SOIL COMMUNITY SHIFTS, PLANT-SOIL FEEDBACKS, AND IMPACT OF NITROGEN FERTILIZATION IN POTENTIAL BIOFUEL CROP *PANICUM VIRGATUM*

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ABSTRACT OF THE DISSERTATION

Soil community shifts, plant-soil feedbacks, and impact of nitrogen fertilization in potential biofuel crop *Panicum virgatum*

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Three concepts form the foundation of the hypotheses of this dissertation. First, that the partnership between switchgrass (*Panicum virgatum*) and arbuscular mycorrhizal fungi (AMF) allows the plant to grow with very limited agricultural inputs, which makes it an excellent candidate for a biofuel crop. Second, that the soil community including soil arthropods and nematodes also functions in conjunction with AMF to support the resilience of biomass production in switchgrass. Third, that annual N fertilization will disrupt the switchgrass-AMF relationship and cause changes in the soil community that might affect the biomass production in the long run. Commercial production is projected to harvest fields for up to 20 years.

The experiments in this dissertation measured multiple measures across soil community to capture any effects of the experimental treatments that may have cascaded across trophic levels. Soil community measures included AMF colonization, the communities of soil microarthropods, nematode abundance, and microbial community function (enzyme profiles) through BIOLOG ecoplates. In addition, soil measurements of plant available N and P were taken to measure impact of experimental treatments on fundamental soil properties and measurements of biomass yield were taken to capture the

impact of experimental manipulations on plant growth. The three experiments were conducted in field and greenhouse settings. Chapter 2 addresses a study of the shift in the soil community of an established switchgrass field that received annual N fertilization. Chapter 3 describes a greenhouse experiment where sterilized field soil was manipulated with additions of AMF, fungal-feeding nematodes, and N fertilizer. Chapter 4 describes a greenhouse experiment where homogenized field soils from " prime ", "marginal", and "poor" soils (rated for farming purposes by the Natural Resources Conservation Service) were manipulated with additions of AMF and N fertilizer.

For the first experiment (Chapter 2), a switchgrass field that was established in 2008 was studied for three years from 2013 - 2015. It was investigated whether yearly additions of nitrogen (N) fertilizer at 100 lb/ac (112.1 kg/ha) changed the soil community. This level of N fertilization fell within the range of best-management practices. Measurements from the established switchgrass field were compared to adjacent unplanted farmland as representative of the soil community prior to switchgrass establishment. The results showed statistically significant impact of N fertilizer on the soil community, but more occurrences of statistically significant differences between the reference area and the established switchgrass field. Soil arthropod communities were statistically different between fertilized and unfertilized plots on 3 of 6 sampling dates. In comparison, the planted areas combined differed from control areas on 4 of 6 dates. Similarly, mycorrhizal structures were statistically different between planted and unplanted plots on 2 of 9 dates, whereas reference area was different from planted areas on 5 of 9 dates. There were statistical differences in nematode abundance and microbial community function as measured by BIOLOG ecoplates. However, further testing showed the differences were in the comparison of the reference area to the planted plots rather than between the planted areas and due to N fertilization.

These results suggest that the impact of fertilization is less than that of the change due only to plant establishment. Soil extractable nutrients showed significantly higher amounts of NH_4 and NO_x and significantly lower amounts of PO_4 in fertilized plots. There was evidence that NO_x was increasing over time in the soil. Above-ground biomass yields as well as N content of stem biomass were different at the P = 0.1 level, with 75% higher biomass yields as well as 20% higher N content in the fertilized areas. Therefore, since the soil extractable nutrients were statistically different, and the plant measurements between fertilized and unfertilized areas are less statistically supported, soil changes do not directly translate into plant growth. These results suggest that switchgrass is insensitive to manipulations of N levels, as the factors related to the response of the plant to fertilization were not significant at the P = 0.05 level. The results also show that the soil community is similarly insensitive to the changes due to fertilization because the soil community responded less often to fertilization than to the difference between planted and reference areas.

