Krumholz et al., 1982). The institutions should be knowledgeable of the needs and experiences of the communities identified in Step 2 and be able to connect with individuals from those

communities. Note that some institutions, particularly government bodies and business interests, may be mistrusted by marginalized communities (Williams, 2024).

Step 4: Gather data on relevant group and individual experiences, needs, and priorities related to your project. Activities in this area may involve interviews or focus groups with institutional representatives that can speak knowledgeably about a particular population of interest ("grass tops"), surveys, interviews, or focus groups conducted with members of those community groups ("grass roots"), or a combination of these activities. Institutions identified in Step 3 are a valuable resource for reaching affected populations themselves.

Step 5: Faithfully use what has been learned. Engagement must include learning from the engaged communities. This includes acknowledging points of accord and points of discord within and between community groups and recognizing when and where their ideas have been incorporated (Forester, 1980).

Step 6: Build feedback loops. For true community empowerment and engagement (not just consultation), be sure to return to the communities with findings to gather feedback, make corrections, and provide more opportunities for input. This both provides more community control and reflects back to community members that their voices are being heard and their input impacts the final outcome (Arnstein, 2019). All in all, an inclusive process is necessary to produce an inclusive outcome.

BWtE Case Study

The Lower Eastern Shore of Maryland, USA (Somerset, Wicomico, and Worcester counties) faces two converging environmental crises: excess nutrients from poultry waste and saltwater intrusion (SWI). This region produces over 220,000 tons of nutrient-rich poultry litter

annually. While this waste has historically been land-applied as fertilizer, about 69% of agricultural land on the Lower Eastern Shore has restrictions for applying poultry litter due high phosphorus levels based on the Phosphorus Fertility Index (FIV) threshold (FIV >150), where phosphorus becomes mobile and poses a significant runoff and leaching risk (Schulenburg et al., 2024; Hogan et al., 2017). This nutrient imbalance is compounded by the fact that many poultry farms are not co-located with land that can receive the litter under current nutrient management rules (Hogan et al., 2017).

Additionally, the region is experiencing SWI at about three times the global average (Sallenger et al., 2012). As SWI moves inland ahead of sea level rise, salinizing soils and reduced yields devastate local staple row crops like corn and soybean (Tully et al., 2019; Mondal et al., 2023). These challenges present a unique opportunity to develop a localized circular economy that utilizes poultry litter for use as a feedstock for AD while simultaneously reclaiming salt-impacted production sites through the cultivation of salt-tolerant bioenergy crops, such as perennial grasses, as co-feedstocks for AD. This case study follows the approach outlined above for BWtE circular economies with localization, closed-loop nutrient cycling, community engagement, and economic support to assess how a poultry litter-based circular economy AD system coupled with switchgrass cultivation could address these regional challenges.

Localized systems for regional resilience

As an input for AD, salt-tolerant perennial crops such as switchgrass (*Panicum virgatum*) can be cultivated on land affected by SWI. Switchgrass offers multiple ecosystem benefits through sequestering soil carbon through its deep root systems, controlling erosion, and

increasing AD efficiency by improving feedstock carbon-to-nitrogen ratio (C:N when co-digested with nitrogen-rich poultry litter) (Liebig et al., 2005; Lee et al., 2007, Rana et al., 2025). Salt patches resulting from SWI represent areas where typical crops (e.g., corn and soybean) experience minimal to no productivity. Using a 100 m buffer around these salt patches on the Maryland Lower Eastern Shore, our previous analysis (2017) estimated that 9,075 hectares of agricultural land were at-risk and likely no longer suitable for traditional crops but could benefit from conversion to switchgrass cultivation (Mondal et al., 2023). With switchgrass generating 7 to 11 metric tons of biomass per hectare, depending on harvest frequency (Liebig et al., 2008), growing switchgrass on SWI areas on the Lower Eastern Shore of Maryland could produce 63,525 to 99,825 metric tons of switchgrass biomass annually (Wang et al., 2015).

Our prior work showed poultry litter on the Maryland Lower Eastern Shore averaged 222,455 metric tons in 2017 (Hassanein et al., 2022). Using a ratio of 2:1 (poultry litter: switchgrass) and a specific methane yield of 0.195 m^3 CH_4/kg TS added for poultry litter and 0.288 m^3 CH_4/kg TS added for switchgrass (Hassanein et al., 2024; Montoya et al., 2025), the region could generate 549,000 –863,000 MMBtu/yr of renewable energy annually with co-digestion of locally generated poultry litter with switchgrass grown on marginal lands.. The biogas produced is enough to provide the necessary electricity to 6,608 to 10,384 residential homes using an average of 76.8 MMBtu/year, which is equivalent to supplying 4% to 6% of all electricity needs for the residential homes on the Maryland Lower Eastern Shore, while providing an equivalent of 362,000 MMBtu/yr of heat to industry through co-generation (FindEnergy, n.d.; US Energy Information Administration, n.d.).

Our prior LCA research evaluated the environmental trade-offs associated with transporting poultry litter to/from an AD system and food waste transported for co-digestion to a dairy manure AD system (Mahoney et al., 2024). These analyses showed that transportation of 40 km, which is the average distance to drive poultry litter across one county in the Maryland Lower Eastern Shore was more sustainable than the average of 110 km used in transporting food waste or poultry litter across state lines for field application or other disposal. Maintaining lower transportation distances and using local resources reduces carbon emissions. The distances to and from BWtE sites for feedstocks and byproduct utilization must be considered alongside the carbon sequestration potential of switchgrass and alternative pathways for the poultry litter when planning circular bioeconomy systems, or potential benefits could be lost.

Biogas (55-75% CH_4) use should be carefully planned to optimize biogas utilization with local energy needs and resources (Lansing et al., 2023). Biogas can be used directly in a combined heat and power system to generate electricity and heat. Renewable natural gas (RNG) is formed when biogas is upgraded to >99% CH_4, with the RNG injected into a natural gas pipeline or used in any natural gas installation, including a vehicle. Geospatial location can be used to determine where power can be added to the grid, available pipeline injection sites, or RNG vehicle fleets to determine the best use based on community input and local energy infrastructure.

Closed-loop nutrient flows

Although part of the chicken feed used in local CAFOs is produced on the Maryland Lower Eastern Shore, much of it is imported, creating a disconnect between where nutrients originate and where they are recycled. The poultry litter generated from these operations often

does not return the nutrients to the soil used to grow the feed, contributing to regional nutrient imbalances (Figure 1.2A).

The AD process can address this challenge by transforming poultry litter into renewable energy *and* digestate. Digestate can be applied to switchgrass and local grain fields at the same rate that biomass is removed for the anaerobic digestion to close the nutrient loop directly. Solids separated from digestate can simultaneously be packaged and sold as a value-added product for distribution to nutrient-deficient soils (Figure 1.2B). This approach not only reduces phosphorus runoff but also supports in-region nutrient recycling (Figure 1.2B).

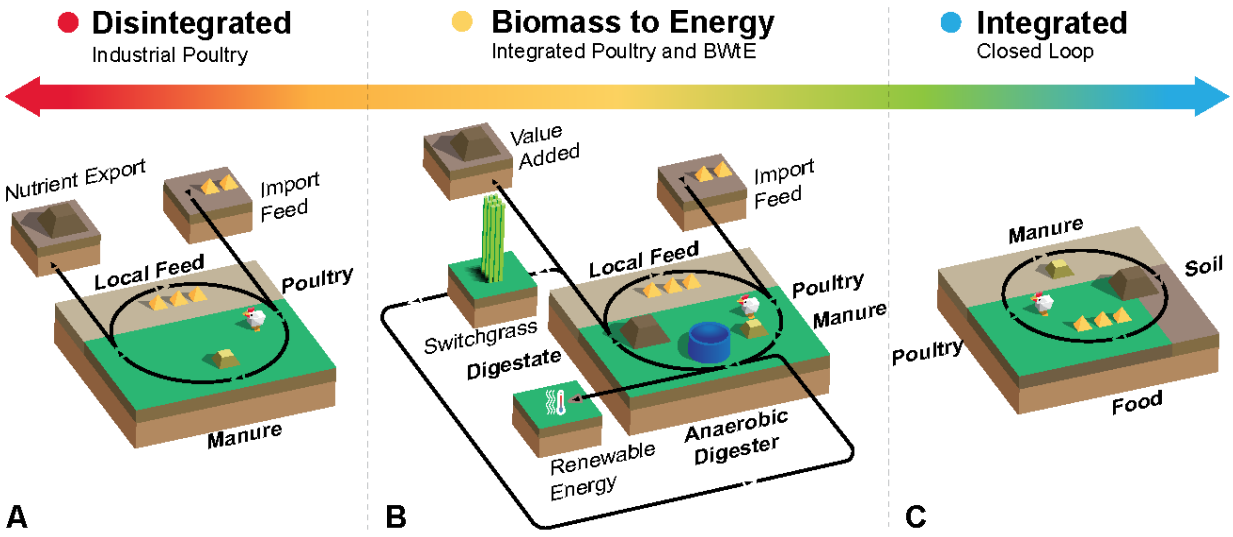

Figure 1.2. *Spectrum of poultry production integration on the Maryland Lower Eastern Shore. (A) Current poultry production nutrient system. (B) Proposed BWtE system - locally-sourced poultry manure and switchgrass serve as co-feedstocks for renewable bioenergy production. Digestate is applied locally to support feed and feedstock productivity and can also be shipped out-of-loop as an economic and nutrient benefit for the system. (C) Totally integrated system.*

Economic viability

The economic opportunity cost of continuing to grow salt-sensitive crops is high. Potential losses, assuming zero profits on salt patches and a 100 m buffer around the salt patches, for the Maryland Lower Eastern Shore are $4.9 million (Mondal et al., 2023). Assuming crop

insurance will not indefinitely recoup the costs, planting salt-tolerant perennial species, such as switchgrass, may represent a potential alternative and create a new commodity for farmers challenged by SWI in the region. However, for the implementation of such a new crop, subsidies and support may be necessary, considering initial capital costs. Equipment such as grass seeders and harvesters would be required initially. Cooperative models for shared equipment may help mitigate these costs since switchgrass establishment is typically a one-time investment and cultivation requires little management aside from harvesting.

Developing a circular system that utilizes poultry litter and salt-tolerant switchgrass as co-feedstocks for AD may offer an alternative use for land affected by SWI and for the surplus nutrient generated in the local poultry production. This approach already aligns with some local policy and program priorities related to nutrient management, GHG reductions, and alternative animal waste advancements. For example, Maryland has a clean energy goal to reduce GHG emissions by 60% below 2006 levels by 2031, and BWtE technologies can play a critical role in achieving that goal (Lansing et al., 2023). Maryland also has an Animal Waste Technology Fund that provides financial support for implementing proven technologies for managing animal waste to reduce nutrient levels and transform the waste into alternative products, such as the implementation of AD systems (Lansing et al., 2023). Additionally, there is the Manure Transport Program that provides financial assistance to help farmers transport excess animal waste, mainly poultry litter, from land bases with surplus nutrients to areas where the nutrients can be safely and effectively utilized. Thus, a set of policy levers and economic support exists to support BWtE circular systems in the region.

Investment, research, and implementation priorities

The realization of this circular model requires a thorough economic evaluation of the entire system based on local prices, community needs, energy infrastructure, transportation distances, state and local land use and development restrictions, and available grant funding. Additionally, while digestate can serve as a nutrient-rich soil amendment and provide an avenue to close nutrient loops, the economics of field application and viable sale as a soil amendment in or out-of-region must be assessed. Governmental and state investment through research grants, pilot programs, and market development initiatives are essential in the initial implementation of circular BWtE economies. Finally, targeted research is needed to optimize feedstocks, assess by-product management, and develop context-specific strategies for integrating circular BWtE systems into local communities, ensuring these systems are both environmentally effective and socially equitable.

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Chapter 2: Harvest frequency impacts arbuscular mycorrhizal fungal community composition in rhizosphere and bulk soil of switchgrass

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Note: This chapter was prepared as a short communication piece. Therefore, the bulk of the methods and results are in a supplemental material section at the end of this chapter per journal style guidelines.