The second set of experiments (Chapter 3) tested the hypothesis that plant-soil interactions exist in agriculturally-produced switchgrass with two related greenhouse experiments. The soil community and edaphic conditions were manipulated in a fully factorial design to see how commercial mycorrhizal inoculum, fungal feeding nematodes, and N fertilizer impacted biomass yield of switchgrass during one growing season. A subset of the samples was overwintered and grown for a second season to measure any lag effects from the initial treatment, since switchgrass is a perennial crop. Plant biomass and plant N content showed no statistically significant differences due to the experimental manipulations in the one-season or two-season experiment. There were no clear trends in the response of mycorrhizal colonization and structure formation based on experimental treatments in either experiment. The soil arthropod community changed due to N fertilization in the one-season

experiment, but not in the two-season experiment. Nematode abundance showed no differences in response to experimental treatments in both experiments. When all soil response variables were combined in an NMDS analysis, none of the experimental treatments consistently affected the distribution of results for either experiment. These results suggest that there are not any strong plant-soil feedbacks in this system. Although there was a limited response of the soil community to experimental treatments, there was no statistically significant response in the plants that could be attributed only to the experimental manipulations.

The third set of experiments (Chapter 4) investigated whether *P. virgatum* growth responded differently to soil manipulations (adding N fertilizer and commercial mycorrhizal inoculum) in three soils: "prime farmland", "farmland of local importance", and "not prime farmland" as rated by the Natural Resources Conservation Service farmland classification system. Additionally, the soil was used for two consecutive years with no additional manipulation to test for soil exhaustion. A 3x2x2 factorial design greenhouse experiment was conducted to test the hypothesis that switchgrass biomass yields and soil community would respond with different effect size or direction to edaphic manipulations in three soils. In the first year, measurements of soil factors were different with statistical significance due to soil type. Higher extractable NO_3 in fertilized treatments additionally was statistically significant. Statistically significant differences in measurements of plant factors (biomass and N content) were primarily due to soil type. While both stem and root N content were higher in fertilized treatments, the trend was not statistically significant. Soil community factors (mycorrhizal structures, soil arthropod morphospecies, carbon utilization of the microbial community, and nematode abundance) which were primarily multivariate data, were only different with statistical significance due to soil type, not inoculation or fertilization. In the second year,

when no additional manipulation occurred, soil extractable nutrients were lower than in the first year ($P < 0.05$). Within the second-year-only analyses of soil factors, statistically significant differences were primarily due to soil type. However, lower amounts of extractable PO_4 were statistically significant in inoculated treatments. Plant factors were different with statistical significance only for soil type. Soil community factors showed stronger response to inoculation than in the first year, in addition to the statistically significant differences attributable to soil type. These results showed the resilience of switchgrass and the soil community to perturbation across all soil types. While there were signs that the soil was approaching a state of exhaustion, this was not reflected in plant biomass yields. The lag response that was found suggests that treatment effects build up over time; since inoculation was not statistically significant in the first year but was in the second year for BIOLOG ecoplate results and nematode abundance. This building effect is worthy of further investigation.

In general, the manipulations to the soil resulted in changes to the soil extractable nutrients. However, the soil community did not consistently change in response to experimental manipulations. Similarly, plant biomass and plant N content generally did not respond to these experimental manipulations. These results speak to the resilience of the soil community to perturbation, and the physiological resilience of switchgrass to a variety of growth conditions. These results show that switchgrass is an excellent candidate for biofuel production. Best-management practice of annual N fertilization should be reconsidered to reduce the energetic cost of fertilizer to ensure net energy gain in the production of biofuel. However, the results also indicate that the current best-management practice of applying low levels of N fertilizer is not outweighing the benefit to the soil and soil community of the extensive root system of switchgrass.

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Introduction

The seductive promise of biofuel crops is sustainable energy achieved from renewable, carbon neutral sources. However, "sustainable biofuel" is a complex idea, relies on complex processes, and connects to other large challenges facing the world such as food production. This dissertation aims to demonstrate that the ecological questions at the micro-scale of how biofuel crops interact with the soil in agricultural settings are relevant to managing the complex challenges of climate change.

As agricultural practices expand from food production to producing crops for biofuel purposes, criteria must be developed for meeting new sustainability standards. This dissertation defines "sustainability" of biofuel crops to be a net zero or reduction in greenhouse gas (GHG) emissions in addition to a net energy gain in the production of the final fuel product. Most previous research combining agriculture and ecology has gone into understanding factors most important to humans, such as agricultural food webs that preserve crop health via pest control. However, ecology will have relevance to these new sustainability goals of agriculture. Ecological processes that affect the carbon (C) cycle are important to study for GHG reduction because carbon dioxide is considered the main driver of climate change through its consistent increase in the atmosphere over the last few hundred years. Ecological processes related to the nitrogen (N) cycle relate to the use of synthetic N fertilizer, which is tied indirectly to GHG emissions during its production, and directly to emissions of nitrous oxide during application. Soil communities that drive decomposition are crucial in the C and N cycles. However, because the soil community primarily comprises hard-to-observe micro-flora and -fauna, it has been under-studied in the past. Thus, through their impact on the C and N cycles, the soil community in agricultural

production of biofuel crops is relevant to questions of GHG reduction and the field of ecology.

Three concepts form the foundation of the hypotheses of this dissertation. First, that the partnership between switchgrass (*Panicum virgatum*) and arbuscular mycorrhizal fungi (AMF) allows the plant to grow with very limited agricultural inputs, which makes it an excellent candidate for a biofuel crop. Second, that the soil community including soil arthropods and nematodes also functions in conjunction with AMF to support the resilience of biomass production in switchgrass. Third, that annual N fertilization will disrupt the switchgrass-AMF relationship and cause changes in the soil community that might affect the biomass production in the long run. Commercial production is projected to harvest fields for up to 20 years. Chapter 1 reviews the research that supports these three concepts and introduces the questions that lead to the three experiments in this dissertation.

In Chapter 2, switchgrass is considered from the perspective of harvesting a field for 20 years. Will there be changes due to yearly fertilization that affect edaphic conditions and lead to a decrease in sustainability of the crop? Recommendations for growth of switchgrass do not focus on cultivating a diverse field or for benefiting AMF, they focus on fertilizer, herbicide, and harvesting methods (McLaughlin & Kszos, 2005; Mitchell et al., 2010; Vogel et al., 2002). A 5-year-old, established switchgrass experiment was measured over a 3-year period to see if there were changes to the soil, AMF colonization, and the soil microarthropod community over time due to N fertilization.

In Chapter 3, it is proposed that that switchgrass is subject to plant-soil feedbacks because of its relationship with AMF. A 2-year greenhouse experiment was conducted to test for and measure interactions between soil manipulations and plant growth. Although Ehrenfeld et al. (2005) criticize the assumption that plant-soil feedback loops drive plant

communities across all ecosystems, switchgrass may be one of the unique species where soil communities do impact long-term health of the plant. Because it is reliant on mycorrhizal associations, nematode and microarthropod communities may impact plant growth indirectly through feeding choice. In this study, impacts on plant biomass yields will be measurable if plant-soil feedbacks exist.

In Chapter 4, switchgrass was grown in three soils to test if edaphic manipulations produce different results in different soils. The studies in chapters 2 and 3 were conducted on good farm soil. Ideally, switchgrass will be planted on marginal farm land, thus reducing negative impacts from competing with or displacing food crops and potentially building greater C storage since the soil may be in a degraded state (Fargione et al., 2010). Because nutrient availability is different in different soils and the interaction of plants with fungi is dependent on nutrients, it was hypothesized that different effect sizes or even opposite trends would emerge in response to edaphic manipulations in nutrient-limited soils compared to the good farm soil from chapters 2 and 3.

The research presented here attempts to study complex interactions by measuring multiple measures across fields of ecological research that has historically been independently studied. The integration across traditional divides in research methods is the key to further our understanding of complex ecological interactions in a world that is confronting complex challenges such as climate change.