Abstract

Arbuscular mycorrhizal fungi (AMF) play critical roles in plant growth, nutrient acquisition, and stress tolerance, yet their responses to aboveground agricultural management practices remain poorly understood, especially in bioenergy cropping systems. Switchgrass (*Panicum virgatum*) is a perennial bioenergy crop, which can be grown on marginal lands and forms a strong association with AMF. In this study, we investigated how aboveground harvest frequency influences the AMF communities associated with switchgrass on the Delmarva Peninsula, USA. Using high-throughput amplicon sequencing, we analyzed AMF diversity and characterized differentially abundant genera across harvesting regimes and soil depths within the bulk and rhizosphere soils and root tissue. Our findings demonstrate that after two years of stand establishment and one year of harvest treatments, there was a limited, yet statistically significant, impact of harvest frequency on AMF α-diversity in the rhizosphere, and a statistically significant effect on β-diversity in the rhizosphere and bulk soil, underscoring the importance of understanding the selective pressures imposed by agronomic management on plant-soil-AMF systems.

Importance

Arbuscular mycorrhizal fungi (AMF) are essential to ecosystem function, yet little is known about how agricultural management practices influence these vital communities. Protecting and managing AMF is increasingly important in the face of climate change, as they enhance plant tolerance to salinity, drought, and extreme temperatures. Switchgrass, a perennial grass that forms symbiotic associations with AMF, is a promising candidate for biofuel production on expanding marginal lands due to its low input requirements, high carbon sequestration potential, and adaptability. Understanding how harvest management of switchgrass affects AMF communities is critical not only for conserving AMF biodiversity but also for sustaining the functional benefits that AMF provide for switchgrass growth, productivity, and ecosystem resilience. To our knowledge, no studies have examined how harvest frequency shapes AMF communities in switchgrass systems. Here, we use high-throughput amplicon sequencing to assess the effects of harvest frequency on AMF α-diversity, β-diversity, and taxonomic distribution across the root, rhizosphere, and bulk soil.

Observation

Arbuscular mycorrhizal fungi (AMF) are important plant symbionts that form relationships with >70% of terrestrial plant species (Hawkins et al., 2023). These fungi play critical roles in supporting plant growth and productivity (Begum et al., 2019) and confer protection against environmental and anthropogenic stressors such as salinity, herbivory, agricultural intensification, drought, and extreme temperatures (Borde et al., 2017; Faghihinia et al., 2020). Concurrently, the frequency and intensity of ecological stressors are increasing, often acting in concert (Custer & Dini-Andreote, 2022), thus leading to an elevated dependence of plants on their mycorrhizal counterparts (Dini-Andreote, 2025).

Most traditional annual row cropping systems are not resilient to changing environmental conditions (Su et al., 2025; Mondal et al., 2023; Abdalla et al., 2023), and this has driven the need for alternative cropping systems to maintain agroecosystem and economic productivity. Against this backdrop, switchgrass (*Panicum virgatum*) presents itself as a valuable alternative for marginal lands that are no longer suitable for food and feed production (Fan et al., 2020). Switchgrass is a deeply rooted, perennial grass widely cultivated for forage and as a potential bioenergy crop, thus promoting sustained productivity on degraded lands, the sequestration of carbon in the soil profile, and an array of benefits and ecosystem services (Bai et al., 2022). While previous studies have documented switchgrass-AMF relationships and their importance for plant growth, nutrient acquisition, and drought or salinity tolerance (Basyal & Walker, 2023), little is known about how aboveground management practices, such as harvest frequency, shape AMF community composition at the root-soil interface (Hestrin et al., 2021). To address this knowledge gap, we set out to examine the influence of aboveground biomass harvest frequency on AMF communities across soil depths and three belowground compartments (e.g., root, rhizosphere, and bulk soil) in a switchgrass bioenergy system. We hypothesized that, analogous to patterns observed under varying grazing intensities (Faghihinia et al., 2020; Dudinszky et al., 2019), the most intensive harvest treatment would result in the lowest AMF diversity, whereas moderate harvest frequency would yield the highest AMF diversity since removal of moderate aboveground biomass can stimulate belowground carbon allocation and potentially enhance carbon availability to AMF (Faghihinia et al., 2020).

To address this hypothesis, we implemented a randomized block design with three switchgrass harvest treatments: 3-cut (harvests in June, August, and October), 2-cut (harvests in August and October), and a 1-cut (harvest in October). These harvest regimes reflect realistic

management options available to switchgrass bioenergy producers (Rivera-Chacon et al., 2023; Collins et al., 2010). Switchgrass var. 'Alamo' was planted in May 2023, and the harvest treatments began June 2024 (~ one-year post planting). In November 2024, after one season of harvest treatments, soil and root samples were collected to a depth of 60 cm and separated into four distinct depth horizons (e.g., 0–10 cm, 10–20 cm, 20–30 cm, and 30–60 cm).

Bulk soil, rhizosphere, and root compartments were then separated within each depth and DNA was extracted from each subsample using Qiagen Power Soil for bulk soil and rhizosphere samples and the PowerPlant kits for root samples. Extracted DNA was amplified using the AMF-specific primer pair NS31f_il and AML2r_il (Morgan et al., 2017), and sequenced on the Illumina MiSeq platform (2×250 bp). Sequence data were demultiplexed and processed using dada2 (v1.16) (Callahan et al., 2016) and Phyloseq in R (v4.5.2) (R Core Team, 2020). Taxonomy was assigned using the MaarjAM database (Öpik et al., 2010). The effect of harvest frequency and depth on AMF diversity was assessed using analysis of variance (ANOVA) and permutational multivariate analysis of variance (PERMANOVA). To understand AMF taxa that may account for compositional differences among the treatment frequencies, we implemented the Boruta algorithm (Kursa & Rudnicki, 2010). Please see supplemental materials within this chapter for a full description of the experimental design and methods.

To understand the influence of harvest frequency on AMF diversity we examined Shannon diversity (H') and observed amplicon sequence variant (ASV) richness across soil depths and compartments. We show that the harvest frequency did not significantly affect observed AMF richness in any of the compartments (e.g. bulk soil, rhizosphere soil, and root). However, a significant effect of harvest frequency on Shannon diversity was observed in the rhizosphere soil ($p \leq 0.05$). Contrary to our hypothesis, the treatment with the lowest H' was the

2-cut treatment (2.915) and the 1-cut and 3-cut had similar H' (3.359 and 3.246, respectively). Statistical pairwise differences were observed in only the 0-10 cm depth between the 1 and 2-cut treatments ($p \leq 0.01$) and the 1-cut and 3-cut treatment ($p \leq 0.05$), while other pairwise comparisons showed no statistical difference (Figure 2.1). The impact of harvest frequency on Shannon diversity was detected only in the rhizosphere, likely because this zone is highly dynamic and is directly influenced by root exudation and carbon allocation (Faghihinia et al., 2020; Dai et al., 2016), both of which are sensitive to aboveground harvest and disturbance. The lack of impact on the bulk soil and root compartments indicate that over short periods, additional cutting and harvest does not suppress AMF α-diversity in switchgrass systems.

In contrast, we report decreased α-diversity as depth increased, and this trend was observed within each compartment (e.g., bulk soil, $p \leq 0.001$; rhizosphere soil, $p \leq 0.001$; and root, $p \leq 0.001$) (Figure 2.1). Previous studies have demonstrated important edaphic factors that change across soil depth, such as pH, moisture content, and organic matter, which strongly influence soil fungal communities. As expected, soil depth thus influenced observed richness and Shannon diversity (Ko et al., 2017) in our system.

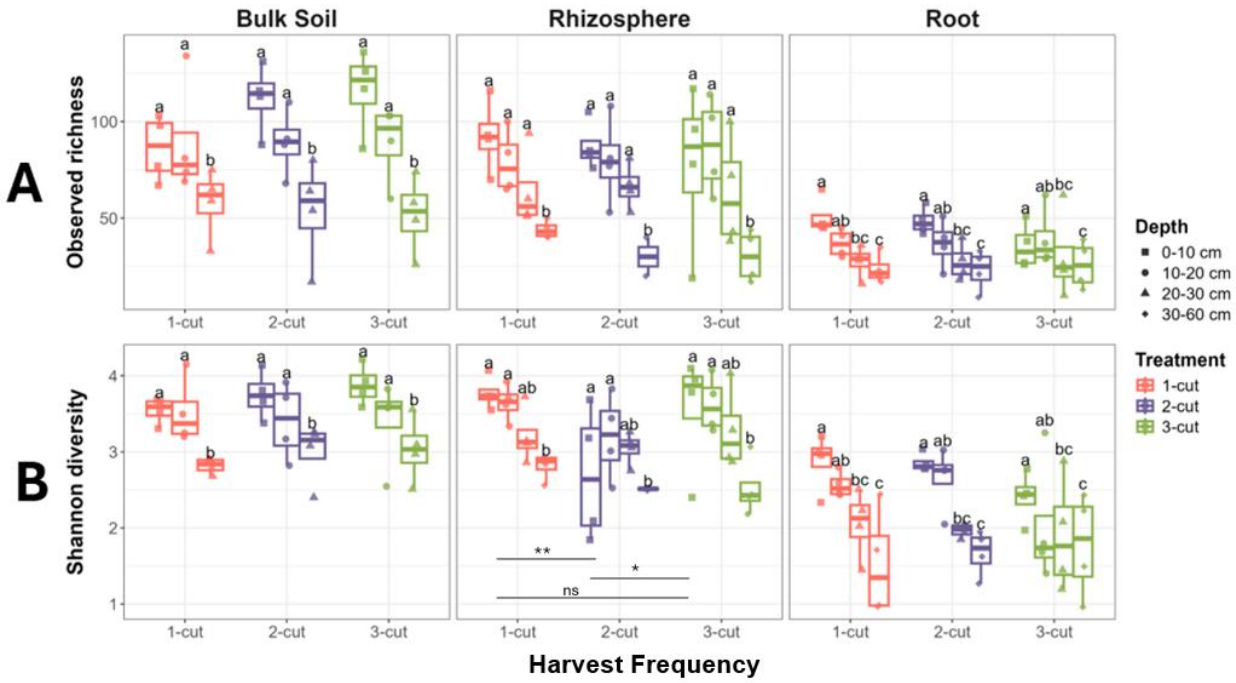
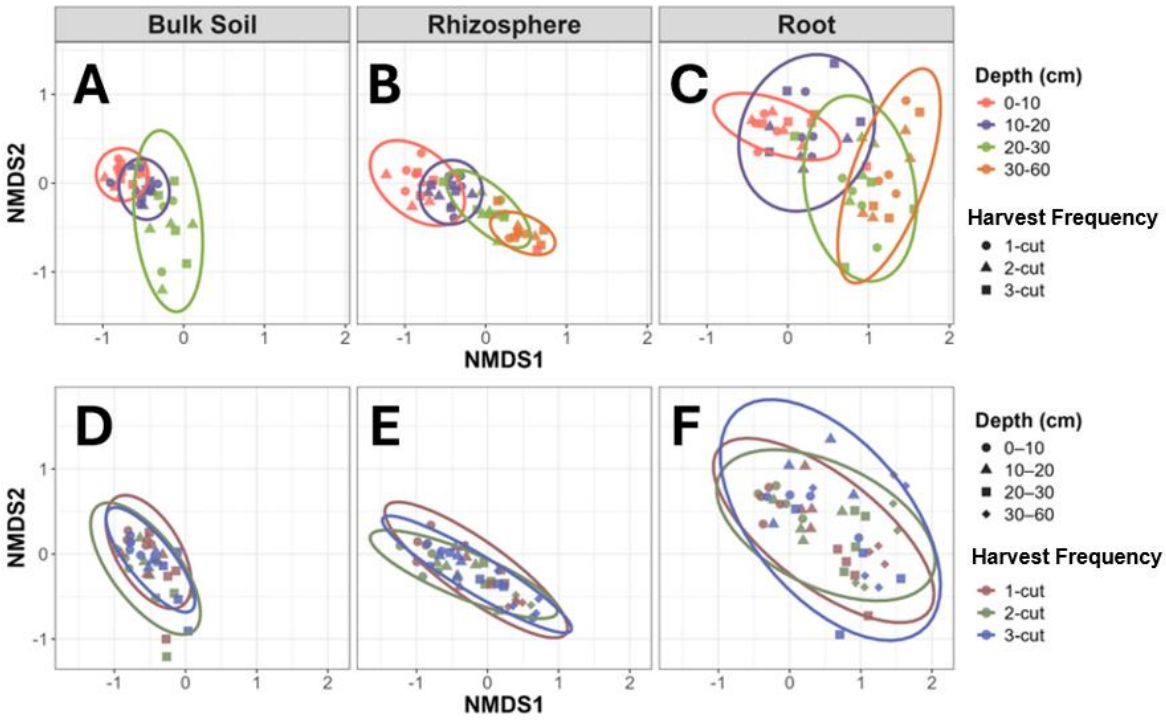