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Chapter 1: Literature review of potential biofuel crop *Panicum virgatum* and its associated soil community

Switchgrass is a promising bioenergy crop that has been researched in the United States since the 1980s (McLaughlin & Kszos, 2005). Biofuel produced from switchgrass could be C neutral in addition to having a net energy gain when fermented into ethanol (Schmer et al., 2008). However, best management practices for switchgrass production suggest the need for a yearly addition of N fertilizer (McLaughlin & Kszos, 2005), which may undermine the sustainability of the crop and subsequently produced biofuel. Three questions about the switchgrass system are fundamental to this dissertation: (i) Does the soil community influence plant growth and promote sustainability of the crop? (ii) Does yearly fertilization change the soil community? (iii) Does fertilization affect the long-term sustainability of this plant as a crop through its effect on the soil community? In this chapter there are two sections and five subsections to address the research that pertains to these fundamental questions: (I) Background: (1) Biofuels, biofuel crops, GHG reduction (2) Soil community (II) Questions: (3) Agricultural effects on soil communities, (4) Nitrogen—nutrient cycling, and ecological impact (5) Ecological context—Feedbacks, drivers, and passengers.

Background: Biofuels, biofuel crops, and GHG reduction

There are three large challenges that relate to the practice of using crops to produce energy: energy security, food security, and GHG reduction. Energy security references the fact that the strongest condition of bioenergy production is the requirement that bioenergy crops have a net energy gain in order to make production feasible. Switchgrass fits this constraint—predictions of energy gain for switchgrass end-products are reliable and positive. Food security references the fact that food production is itself a complex challenge. The

world has finite resources and a growing human population. In addition, climate change threatens food crops directly through changes to water regimes and extreme weather events (Cribb, 2010; Hallegatte et al., 2017; Treidel et al., 2011). Using arable land to produce energy crops will affect food crops, although the details and the final outcomes are debatable (Cushion et al., 2010; Fargione et al., 2010). It is hypothesized that bioenergy crops will be less threatening to global food security if they are produced from non-food crops. Thus, switchgrass as a non-food perennial crop fits this constraint. Finally, GHG emissions are considered the driving force of climate change. In the simplest terms, biofuel crops will reduce GHG emissions if the carbon dioxide (CO_2) absorbed by the plant during growth is greater than the CO_2 released both in the process of converting the raw plant material into usable fuel and in subsequent use of the fuel. Although this dissertation addresses only indirect GHG reduction as a result of making ecologically advised changes at the production-level of one crop, energy security and food security are a crucial part of biofuel production. Thus, the larger context of studying switchgrass is that switchgrass is a good choice for a biofuel crop because it does not exacerbate the critical issues of energy security and food security.

As previously mentioned, should potential biofuels ultimately require more energy in their production than is actually usable in the end product, they would fail to meet the criteria to be considered effective biofuels. Similarly, if biofuels produce more GHG emissions than traditional fuels then the fundamental purpose of biofuels would be completely undercut. Biofuel ethanol already is added to gasoline, which helps it burn more efficiently and thus reduces GHG emissions (Yee et al., 2013). However, the current trend in the United States is creating ethanol from corn. Corn ethanol is problematic because corn is a fertilizer-dependent annual crop that tends to be produced in industrialized farm systems

(de Vries et al., 2010; Demain, 2009; Nguyen et al., 2019; Varvel et al., 2008). Thus, ethanol produced from corn potentially doubles the GHG emissions of traditional fuels (Searchinger et al., 2008). However, the proposed alternative of creating cellulosic ethanol, from any cellulosic material, requires greater energy to break cellulosic structures into sugars that can then be fermented into ethanol than the process already commercialized for corn (Demain, 2009; Dien et al., 2018; Galán et al., 2019; Rabbani et al., 2018).