Figure 2.1*. Alpha diversity of AMF communities across treatment and soil depths. (A) Observed richness and (B) Shannon diversity are shown for three compartments: bulk soil, rhizosphere, and root under harvest frequencies of 1-cut, 2-cut, and 3-cuts. Letters above the boxes indicate significant differences between depths within each compartment, as determined by a one-way ANOVA followed by Tukey's Honest Significant Difference (HSD) test. Groups sharing at least one letter are not significantly different. In the rhizosphere section of panel B, asterisk (*) indicates significance between harvest frequencies in the 0–10cm depth at *p < 0.05 and **p < 0.01.*

Next, we examined the influence of harvest frequency on AMF community composition using pairwise Bray-Curtis dissimilarities (i.e., β-diversity). Beta diversity differed significantly among harvest frequencies in both the bulk ($p \leq 0.05$) and rhizosphere soils ($p \leq 0.01$) (Figure 2.2D & 2.2E). Results from pairwise comparisons in community composition within each soil compartment, with depth included as a blocking factor, showed significant differences among all treatment pairs in the bulk and rhizosphere soil ($p \leq 0.001$). In root samples, only the comparison between the 1-cut and 2-cut treatments was significant ($p \leq 0.01$).

The discrepancy of significant shifts in β-diversity among treatments without corresponding changes in α-diversity among treatments in the bulk and rhizosphere soil suggests that community composition changed among treatments even though overall AMF species richness and within-sample Shannon diversity remained relatively stable. This change, or lack thereof, may be particularly important as we consider community structure-to-function relationships in soil AMF communities and the functional similarity of replaced taxa (Eisenhauer et al., 2023). Should α-diversity remain unchanged and β-diversity shift, it is plausible that the compositional shift in taxa includes new taxa with differing functional traits. The introduction of new functional traits may have cascading impacts on critical ecosystem functions and benefits (e.g., soil carbon storage), underscoring the need to fully appreciate these delicate relationships in plant-soil systems, especially amid agricultural intensification or other environmental stresses. In consensus with the α-diversity, β-diversity differed significantly among depths in all three compartments (Figure 2.2A, 2.2B, and 2.2C). Please see the supplemental material within this chapter for a full description of the analysis and results.



	P-value	F-value	R2	P-value	F-value	R2	P-value	F-value	R2
Depth	0.001	4.796	0.224	0.001	5.600	0.275	0.002	2.517	0.149
Treatment	0.019	1.777	0.082	0.006	2.304	0.075	0.357	1.065	0.042

Figure 2.2*. Non-metric multidimensional scaling (NMDS) of fungal community composition in three compartments: bulk soil, rhizosphere soil, and root. Panels A-C are colored by depth, with hulls highlighting depth groupings, illustrating that community composition varies across depths. D-F panels are color-coded by treatment to show shifts in community structure associated with each harvest frequency. A PERMANOVA table below the NMDS plot summarizes the adonis2 model used to test the effects of harvest frequency on community composition, reported in the table below the figure.*

Despite 501 unique ASVs in the bulk soil, 541 ASVs in the rhizosphere, and 475 ASVs in the root, the Boruta feature selection identified only one genus per compartment as a statistically relevant predictor of treatment differences. In root and bulk soil, *Clariodeoglomus* was the lone taxa associated with the different harvest frequencies, while in the rhizosphere, it was the genus *Glomus.* For further information on the primary taxa driving the treatment

differences and relative abundance of each taxon, please see the supplemental materials within this chapter.

We show that after a single year of contrasting harvesting frequencies, small but detectable and statistically significant shifts occurred in AMF diversity and community structure, especially in the rhizosphere, highlighting the importance of aboveground management decisions and the influence they have on AMF communities. Additional research focused on linking harvest regimes to belowground functional profiles and carbon storage is recommended to develop and implement informed agricultural management decisions aimed at sequestering carbon and fostering diverse microbiomes in a changing world.

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Chapter 2 Supplemental Material

Materials and Methods

Study site and experimental design

This research was conducted at the University of Maryland Lower Eastern Shore Research and Education Center (LESREC) near Quantico, MD in Wicomico Co. (38.3549° N, −75.7768°W: 3.6 meters above sea-level). The soils are classified as Typic Hapludults and mean annual rainfall is 1016 mm and mean annual temperature is 12°C.

Switchgrass (*Panicum virgatum)* was planted in a randomized complete block design in May 2023. The experiment consisted of three harvest frequency treatments, with four replicates per treatment (total of 12 plots). Plots were 9 m wide by 15 m long and planted with the 'Alamo' cultivar using a no-till seed drill (John Deere 1590) at a seeding rate of 7.1 lb $acre^{-1}$. After the first season of establishment, all plots were mowed in November 2023 with the residue left on the field. In 2024, the three harvest frequencies were employed: three harvests ("cuts") per season (3-cut), two cuts (2-cut), and a single cut (1-cut). Harvest was conducted with an RCI 36a small plot harvester set at a 15 cm cut height. The 3-cut harvest plots were harvested in June, August, and October. The 2-cut harvests were conducted in August and October. The 1-cut harvest was in October. The interval between each harvest was an average of 1677 year-to-date growing degree days (YTD GDDs).

Sample collection and processing

In November 2024, after one season of implementing the harvest frequency treatments, bulk soil, rhizosphere soil, and root samples were collected using a 6.35 cm diameter stainless steel corer. Samples were collected at stratified depths (0–10, 10–20, 20-–30, and 30–60 cm) in

three locations per plot, about 5 cm from the base of a switchgrass plant. Plot samples for each depth were separated in the field and composited in a labeled gallon Ziplock bag. Samples were transported on ice to the University of Maryland Eastern Shore (UMES) for further processing.

Each Ziplock bag contained bulk soil, roots, and rhizosphere soil. Roots were separated from bulk soil by hand using ethanol and flame sterilized forceps, retaining a thin layer of soil (< 2 mm). The roots and rhizosphere soils were transferred to a sterile 50 mL centrifuge tube and submerged in chilled (4°C) 1× phosphate buffered saline (PBS; pH 7.4). Samples were vortexed for 15 seconds and sonicated in an ultrasonic bath (Bransonic 220) for 10 minutes at 4°C with pulsed cycles (160 W, 30s on/off) to dislodge adhering soil. Using sterilized forceps, the roots were transferred to a new clean tube and the PBS solution containing the rhizosphere soil was set aside for further processing. In the clean tube the roots were washed twice with ~20 mL chilled (4°C) sterile water by vigorously shaking for 15–30 seconds per wash. The wash liquid was drained and the roots were flash-frozen in liquid nitrogen and finely ground using a ceramic mortar and pestle. The remaining PBS solution containing the rhizosphere soil was centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was decanted and the soil pellet was stored at –80°C until DNA extraction. The remaining bulk soil was sieved (2 mm mesh), aliquoted into 50 ml tubes, and stored at –80°C until DNA extraction. DNA was extracted from 250 mg of bulk soil and rhizosphere samples separately using the Qiagen DNeasy PowerLyzer PowerSoil Kit following the manufacturer's protocol. Root DNA was extracted from 100 mg of the ground tissue using the Qiagen DNeasy Plant Pro Kit following the manufacturer's protocol. DNA extracts were stored in a microcentrifuge tube and kept at –20°C until amplification and library preparation.

DNA Quality Control and Processing

Total genomic DNA of 132 samples were sent to Oak Ridge National Laboratory (ORNL). At ORNL, DNA concentration was quantified using a Qubit fluorometer (Invitrogen, USA). DNA extracts were normalized to a concentration of 6.66 ng μL^{-1} where possible, with the intent of using 50 ng of DNA per PCR reaction. Samples with DNA concentrations below 6.66 ng μL^{-1} were used without further dilution. All DNA samples were stored at −20°C until downstream processing.

PCR Amplification of AMF SSU rRNA Gene

Arbuscular mycorrhizal fungal (AMF) communities were characterized by amplification of a fragment of the small subunit (SSU) rRNA gene using the AMF-specific primer pair NS31f_il (Morgan et al., 2017) (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTGGAGGGCAAGTCTGGTGCC-3′) and AML2r_il (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAACCCAAACACTTTGGTTTCC-3′), which included Illumina overhang adapters.

PCR reactions were performed in a total volume of 25 µL containing 7.5 µL of template DNA, 12.5 µL of Q5® High-Fidelity 2× Master Mix (New England Biolabs), 0.08 µM of each primer, and nuclease-free water. Thermal cycling conditions consisted of an initial denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 63.1°C for 30 s, and extension at 72°C for 60 s; followed by a final extension at 72°C for 1 min. No-template negative controls were included in all PCR runs to monitor potential contamination.

Library Preparation and Illumina Sequencing

PCR products were visualized by agarose gel electrophoresis and purified using AMPure XP magnetic beads (Beckman Coulter) following the Illumina protocol. Dual-index sequencing adapters were added during a second limited-cycle PCR following Illumina's standard library preparation protocol. Resulting libraries were quantified, normalized, and pooled in equimolar concentrations prior to sequencing.

Sequencing was performed on an Illumina MiSeq platform using 2 × 250 bp paired-end chemistry at ORNL. PhiX control DNA was spiked into the sequencing run at 20% to increase sequence diversity. The metagenomic workflow from Illumina automatically demultiplex's the samples, resulting in .fastq files without adapters or primers.

Sequence data processing

Sequenced data was processed in R v4.5.2 using the dada2 pipeline (v1.16) (Callahan et al., 2016). First, the quality of the reads was inspected. Given that the AMF amplicon (expected total length ~600 bp), paired-end reads could not be reliably merged, only forward reads were retained for downstream analyses. The forward reads were filtered using filterAndTrim() function with the following parameters: trimLeft = 21, maxN = 0, maxEE = 2, truncQ = 10, and rm.phix = TRUE. Next, we used the learnErrors function to determine the frequency of sequencing errors and errors were corrected using the dada2 function. A sequence table was constructed from de-noised forward reads and amplicon sequence variants (ASVs) were further filtered to retain sequences with lengths between 227 and 230 bp. Chimeras were removed using the "consensus" method. Taxonomic assignment of the non-chimeric ASVs was performed using a dada2 formatted MaarjAM reference database (Öpik et al., 2010).

Statistical analysis of microbiome data

The tables produced by the dada2 pipeline were imported into Phyloseq for statistical analysis (McMurdie et al., 2013). Sample metadata were constructed by parsing sample filenames to extract compartments (bulk soil, rhizosphere, and or root), treatment, and depth, which were compiled into a sample metadata table and merged with the ASV table and taxonomy table. Taxonomic rank names were standardized to Class, Order, Family, Genus, and Species, and ASVs lacking class-level assignments were removed along with ASVs with zero counts across all samples. An iterative rarefaction approach was applied following the recommendations of Schloss (2024). The ASV table was rarefied to 4,000 reads per sample across 100 permutations. The rarefied dataset consisted of 130 samples with two samples failing to sequence.

Alpha-diversity

Alpha diversity was quantified as observed richness (i.e., the number of unique ASVs observed in each sample) and Shannon diversity (H`) using the estimate_richness function in Phyloseq. Diversity metrics were analyzed separately for bulk soil, rhizosphere, and root compartments. Prior to testing, assumptions of normality were evaluated using Levene's test (Levene et al., 1960). Differences in α- diversity among depths and treatments were assessed using analysis of variance (ANOVA), and the normality of residual error was assessed visually. When ANOVA results were statistically significant ($\alpha = 0.05$), pairwise comparisons were performed using Tukey's honestly significant differences (HSD) tests at $\alpha = 0.05$. Results were visualized in ggplot2 (Wickham et al., 2016) using boxplots with individual sample points overlaid and letters added to show significant statistical differences.

Beta-diversity

AMF community composition was analyzed using permutational multivariate analysis of variance (PERMANOVA) based on pairwise Bray-Curtis dissimilarities. This test was implemented using the adonis2 function in the vegan package. Analysis was conducted separately for bulk soil, rhizosphere, and roots compartments. In the first model, the effects of treatment, depth, and their interaction on community composition were tested (dist ~ treatment * depth). In the second model, the effect of treatment was evaluated while controlling for depth, and the permutations for the treatment effect were restricted to each depth independently (dist ~ treatment, strata = depth).

Identification of treatment associated taxa

To identify fungal taxa strongly associated with harvest treatment, we applied the Boruta algorithm, a random forest-based method that evaluates the relative importance of each taxon (Kursa et al., 2010). Analysis was performed separately for each soil compartment (e.g., bulk, rhizosphere, root) and at both the genus and species level. Prior to modeling, ASVs sharing the same taxonomic classification were merged using tax_glom function at both the genus and species level. Taxa tables from the Phyloseq objects were used as input, with treatment as the grouping variable. Boruta iteratively compares the importance of each taxon to randomized "shadow" features generated by permuting the original data to establish a noise baseline (Kursa et al., 2010). The model was constructed using 1,000 trees (ntree = 1000) and the procedure was repeated for up to 1,000 iterations (maxRuns = 1000). For each taxon, importance scores were averaged across runs, and taxa whose importance exceeded the shadow's features were classified as informative. These taxa were subsetted from the original Phyloseq object for downstream analyses and visualization. Heatmaps of Boruta-selected genera for bulk soil and rhizosphere soil

were generated to visualize relative abundance across samples using a standardized z-score transformation color scale.

Results

Alpha-diversity

Observed richness differed significantly between depths within each compartment (ANOVA: bulk soil, $p \leq 0.001$; rhizosphere, $p \leq 0.001$; root, $p \leq 0.001$). Alpha-diversity was highest in the topsoil and generally decreased with depth. There was no significant difference among harvest treatments within each compartment. Shannon diversity differed significantly between depths within each compartment (ANOVA: bulk soil, $p \leq 0.001$; rhizosphere, $p \leq 0.001$; roots, $p \leq 0.001$). No significant differences in Shannon diversity were observed between treatments in bulk soil or roots. In contrast, Shannon diversity in the rhizosphere was slightly, but significantly affected by treatment ($p = 0.044$). Shannon diversity was lowest in the 2-cut treatment and highest in the 1-cut treatment.

Beta-diversity

In both PERMANOVA models, β-diversity differed significantly among treatments for bulk soil ($R^2 = 0.083$, F-value = 1.772, $p = 0.019$, $df = 2$) and rhizosphere communities ($R^2 = 0.075$, F-value = 2.303, $p = 0.006$, $df = 2$), while no significant treatment effect was detected in root communities. Depth, however, had a significant impact on β-diversity across bulk soil, rhizosphere soil, and root samples ($p = 0.001$, $p = 0.001$, and $p = 0.002$, respectively).

Boruta analysis

Boruta identified a small set of taxa that were associated with harvest frequency within each compartment. At the species level, 1 species was selected in rhizosphere soil, 2 in the root, and 3 in the bulk soil. The taxa identified in the rhizosphere soil was Glo3b, which has previously been identified, according to the MaarjAM database (Öpik et al., 2010), in the roots of a *Tetragastris panamensis*, an evergreen canopy tree, in Barro Colorado Island. The rhizosphere had ORVINGLO4, MO-G17, and IH1 (each previously noted only once before in a vineyard, borneo-nemoral forest, and alvar grassland, respectively). The root had MO-G8 and MO-G56. MO-G8 had each previously been noted on MaarjAM database once before in a temperate grassland in Estonia and MO-G56 on 3 occasions, all in a subtropical forest or grassland in South Africa, Australia, and French Guiana.

On the genus level, *Glomus* was selected for the rhizosphere and *Clarioreglomus* for the root and bulk soil. The small number of taxa identified by Boruta is not surprising given that only one year of harvest frequency treatments were implemented, therefore only very sensitive taxa would likely respond. The limited sample size may also further obfuscate trends in differentially abundant taxa due to the heterogeneous nature of soil microbial communities.

Taxa abundance

We observed *Glomus, Paraglomus*, and *Claroideoglomus* as the most abundant taxa across all treatments in the bulk soil. The top three most abundant taxa in the rhizosphere were *Glomus, Diversipora*, and *Paraglomus* in the 1-cut and 2-cut treatments while *Glomus, Claroideoglomus*, and *Diversipora* were the most abundant in the 3-cut rhizosphere. In the root, the 1-cut had *Glomus*, *Claroideoglomus*, and *Paraglomus* as the top three most abundant and, in

the 2-cut and 3-cut, the most abundant were *Glomus, Paraglomus*, and *Claroideoglomus*, respectively. These results align with a previous study that found all three of these taxa in the community structure of AMF (Dirks & Jackson, 2020). Additionally, a study exploring the communities in corn, switchgrass, and prairie cropping, *Paraglomus* was one of only two genera detected in the switchgrass sites (Jesus et al., 2016). *Glomus,* and other genera in the *Glomeraceae* have high growth rates and are tolerant of harsh conditions. This trait may explain why *Glomeraceae* increases with depth (Figure S2.1). Interestingly, *Gigasporaceae*, which is present in very low abundance, is known to be intolerant to disturbances such as grazing and harvests (van der Heyde et al., 2017). *Gigasporaceae* was found almost exclusively in the roots (Figure S2.2), indicating potentially more tolerable conditions for this family which requires time and high resource investment into generating (Heyde et al., 2017).

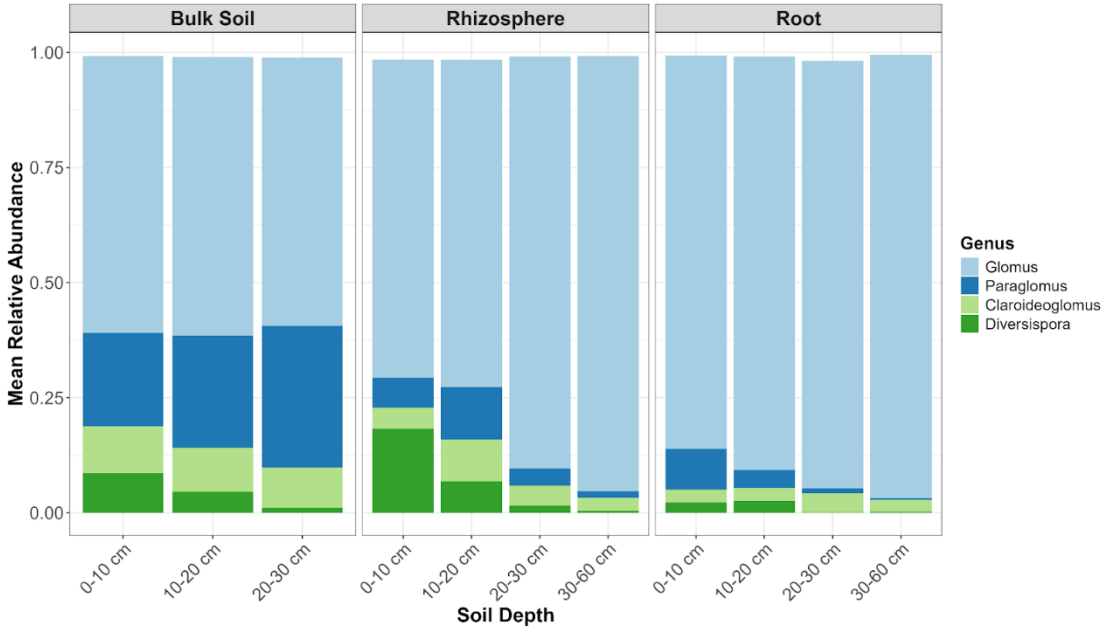

Figure S2.1. *Mean relative abundance of each genera comparing depth by compartment.*

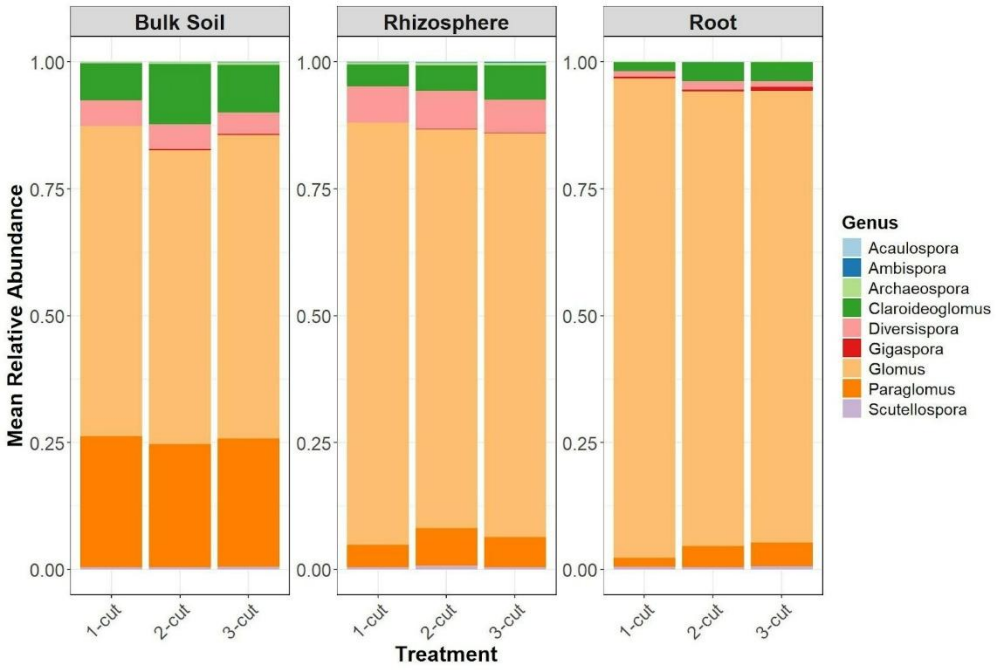

Figure S2.2. *Mean relative abundance of each genera comparing treatment by compartment.*

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Chapter 3: Arbuscular mycorrhizal fungi, harvest frequency, and their influence on soil aggregates and carbon in a switchgrass system

Abstract

Perennial grass systems, such as switchgrass (*Panicum virgatum*), have potential for sustainable biofuel production and soil carbon sequestration. Effective management of these systems depends on understanding how practices such as harvest frequency influence plant-microbe interactions, especially symbioses with arbuscular mycorrhizal fungi (AMF), which can promote soil carbon stabilization and aggregate formation. In this study, we evaluated three harvest frequencies commonly used in switchgrass bioenergy production to assess their effects on AMF colonization, soil aggregate size distribution, and total aggregate carbon. Root samples were stained and quantified for AMF colonization, and soils were fractionated into four aggregate size classes (large macroaggregates, small macroaggregates, microaggregates, and silt and clay) to determine carbon content. Harvest frequency significantly affected small macroaggregates (0.25 mm to 2 mm) in the 0-10 cm soil layer, with lower frequency harvests promoting greater aggregate formation. However, AMF colonization was not significantly associated with aggregate size classes or carbon content under any harvest frequency. These findings suggest that short-term harvest management can influence soil aggregation and carbon distribution, but longer-term studies and alternative methods for quantifying AMF may be necessary to fully capture the role of AMF in soil carbon stabilization in perennial bioenergy systems.