The process of creating biofuels from cellulosic materials is complex and requires both energy and GHG emissions (Demain, 2009; Dien et al., 2018; Galán et al., 2019). Since every part of the process of commercial production will necessarily use energy, the added complexity of converting cellulosic material (compared to corn or sugarcane processes) into ethanol means that reduction of energy use and GHG emissions at other stages of production is more critical. Farm practices use energy as well (Lal, 2004; Pelletier et al., 2011). Thus, for cellulosic materials, it is critical to reduce emissions throughout the entire process, including in the first stage—farming practices—in order to ensure a net neutral or negative CO_2 release. The farming practices where care should be taken for emissions include: fertilizer applications, irrigation, tillage or seedbed preparation, harvesting, and the emissions from pesticide, herbicide, or fungicide application (Lal, 2004; Pelletier et al., 2011). Notably, by reducing these applications for purposes of reducing GHG emissions, the net energy gain of the crop should also be positively affected. Therefore, the focus on ecological processes at the farm level that relate to GHG emissions is reasonable in order to ensure that the lifecycle analysis of the biofuel crop is sustainable.

Although switchgrass requires relatively low farming inputs, N fertilization is recommended, which is problematic from the perspective of the energetic cost of fertilizer production. Switchgrass has low requirements for fertilizer, herbicide, pesticide, fungicide,

irrigation and tillage (Bouton, 2007; Casler et al., 2018; Casler & Vogel, 2014; McLaughlin & Kszos, 2005; Mitchell et al., 2010; Sarath et al., 2008; Schmer et al., 2008) and thus is a good candidate for having net C absorption. As a perennial crop, tillage is only required in the initial field set up, and then switchgrass fields are proposed to be harvested for at least 10 and up to 20 years (McLaughlin & Kszos, 2005; Sanderson et al., 2006). Switchgrass has an aggressive root system, and thus typically needs herbicide only during establishment (McLaughlin & Kszos, 2005; Mitchell et al., 2010). Similarly, switchgrass has a lower burden of pests compared to traditional crops, thus the lower requirement for pesticide and fungicide (McLaughlin & Kszos, 2005). Irrigation needs are reduced based on the extensive research and breeding that has gone into the plant, thus a proper variety can be selected to match the local water conditions (McLaughlin & Kszos, 2005; Sanderson et al., 2006; Sanderson & Reed, 2000; Zhang et al., 2019). Switchgrass does not require fertilizer to grow (and does not respond to phosphorus (P), potassium, or calcium fertilization), though recent research has suggested that P- and K-limited soils reduce biomass yields (Ashworth et al., 2019). However, the consensus is that yearly N fertilizer application increases and stabilizes yields (Ameen et al., 2018; Guretzky et al., 2011; McLaughlin & Kszos, 2005; Vogel et al., 2002). Ammonia fertilizer is derived from natural gas in the Haber-Bosch process, which Pfromm (2017) estimates to cause 2.5% of worldwide fossil fuel emissions. Pelletier et al. (2011) suggest that energy requirements for N fertilizer production is the largest part of the energy use/intensity of agriculture; for example, it represents about 45% of energy use in agriculture in China. Thus, there is a fundamental flaw in the setup of switchgrass as a sustainable biofuel if it relies on yearly application of ammonia fertilizer derived from natural gas.

Switchgrass is also interesting from a biofuel perspective because of its extensive root network that can lead to C storage. Liebig et al. (2008) found that switchgrass increased soil C over 5 years at accrual rates of 1.1 Mg/ha in the top 30 cm of the soil profile. Other research found that switchgrass improved multiple measures of soil quality over 9 years when compared to no-till corn production (Stewart et al., 2015). Understanding ecological dynamics of this extensive root network is important for understanding switchgrass sustainability and is relevant to the field of soil ecology.

In conclusion, switchgrass is a promising biofuel crop, thus the ecological focus of this dissertation on the soil community of switchgrass is relevant to the larger picture of climate change through its connection to the GHG emissions of switchgrass biofuel production. The facts mentioned in this section are the foundation for the relevance of the entire dissertation, support the assumption that the switchgrass-AMF relationship supports plant growth without need for a lot of fertilization, and connects to a secondary assumption that will be explored in the Questions section that there is long-term damage done to soil in agricultural settings that may impact consistent biomass production.