Introduction

Soil carbon sequestration is increasingly recognized as a natural climate mitigation strategy, capturing atmospheric carbon dioxide (CO_2) and storing it in soil organic carbon (SOC) pools (Rodrigues et al., 2023). Perennial bioenergy crops, such as switchgrass (*Panicum virgatum),* can enhance SOC over time due to its high biomass production and deep rooting systems. Further, if grown on marginal or degraded soils, switchgrass offers the potential to provide renewable energy feedstocks and soil carbon sequestration without displacing food and feed production (Kim et al., 2023). However, maximizing SOC sequestration in switchgrass production is complex, as aboveground management, including harvest frequency, influences belowground processes that govern how carbon is allocated, stabilized, and retained in soils (Lee et al., 2007; Faghihinia et al., 2023). Understanding these interactions is essential for balancing biomass production with SOC storage.

Like many perennial grasses, switchgrass relies on symbioses with arbuscular mycorrhizal fungi (AMF; phylum *Glomeromycota*) to acquire nutrients, primarily phosphorous, in exchange for photosynthetically-derived carbon (Wahab et al., 2023). Beyond nutrient uptake, AMF confers increased tolerance to drought, salinity, and temperature stress - factors particularly relevant under climate variability and marginal land conditions (Basyal & Walker, 2023; Boorboori & Lackóová., 2025; Emery et al., 2022; Kivlin et al., 2013). Furthermore, by directly contributing to mineral-associated organic matter (MAOM) and facilitating the formation of aggregates that physically protect soil organic matter (SOM), AMF also plays a critical role in SOC storage.

Globally, over 70% of terrestrial plants form associations with AMF, allocating an estimated 1.66 Gt CO_2 equivalents of herbaceous photosynthetic carbon to the fungal mycelium

(Hawkins et al., 2023). Isotope tracer studies indicate that, on average, herbaceous plants allocate 6.2% of assimilated carbon to AMF, with values reaching as high as 20% (Hawkins et al., 2023; Jansa & Treseder. 2017). Of the carbon allocated to AMF, up to 25% of the AMF-transferred carbon has been traced to the mineral-associated fraction (Kakouridis et al., 2024), highlighting one pathway by which AMF may contribute to long-term soil carbon storage. Of critical importance, the mineral-associated organic carbon (MAOC) fraction of soil organic matter (SOM) has slower turnover than other fractions due to the strength of organo-mineral bonds (Cotrufo et al., 2013) and is therefore desirable when managing for long-term soil carbon storage.

Numerous studies and reviews have proposed additional mechanisms by which AMF contribute to SOC stabilization (See at al., 2022; Shukla et al., 2025; Huang et al., 2021). Across these studies, there is consensus that AMF hyphae physically enmesh organic matter and soil particles to form aggregates, providing protection to all forms of carbon including MAOC (Shukla et al., 2025).

Previous studies have demonstrated that there is a positive relationship between AMF and the abundance of microaggregates and macroaggregates (Morris et al., 2019, de Goede et al., 2025; Rillig & Mummey, 2006). Factors that influence the abundance of mycorrhizae in agricultural systems, and therefore their ability to contribute to soil aggregation, are wide-ranging. Some of these factors include fertilization (Mader et al., 2000), tillage (Sale et al., 2015), and grazing (Heyde et al., 2017). For example, tillage mechanically destroys the hyphal network, and fertilization can reduce the need for symbiosis by providing readily available nutrients, potentially weakening the AMF-plant relationships (Mader et al., 2000). However,

comparatively little is known about how harvest frequency influences this association and what frequency of harvest affects the AMF-plant relationship most dramatically.

Harvesting switchgrass for feedstock biomass is a disturbance analogous to that of herbivory (minus animal excrement), since the removal of aboveground biomass reduces residue inputs and alters resource allocation between shoots and roots (Kering et al., 2024). Belowground carbon dynamics are particularly relevant for AMF, whose abundance and activity depend on host-derived carbon. By influencing belowground carbon allocation, harvest frequency has the potential to shape AMF populations and their contributions to soil aggregation and carbon cycling (Vályi et al., 2014). Framing this within the intermediate disturbance hypothesis (Moi et al., 2020), moderate harvest frequencies may enhance ecosystem function by stimulating regrowth and maintaining productive interactions with soil microbiota, while very frequent or infrequent harvests can constrain productivity and ecosystem function.

Despite the potentially important relationship among AMF and soil carbon stabilization, few studies have explicitly examined the role of AMF in mediating carbon sequestration under different harvest regimes or identified which soil aggregate size classes are most responsive to management. The primary objective of this study was therefore to determine how three different aboveground harvest frequencies (e.g., 3-cut, 2-cut, or 1-cut) affect AMF root colonization, and SOC distribution among aggregate size classes, and to understand the role AMF may have in mediating carbon accumulation within aggregate size classes. We hypothesize that (1) the 2-cut harvest frequency will sustain higher AMF colonization, (2) the 2-cut harvest frequency will promote more macroaggregates (large and small) and greater SOC retention and (3) plants with higher AMF colonization will have a positive influence on SOC across aggregate size classes.

Methods

Study site and experimental design

This research was conducted at the University of Maryland Lower Eastern Shore Research and Education Center (LESREC) near Quantico, MD in Wicomico Co. (38.3549° N, −75.7768°W: 3.6 meters above sea-level). The soils are classified as Typic Hapludults and mean annual rainfall is 1016 mm and mean annual temperature is 12°C.

Switchgrass (*Panicum virgatum)* was planted in a randomized complete block design in May 2023. The experiment consisted of three harvest frequency treatments, with four replicates per treatment (total of 12 plots). Plots were 9 m wide by 15 m long and planted with the 'Alamo' switchgrass cultivar using a John Deere 1590 no-till seed drill at a seeding rate of 7.1 lb $acre^{-1}$. After the first season of establishment, switchgrass was mowed in November 2023 and the residue was left on the plots. In 2024, the three harvest frequencies were employed: three harvests per season (3-cut), two harvests (2-cut), and a single harvest (1-cut). Each cutting was conducted with an RCI 36a small plot harvester set at a 15-cm cut height. The 3-cut harvest plots were harvested in June, August, and October. The 2-cut harvests were conducted in August and October. The 1-cut harvest was in October. The interval between each harvest was an average of 1677 year-to-date growing degree days (YTD GDDs).

Soil Sampling and Processing

Bulk density cores were collected using the carving method one year prior to establishing the study site (Gradwell., 1972; Blake & Hartge, 1986). Three soil pits were excavated to a depth of 55 cm at the study location. At each pit, a 5-cm aluminum core (of a known weight) was inserted horizontally into the pit wall in increments of 0-10 cm, 10-20 cm, 20-30 cm, and 30-60. Cores were carefully extracted from the side of the pit using a hand trowel to ensure that the core

was completely filled with soil. Three replicate cores were collected at each depth increment and in each pit. Cores were wrapped in aluminum foil, labeled, and placed in Ziplock bags, then they were weighed before being placed in the oven. Cores were carefully unwrapped and placed in a pre-weighed aluminum weigh-boat. Cores were dried at 105 °C for 7 days and re-weighed. Dry weights were corrected by removing the weights of the ring and weigh-boat. As soils were removed from the rings, they were inspected for rocks. Rocks were weighed and the volume was determined by water displacement in a graduated cylinder. Corrected dry weights and core volume were used to calculate bulk density (g cm^{-3}). Bulk density values were then averaged across pits for each depth, and these mean values were used in subsequent soil carbon stock calculations.

Soil samples for fractionation and carbon analysis were collected in March 2025 using a 1-inch diameter probe to a depth of 60 cm. Within each plot, five cores were collected at randomized locations and stratified by depth (e.g., 0-10 cm, 10-20 cm, 20-30 cm, and 30-60 cm). Cores from the same depth within a plot were composited into a single sample, resulting in one composite sample for each depth per plot. Soils were transported back to the lab and stored in the cold room to retain field moisture, until further analysis. Within 48 hours, soils were sieved through a 4.75 mm screen to homogenize the sample and remove large rocks, debris, and roots. The homogenized soil was then air-dried at 27°C until further processing.

Random subsamples from each depth and block were tested for the presence of carbonates in the bulk soil. Approximately 2 g of soil were placed in a glass dish and treated with a few drops of 1 *M* HCl to assess bubble formation, indicative of carbonate content (Nelson and Sommers, 1996). No bubbles were observed, confirming the absence of carbonates.

Aggregate Size Class Fractionation

In soil organic matter (SOM) research, chemical extraction methods have been widely used. However, this approach has been shown to produce artificial products (humic substances) that do not accurately reflect natural SOM dynamics (Lehmann & Kleber, 2015). As a result, physical fractionation approaches based on density or particle size have become recognized as best practice (Lavellee et al., 2019). Soil aggregates form hierarchically; microaggregates (0.053 - 0.25 mm) arise from the binding of silt and clay particles (<0.053 mm), and macroaggregates (>0.25 mm) results from the aggregation of microaggregates (Six et al., 2000). Since this paper is specifically focused on AMF's role in aggregate formation as a pathway for SOC protection and therefore stabilization, we selected the aggregate size class fractionation method developed by Six et al., (2000).

A 100-g subsample of soil from each depth and plot was fractionated into four size classes using the wet sieving method developed by Six et al., (2000). Each sieve was placed in a wash bin and submerged with ultrapure deionized (DI) water to a depth of 2 cm above the mesh. The 100-g sample was gently poured directly onto the 2-mm sieve, taking care to ensure no splashing outside of the sieve walls. The sample was allowed to settle for 5 minutes to break apart any unstable soil aggregates. The sieve was then slaked at 25 beats per minute for 2 minutes. The soil retained on top of the 2 mm sieve at the end of the slaking process was classified as large macroaggregates (LM) and transferred using a lab wash bottle with ultrapure DI water to move the aggregates into a pre-weighed tin. The soil that passed through the 2 mm sieve was sequentially wet-sieved at 0.25 and 0.053 mm to isolate small macroaggregates (sM) and microaggregates (m), respectively. The <0.053 mm fraction was designated as silt and clay (sc). Each fraction was transferred from the sieve to a pre-weighed, aluminum tin using ultrapure

DI water. Floating particulate organic matter (fPOM) was removed from LM and sM fractions prior to drying and weighed.

All fractions were air-dried, then oven-dried at 40°C until constant mass was achieved. Final dry mass was recorded, and the pre-weighed tin weights were subtracted from the final weight. Then, the dried soil from each tin was scraped into coin envelopes, and the mass was recorded again to account for any loss during the transfer.

Sand Particle Analysis

Sand content (0.053 – 2 mm) in LM, sM, and m fractions was determined from a subsample by the pipette method (de la Reguera & Tully, 2021). In brief, organic matter was removed from a 2-g subsample by heating it in a hydrogen peroxide (H_2O_2) solution for 12 hrs. Then, the remaining material was placed in a Falcon tube with 2 ml of sodium hexametaphosphate and filled to the 50 ml line with ultrapure DI water. The tube was placed on a rapidly oscillating shaker for 30 mins (~160 rpm). Finally, the sample was wet sieved through 0.053-mm sieve to separate the sand from the silt and clay particles. The sand was dried and weighed. The remaining LM and sM fractions were ground with a mortar and pestle and sieved through a 0.25-mm screen to remove any remaining rocks or coarse fragments (>0.25 mm). These fragments were weighed and subtracted from the aggregate mass. Each fraction (LM, sM, m, sc, fPOM, and rocks) was transferred to a labeled coin envelope for long-term storage. The proportion of sand in the subsample was multiplied by the total aggregate mass (g) to determine the mass of sand (g) in the aggregate size class (Eq. 3.1). The sand-free aggregate mass was determined by subtracting the total aggregate mass (g) by the mass of sand in the aggregate (g) (Eq. 3.2). The aggregate proportion was calculated by dividing the sand free aggregate mass by the sum of all sand free aggregates mass in a given sample (Eq 3.3)

$$Sand\ mass\ per\ aggregate\ (g) = proportion\ of\ sand * aggregate \quad \text{(Eq. 3.1)}$$

$$Sand\ free\ aggregate\ mass\ (g)\ =\ aggregate\ mass - sand\ mass\ per\ aggregate \quad \text{(Eq. 3.2)}$$

$$Aggregate\ proportion = \frac{Sand\ free\ aggregate\ mass}{\sum Sand\ free\ aggregate\ mass\ of\ LM,sM,m,and\ sc\ of\ the\ given\ sample} \quad \text{(Eq. 3.3)}$$

Soil Carbon Analysis

All soil fractions from each sample that had >0.05 g of sample were analyzed using a Thermo Scientific FlashSmart equipped with a thermal conductivity detector (TCD) to quantify total soil carbon by elemental analysis. Given the absence of carbonates, measured carbon was interpreted as soil organic carbon (SOC). To determine the mass of carbon in each aggregate, the sand free aggregate mass was multiplied by the %C obtained from elemental analysis (Eq 3.4). Normalized carbon was calculated by dividing the total aggregate carbon by the sum of the total sand free aggregate carbon from all fractions in that plot and depth (Eq. 3.5). Normalized carbon allows us to compare the proportion of aggregate carbon within the soil.

$$Total\ aggregate\ carbon = \frac{sand\ free\ aggregate\ mass * \%C}{100} \quad \text{(Eq. 3.4)}$$

$$Normalized\ aggregate\ carbon = \frac{Total\ aggregate\ carbon}{\sum Total\ aggregate\ carbon\ of\ LM,sM,m\ and\ sc\ in\ a\ given\ sample} \quad \text{(Eq. 3.5)}$$

To calculate the percent carbon of the bulk soil, we took the sum of the total aggregate carbon and divided it by the sum of the sand free aggregate mass of all fractions in that plot and depth (Eq 3.6). Soil carbon stock was calculated by multiplying the percent carbon of the bulk soil by the bulk density and the depth increment (Eq. 3.7).

$$Bulk\ C\% = \frac{mass\ of\ C\ in\ bulk\ soil}{mass\ of\ bulk\ soil} \quad \text{(Eq. 3.6)}$$

$$Soil\ Carbon\ Stock = Bulk\ C\% * bulk\ density\ (g\ cm^{-3}) * soil\ depth\ increment\ (cm) \quad \text{(Eq. 3.7)}$$

Root Sampling and Processing

Root samples were collected after one season of harvest treatments. Root samples were collected in November 2024, using a 6.35-cm stainless steel corer. Three cores (0-60 cm) were collected per plot at approximately 5 cm from the base of a switchgrass crown. All cores were stratified by depth (e.g., 0-10 cm, 10-20 cm, 20-30 cm, and 30-60) and composited per plot. Cores were transported back to the lab on ice, where roots within the cores were separated from the bulk soil using sterilized forceps, retaining a thin layer of soil (<2 mm). Roots were placed in 50 mL centrifuge tubes and submerged in chilled phosphate-buffered saline (PBS) solution. Tubes were then vortexed for 15 seconds and sonicated in a water bath at 4°C for 10 minutes with pulsed settings (160 W, 30 s on/off). Roots were subsequently transferred to clean 50 mL tubes using sterilized forceps and washed twice more with ~20 mL of chilled sterile water by vigorous hand-shaking for 15–30 seconds per wash (Simmons et al., 2018). After washing, the liquid was drained, and the roots were air-dried until further analysis.

A random sample of roots were selected for each depth and plot for AMF colonization analysis. This subsample of roots was cleared with 10% potassium hydroxide (KOH), rinsed three times with DI water to remove KOH, and stained with a 5% vinegar-ink solution using methods modified from Vierheilig et al. (1998). The roots were destained overnight in a 5% vinegar-water solution and cut into 1-cm segments. Ten random segments per sample were

mounted onto microscope slides for AMF root colonization analysis. Using the magnified intersect method, the percentage of root colonization was determined from 5 fields of view per segment (50 fields of view total per sample) under 100x magnification (Eq. 3.4) on an Olympus CX21 compound light microscope (McGonigle et al., 1990; Hu et al., 2020). If hyphae, vesicles, or arbuscules were present, that field of view received a note of 'presence' (Figure 3.1).

$$AMF\ Colonization\ (\%) = \frac{number\ of\ observations\ with\ AMF\ present}{number\ of\ observation} * 100 \quad \text{(Eq. 3.4)}$$

In addition to noting the presence or absence of AMF structures in each field of view, the intensity of colonization in each view was categorized into five classes. Level 1 (0-1%), level 2 (2-10%), level 3 (11-50%), level 4 (51-90%), level 5 (91-100%), and calculated using the following weighted formula created by Trouvelot et al., 1986.

$$Intensity\ (\%) = \frac{95n5+70n4+30n3+5n2+n1}{N} * 100 \quad \text{(Eq. 3.5)}$$

Where n5 = number of observations scored level 5; n4 = number of observations scored level 4; n3 = number of observations scored level 3; n2 = number of observations scored level 2; and n1 = number of observations scored level 1. N stands for the total number of observations (Eq. 3.5). For example, if 3 observations were classified at level 4, the intensity for that level would be calculated as 70 x 4 = 140. Intensities for each level are summed, divided by the total number of observations (N), and multiplied by 100.

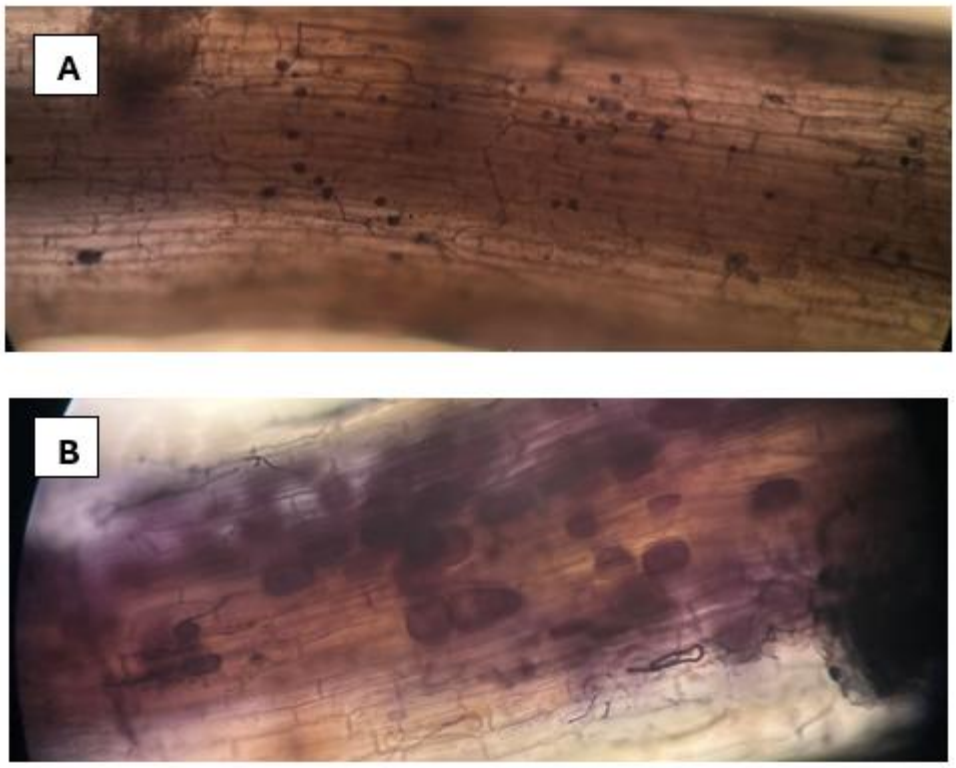

Figure 3.1. *Photos of AMF structures under the microscope at 40x magnification. (A) vesicles and hyphae, (B) vesicles.*

Statistical Analysis

Linear mixed-effect (LME) models were used to evaluate the effects of harvest frequency, depth, and their interaction on soil carbon pools, with block included as a random effect. Linear mixed-effects models were also used to assess the effects of harvest frequency, depth, and their interaction on each aggregate size distribution (%) and total aggregate carbon. Upon inspection, we found LM were rare, and thus this size class was excluded from models due to the low sample size. The other aggregate size classes (sM, m, and sc) were each modeled separately, using three separate LMEs. We assessed if data met model assumptions using diagnostic plots (residual vs. fitted and Q-Q plots), and Box-Cox transformations were applied when necessary to meet normality of residual error assumptions. Models were refitted with

transformed data as needed, and fixed effects were tested using ANOVA to obtain p-values for each model. Tukey Honestly Significant Difference (HSD) *post-hoc* comparisons were performed using the *emmeans* package in R to identify significant differences among treatments and depths. AMF colonization percentages were converted to proportions and logit-transformed prior to analysis to account for the bounded nature of the proportional data. Linear mixed effect models were then used to evaluate the effects of AMF colonization and treatment on total aggregate carbon in each size class, with block as a random effect, modeling each depth separately. Similar models were used to assess the effects of AMF colonization on aggregate size distributions, analyzing each size class and depth separately. Fixed effects were tested using ANOVA to obtain p-values and any significant effects were further investigated with Tukey HSD *post-hoc* comparisons. The same approach was applied using AMF intensity as the fixed effect. All statistical analyses were performed in R (v4.5.2) (R Core Team 2020) using the *lme4* package, and *emmeans* package.

Results

Harvest frequency and depth impact bulk soil carbon percent and stock

There were significant effects of harvest frequency ($p<0.001$), depth ($p<0.001$), and their interaction ($p<0.05$) on bulk soil carbon, indicating that the influence of harvest treatment on bulk soil carbon differed depending on depth. Specifically, at 0-10 and 10-20 cm depths, the 1-cut harvest frequency had higher percent carbon in the bulk soil than the 2-cut and 3-cut

treatments ($p<0.0001$ and $p<0.05$, respectively). The 1-cut also had a higher percent of carbon in the bulk soil than the 3-cut in the 20-30 cm depth ($p<0.05$).