Background: Soil community

The soil community is complex, and generally has more diversity than corresponding terrestrial communities (Sylvan & Wall, 2011; Wardle, 2006). Edaphic communities include plants, microbes, mesofauna, and megafauna. Plants are not described here; a general knowledge of plants is assumed, and the specific ecological processes that are connected to this research are discussed in the Questions section. Megafauna are not described in further detail beyond this paragraph. Within megafauna, earthworms are the most notably omitted group, as they are known to have a major impact on the soil through their burrowing habits (Makeschin, 1997). However, because of their size, they are not limited by the same

heterogeneity and pore structure that makes the smaller members of the soil community so diverse and interesting (Wardle, 2006), and thus they were not included in this research.

Mesofauna typically refers to organisms between 0.1 mm and 2 mm in size; these include nematodes and microarthropods. Nematodes are a critical pest in agriculture; root-feeding nematodes can devastate crops. Parasitic nematodes on animals and plants represent about 48% of nematode genera (Zunke & Perry, 1997). However, nematodes are also important because they represent multiple levels of a food web, including microbivorous, fungivorous, omnivrous, and predatory (Zunke & Perry, 1997). Thus, although nematode agricultural pests are well studied, free-living soil nematode species are also essential for providing plant nutrients through decomposition of organic matter and can provide ecological information through their community structure. Certain sensitive nematode genera can be used as indicators of soil health, showing whether a system has transitioned into a restored or healthier state (Fiscus & Neher, 2002; Yeates et al., 1997).

Acari and collembola are the main groups of soil microarthropods, with oribatid mites in Acari being the most numerous soil mesofauna (Coleman et al., 2004). Both groups can inhabit the litter as well as deeper horizons in the soil (Larink, 1997). Collembola are mainly fungivorous but they also can consume detritus and bacteria (Larink, 1997). Mites have typically been classified as detritivores (mainly oribatid mires) and predators, but recent research suggests that up to 50% of oribatid mites are actually microbivores rather than detritivores (Gan et al., 2014). Both collembola and mites are important in the decomposition and mineralization process (Larink, 1997; Wickings & Grandy, 2011). Microarthropods are accepted as useful bioindicators of soil health (Benckiser, 1997).

Micro-flora and -fauna include bacteria, fungi, archaea, and protozoa. They typically live in water films that surround soil particles (Benckiser, 1997). The link between root and

fungal biomass and fungal and bacterial biomass are well-accepted facts (Coleman et al., 2004). Fungi and bacteria are critical in decomposing matter and immobilizing and extracting nutrients (Bamforth, 1997). Significant research shows that fungi and bacteria typically alternate in dominating the soil microbial community, and bacteria:fungi ratios are often used in plant ecology questions (van der Heijden et al., 2008). Evidence suggests that fungi-dominated communities are associated with characteristics like slow nutrient cycling, low nutrient availability, slow growing plants, late succession, whereas bacteria-dominated communities are associated with fast nutrient cycling, high nutrient availability, fast growing plant species and early succession (van der Heijden et al., 2008). Turner et al. (2019) show that over time in a chrono-sequence dune system, the dominant microbe in soil function switches from bacteria to fungi.

Fungi in soil are primarily saprotrophic, pathogenic and mycorrhizal fungi—with the focus here on mycorrhizae. Mycorrhizal fungi are composed of two major groups: ectomycorrhizae and arbuscular mycorrhizae (AMF). Both form intimate connections with plant roots, but do so in structurally different ways. Switchgrass consistently forms an association with AMF. Hyphae of AMF enter the root cells and form structures within the cells. AMF are obligate symbionts with plants, and form associations with a wide diversity of plants, from angiosperms to sporophytes of pteridophytes (Smith & Read, 2008). The symbiosis involves an exchange of nutrients, typically C to the fungi and P and N to the plants (Smith & Read, 2008). Much research supports the facts that AMF help plants acquire nutrients and generally grow better (Lenoir et al., 2016; Smith & Read, 2008). AMF confer other benefits including protection from disease and abiotic stresses like drought, salinity and pollution (Chandrasekaran et al., 2014; Lenoir et al., 2016; Smith & Read, 2008). Although the mutualistic benefit of AMF is undeniable, the symbiosis can also be parasitic

on plants in some conditions, the benefits are not always linearly positive, and as is true with much of nature, complexity makes these relationships interesting to continue to study (Jach-Smith & Jackson, 2018; Smith & Read, 2008; Yang et al., 2015).