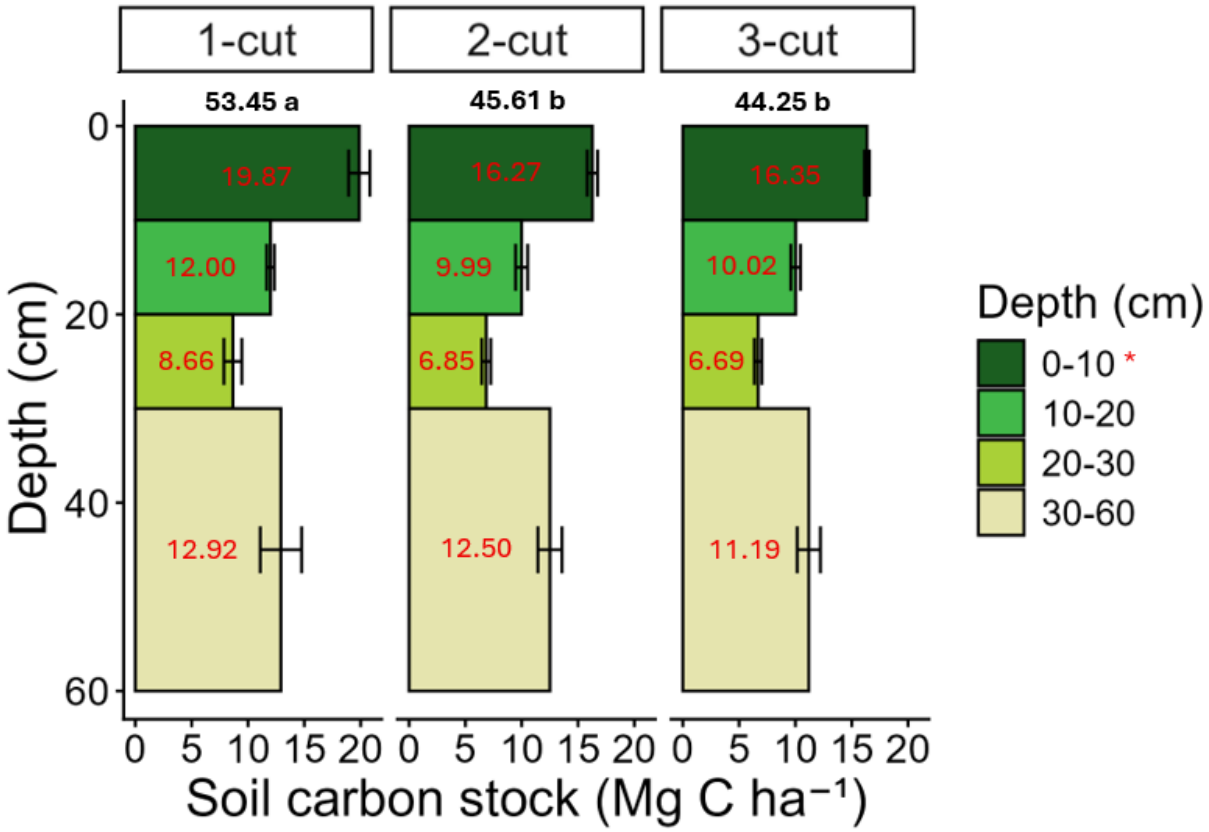

Figure 3.2. *Soil profile chart representing soil carbon stocks (Mg C ha^{-1}) in bulk soils (not fractionated by size class). Error bars are the standard error of the mean. The values above each soil profile represent the average total carbon stock for each harvest frequency. Letters represent statistically significant differences between harvest frequencies at $p < 0.05$ in Tukey's post hoc pairwise comparisons. Asterisks (*) in the legend indicate significance between harvest frequencies by depth at $p < 0.001$ in Tukey's post hoc pairwise comparison. Values in orange within each bar indicate the total soil carbon stock (mg C ha^{-1}) for the given depth increment.*

There was no significant interaction effect of depth and harvest frequency on total soil carbon stocks (Mg ha^{-1}). However, there were significant individual effects of harvest frequency and depth on total soil carbon stocks ($p<0.001$ in both cases). The 1-cut treatment had greater

carbon stocks than the 2-cut and 3-cut treatments ($p<0.01$ and $p\leq0.001$, respectively) (Figure 3.2). Soil carbon stocks were greatest in the 0-10 cm soil layer compared to the deeper depths ($p<0.001$).

Harvest frequency and depth impact on AMF colonization

AMF root colonization (%) ranged from 4% to 88% across all harvest frequencies and soil depths. There was no significant interaction between harvest frequency and depth on AMF colonization. We observed different levels of AMF colonization across soil depths ($p\leq0.001$) with the highest AMF colonization in the 0-10 cm depth compared to all other depths ($p\leq0.01$ in all cases) (Figure 3.3). The average AMF colonization (%) in the 0-10 cm depth was 53.7% (±2.1%). Harvest frequency had no significant effect on AMF colonization.

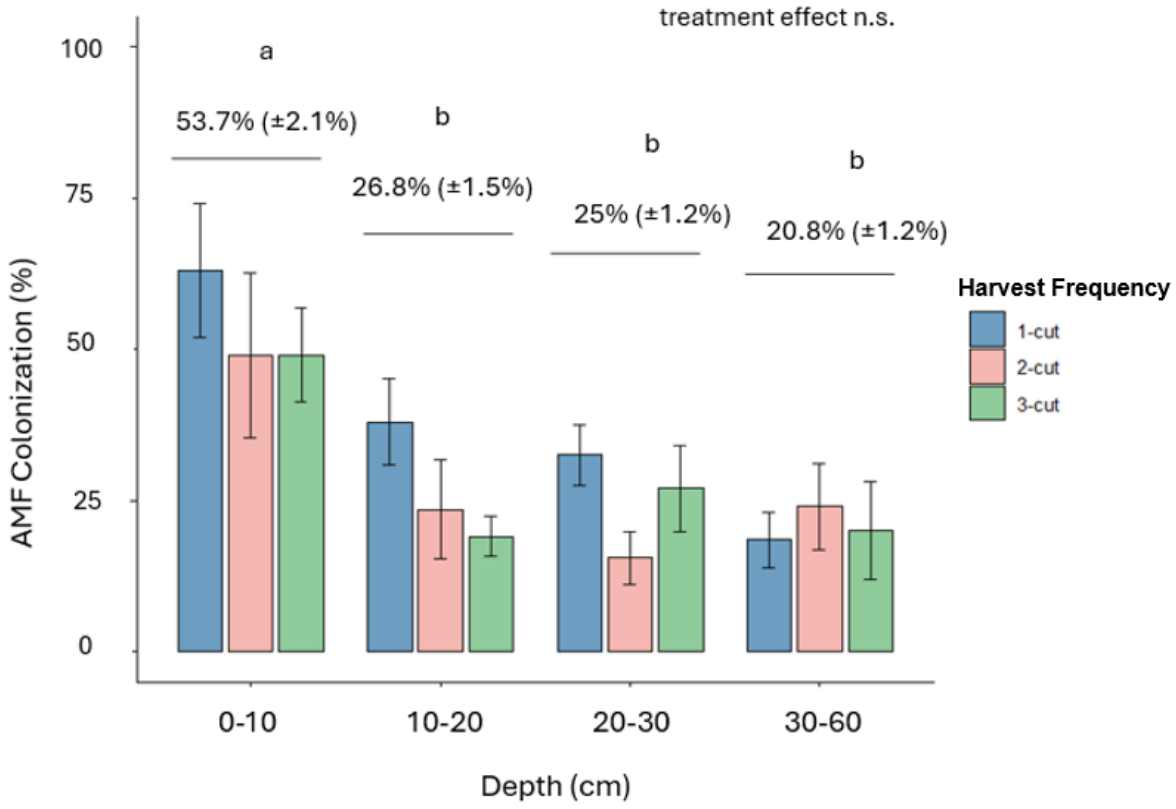

Figure 3.3. *AMF colonization (%) by depth, colored by harvest frequency. Letters above clusters of bars indicate that AMF colonization is statistically significantly different by depth at $p < 0.05$ in Tukey post hoc pairwise comparisons. Percentages above bars are the mean colonization (and standard deviation) per depth.*

Harvest frequency and depth impact on aggregate proportion

There was no significant interaction effect of harvest frequency and depth on aggregate proportion (Eq. 3.3) in any size aggregate class (Figure 3.4). We found a significant independent effect of harvest frequency on the proportion of small macroaggregates and microaggregates ($p<0.01$ in both cases). The 1-cut harvest had a higher proportion of small macroaggregates compared to the 2-cut and 3-cut harvests ($p<0.05$ for both). On the other hand, the 1-cut harvest had a smaller proportion of microaggregates compared to the 2-cut harvest ($p<0.01$).

We found a significant effect of depth on the proportion of all small macroaggregates, microaggregates and silt and clay. As previously mentioned, we did not have a large enough

sample size to examine large macroaggregates. The proportion of small macroaggregates decreased with soil depth. Within the small macroaggregates class, the greater proportion of small macroaggregates in the 0-10 cm depth relative to the 10-20 cm was significant ($p<0.01$), while the greater proportion of small macroaggregates in the 0-10 cm depth relative to the remaining depths were highly significant ($p<0.001$). Depth had the opposite effect on microaggregates, with each successive depth having a greater proportion of microaggregates (Figure 3.4). The proportion of microaggregates in the 0-10 cm depth was significantly smaller compared to all other depths ($p<0.0001$). The proportion of microaggregates in the 10-20 cm depth as compared to the 20-30 cm and 30-60 cm was also smaller ($p<0.05$ and $p<0.001$, respectively). The 10-20 cm increment was characterized as having less silt and clay relative to the 20-30 and 30-60 cm depth ($p<0.05$ and $p\leq0.001$, respectively).

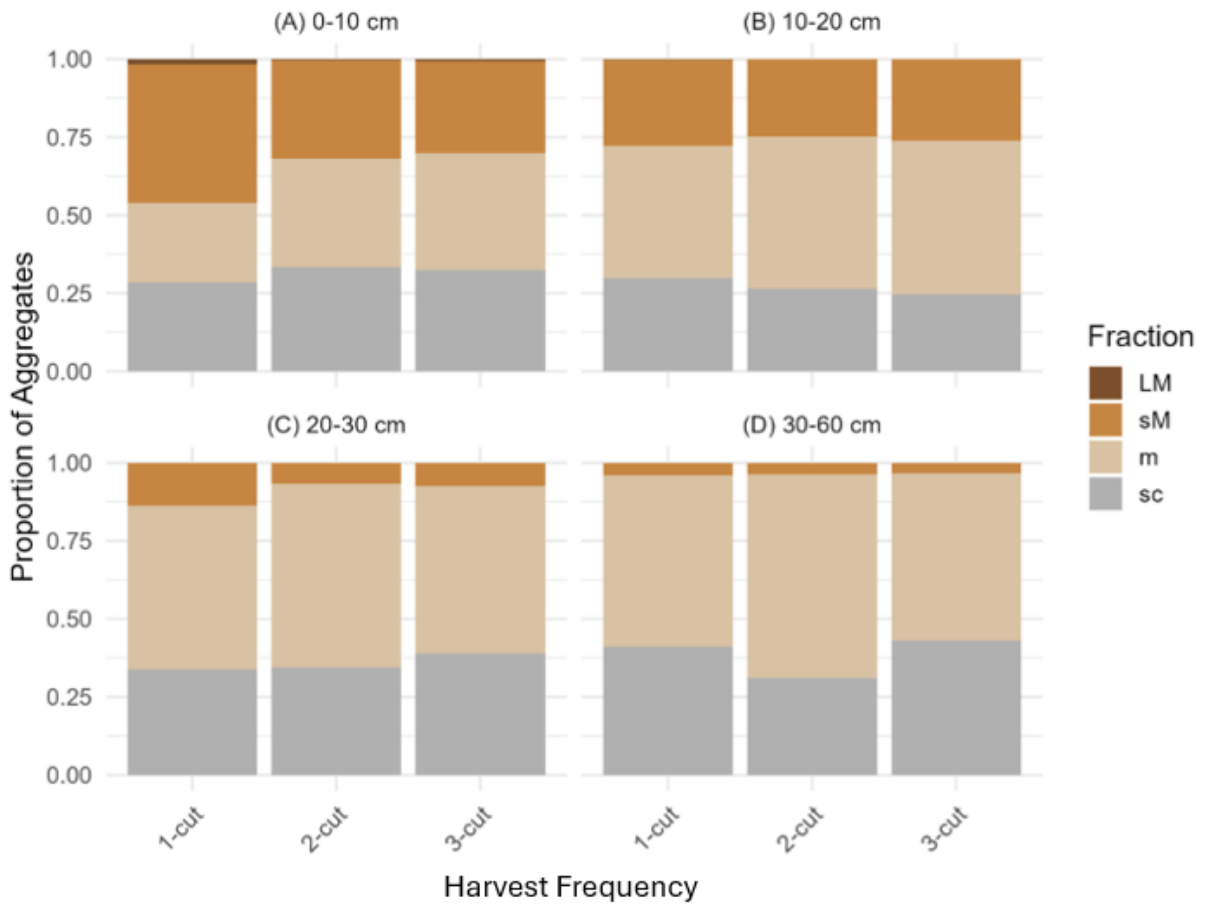

Figure 3.4. *Soil aggregate size distribution as a proportion of bulk soil (%) among harvest frequencies at depth increments: (A) 0-10 cm, (B) 10-20 cm, (C) 20-30 cm, (D) 30-60 cm.*

Harvest frequency and depth impact on total normalized aggregate carbon

The total carbon (g of C) in small macroaggregate was the only aggregate size class with a significant interaction between harvest frequency and depth ($p<0.0001$). The 1-cut harvest had higher total aggregate carbon compared to the 2-cut and 3-cut harvest frequencies in the 0-10 cm depth ($p<0.0001$ for both), although this was not observed in the other depths (Figure 3.5).

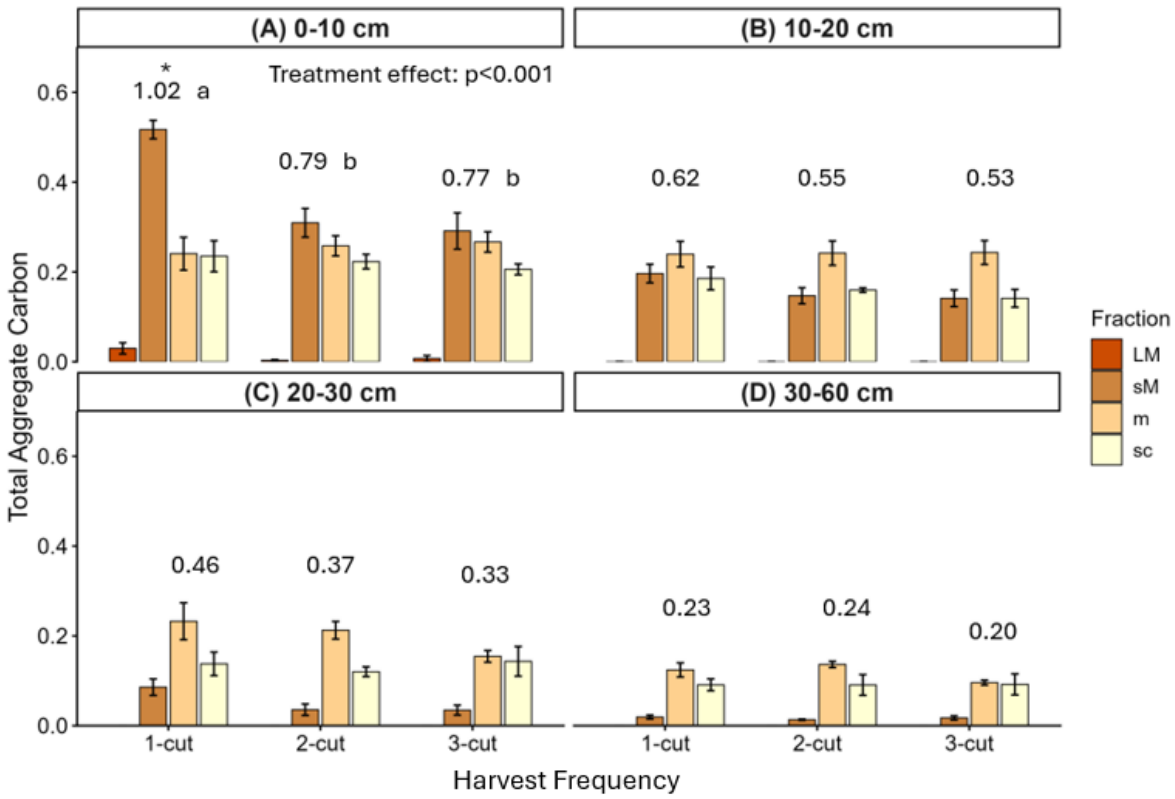

Figure 3.5. *Total aggregate C (g C sand-free aggregate) in (A) 0-10 cm, (B) 10-20 cm, (C) 20-30 cm, and (D) 30-60 cm among each harvest frequency. Error bars are the standard error of the mean. Values indicate the total aggregate C for each treatment. Different letters are statistically significant at p < 0.05 in Tukey post hoc comparisons. Asterisk (*) indicates significance by fraction within a harvest frequency treatment at *p <0.05 in Tukey post hoc pairwise comparison.*

There was no significant interaction effect of harvest frequency and depth on normalized aggregate carbon (g C g^{-1} C) for any size class. Normalized aggregate carbon is the proportion of aggregate carbon normalized to the total aggregate carbon of the given sample. Harvest frequency significantly affected normalized total small macroaggregate carbon and microaggregate carbon ($p<0.01$ and $p<0.05$, respectively). Normalized aggregate carbon in the small macroaggregates was greater in the 1-cut system than the 2-cut and 3-cut system ($p<0.01$ and $p<0.05$, respectively). Normalized aggregate carbon in the microaggregates was smaller in the 1-cut treatment compared to the 2-cut treatment ($p<0.05$).

Depth significantly affected normalized total small macroaggregate carbon, microaggregate carbon, and silt and clay carbon ($p<0.001$ for all). Across all depth increments, shallower layers had significantly higher ($p<0.0001$) normalized small macroaggregate carbon than the layers beneath them, except between the 20-30 and 30-60 cm increments, where the trend was still present but was not significant. Conversely, shallower soil layers had less normalized microaggregate carbon than the layers beneath them, where the 0-10 cm layer had significantly less carbon than all other depths ($p\leq0.001$). Normalized microaggregate carbon was also significantly lower in the 10-20 cm depth than the 20-30 cm ($p<0.05$) and 30-60 cm depths ($p<0.01$). There was less normalized aggregate carbon in the silt and clay in the 0-10 cm depth compared to the 20-30 cm and 30-60 cm depths ($p<0.05$ and $p<0.0001$, respectively). Additionally, the normalized aggregate carbon of silt and clay in the 10-20 cm depth was significantly less than the carbon in the 30-60 cm depth ($p<0.01$).

AMF impact on aggregate distribution and total aggregate carbon

Contrary to our expectations, we found no significant effect of AMF colonization or AMF intensity on aggregate distribution or total aggregate carbon for any of the size classes across all depths. We also did not find a significant effect of AMF colonization or AMF intensity on the bulk carbon content.

Discussion

Harvest frequency impact on aggregates and soil carbon

Our research shows that the 1-cut harvest frequency results in a greater proportion of small macroaggregates and higher total aggregate carbon compared to more frequent harvests

(Figure 3.4 and Figure 3.5, respectively). Soil aggregation is important for not only soil carbon storage but also moisture content, water infiltration, nutrient availability, and long-term sustainability of a cropping system (Bai & Cotrufo, 2022). Macroaggregation is largely driven by binding agents produced by soil microbes and fungi, which are fueled by carbon inputs from residues, root turnover, exudates, and rhizodeposits (Tisdall & Oades, 1982). In the 1-cut system, fewer harvests may allow for more stable root exudation, supporting microbial activity that produces binding agents and promotes macroaggregate formation. These may include AMF extraradical hyphae (ERH) and glomalin-related soil proteins (GRSP), a glue-like protein produced by AMF (Singh et al., 2023). However, neither of which were directly quantified in our study. Other binding agents aside from AMF produced, such as mucilage and polygalacturonic acid, may also contribute to macroaggregate formation (Traore et al., 2008). Our findings align with (Mohammed & Desta, 2017) who found that harvesting switchgrass once a season as opposed to twice a season yielded 16% more macroaggregates.

The greater proportion of small macroaggregates observed in the 1-cut system is also consistent with the aggregate hierarchy model proposed by Six et al. (2000). In this model, macroaggregates are the first aggregation pool to respond to management differences. Macroaggregates initially form around coarse particulate organic matter (POM). Over time, microbial processing (potentially stimulated by the POM itself, or root exudates, and other factors discussed above) reduces this coarse POM into finer POM, which becomes encapsulated by mineral particles and microbial byproducts to form stable microaggregates within the macroaggregates. Eventually, these microaggregates may be released as free microaggregates. In our study, shifts in aggregate proportions were seen primarily between small macroaggregates and microaggregates, while the silt and clay fraction remained steady (Figure 3.4). This indicates

that the soil is actively responding to the 1-cut treatment by forming macroaggregates, whereas the microaggregates pool in the 2-cut and 3-cut systems likely reflects the pre-existing microaggregate pool and the treatment has not influenced new aggregation.

Depth impact on AMF

AMF colonization was significantly higher in the topsoil (0-10 cm) compared to deeper soil layers (Figure 3.3). This is consistent with the fact that, although switchgrass has a deep rooting system, root abundance and density are typically greatest in the topsoil (Adkins et al., 2016). Previous studies have demonstrated that greater root biomass correlates with higher AMF colonization (Wang et al., 2024). These findings suggest that root distribution and density play a key role in shaping the vertical profile of AMF colonization, and that topsoils are critical zones for AMF.

AMF dynamics in early-stage switchgrass systems

Contrary to our hypothesis, AMF colonization did not have a measurable effect on total aggregate carbon or aggregate distribution across any harvest frequency. However, this lack of an observable effect should not be interpreted as evidence that AMF are not involved in soil carbon dynamics in this system. To the contrary, in our study, which was conducted only two years after plot establishment, we observed an average of 53.7% (± 2.12%) AMF colonization in the top 10 cm (Figure 3.3). By comparison, in a study conducted on mature switchgrass stands (5-6 years old) in Michigan on fine-loamy, mixed, mesic Typic Hapludalf soils, AMF colonization rates ranged from 69 to 100% in 0-15 cm (Emery et al., 2018). AMF colonization tends to increase as plants mature (Smilauer et al., 2021), suggesting that AMF colonization in our system will likely continue to increase over time. The early presence of AMF in the

switchgrass roots highlights that the symbiosis is already established and may influence soil processes in a measurable way as the system matures.

Previous work has shown that AMF abundance is higher in switchgrass systems than in corn systems and that switchgrass systems tend to exhibit greater aggregate stability relative to no-till corn, likely due to the AMF relationship (Stewart et al., 2014). Yet switchgrass systems are lower in AMF abundance than in prairie systems (Liang et al., 2012). However, in switchgrass sites established for longer, AMF communities and colonization rates more closely resemble those of prairie systems (Jesus et al., 2015). Over time, managed perennial bioenergy systems may therefore function more similarly to natural grassland systems, which account for about a third of all global terrestrial soil carbon stocks, with AMF playing a critical role in that carbon storage (Bai & Cotrufo, 2022). However, this potential for switchgrass bioenergy swards to approach prairie-like ecosystem functioning may be constrained by harvest management. Harvest frequency can influence the plant-AMF relationship by influencing exudation patterns and root growth, which is likely the mechanisms that would drive early-stage response to different harvest regimes. Additionally, continual removal of biomass over multiple years without additional fertilization, will likely deplete soil nutrients, which may further affect the plant-AMF relationship and its potential to support carbon sequestration and storage. Long-term studies are needed to determine the relationship between AMF and harvest frequency.

Study limitations

Although we took great care to design and execute a robust study, it is possible that we did not observe differences in AMF among harvest frequencies due to our method of AMF quantification, which relied on root staining and microscopy. Certain AMF taxa, including species within the *Paraglomus* genus, yield faint to invisible mycorrhizal structures when stained

(INVAM, n.d.). Since *Paraglomus* had high relative abundance in our site (Chapter 2), it is possible that total colonization was underestimated. Consequently, our measurements may not have fully captured variation in AMF presence among roots, and therefore, the relationship in this study among AMF colonization, soil aggregation, and soil carbon may not offer the full picture.

Though AMF root colonization is a reasonable proxy for estimating extraradical hyphae (ERH) abundance (Barcelo et al., 2020), our measurements were not a direct measurement of ERH, which are the structures of AMF primarily responsible for soil aggregation. Additional methods to quantify AMF ERH such as phospholipid fatty acid (PLFA) assays with AMF-specific markers, or glomalin-related soil protein (GRSP) assays may provide a more complete picture of the role of AMF in aggregation in our system.

Conclusion

In non-production systems left to natural processes, AMF and associated plants maintain a balance where AMF supports plants in exchange for carbon. In agronomic systems, however, management decisions can influence these interactions. Managing agronomic systems to optimize food, fuel, feed, and fodder production, while also fostering healthy and diverse AMF communities may allow plants to better utilize their natural symbiotic relationships, potentially reducing the need for external inputs, such as fertilizers which can lower both economic costs and potential environmental impacts (Begum et al., 2019). We found that a 1-cut harvest frequency results in a greater proportion of small macroaggregates and higher total aggregate carbon compared to more frequent harvests. Our study shows that it may be possible to optimize

agricultural systems for yields while also fostering healthy plant-AMF relationships, which play crucial roles in ecosystem function and resilience, especially in the face of climate change.

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