The importance of mycorrhizae and other fungi in the nutrient cycle is well documented (Coleman et al., 2004). However, there is significant contribution of the entire soil community to nutrient cycling processes (Dighton & Adams Krumins, 2014; Finlay, 2004). Nutrient cycling will be covered more in the next section, however, at the most basic level, nutrient cycling refers to the processes that break down dead organic matter (OM) and make nutrients available for use by other living things; these processes are fundamental for all ecosystems. For example, collembola fecal pellets are thought to increase decomposition by making nutrients more accessible to fungi and bacteria (Siddiky et al., 2012), a positive feedback. A possible negative feedback is that collembolan grazing affects fungal biomass, which can impact the ability of the fungi to perform ecosystem services (Tordoff et al., 2008). Recent research has shown that root exudates can inhibit or stimulate soil nitrification through interactions with AMF and other pathways (Coskun et al., 2017). It seems that diversity itself of the soil community allows for some of the fundamental functions of soil such as nutrient cycling (Bender et al., 2016; Wardle, 2006).

Because soil food webs are complex and have redundancies (Bender et al., 2016; Wardle, 2006), research into drivers of ecological function can be confounding. Some of the complicated responses within the soil may be better understood if multiple trophic levels are included in the measured responses. In one experiment, nutrient pulses caused a trophic cascade that caused an increase in predatory ants who fed on collembola, but no collembola population increase was measured (Milton & Kaspari, 2007). Another experiment found that microarthropod populations responded to the concentration of litter-decomposing fungi,

but increases in fungal hyphal length were not observed (Coleman et al., 2004). Milton and Kaspari (2007) concluded that animals that feed on microbes such as collembola can show response to experimental impact on microbes through trophic cascades.

As previously mentioned, switchgrass is thought to have a limited response to fertilizer additions because of its arbuscular mycorrhizal associations (Brejda et al., 1998; Vogel et al., 2002). The research introduced in this section supports the idea that the entire soil community is relevant to nutrient cycling, that AMF are an important driver of plant growth that connect plant to the soil community, and that soil communities are complex with redundancies that can make understanding shifts in response to changing conditions difficult. Nematodes and other soil fauna feed on mycorrhizal fungi and the rhizosphere is full of fungi and bacteria that are essential to decomposition and nutrient cycling. Thus, this research looks at AMF colonization, the communities of soil microarthropods, nematode abundance, and microbial community function (enzyme profiles) through BIOLOG ecoplates (Burns et al., 2013; Wang et al., 2015), in addition to soil measurements of plant available N and P and measurements of biomass yield. In summary, because of the natural complexity within the soil community, studying multiple members of the soil community is a reasonable method to better understand the sustainability of switchgrass. This multi-pronged approach to the measurements and research is a foundational assumption of this dissertation.

Questions: Agricultural effects on soil communities

Although there is much natural variation in ecological processes relating to plant and soil communities, human activities such as agriculture have a negative impact on soils across the board. This section reviews some of these data and connects them to the questions underpinning this dissertation.

The negative impact of agriculture is clearly shown in diminishing crop yields after the high initial yields following preparation of undisturbed land if exogenous inputs are not applied (i.e. Usher, 1923). There are many reasons that crop yields diminish over time—loss of OM is particularly important—and the local conditions influence which factor is most relevant at each particular geographic point. However, it could be argued that the most fundamentally destructive aspect of farming is disruption of the soil structure. Tillage is at the top of the list; it disrupts natural soil structure, compacts the subsoil and increases the loss of soil organic matter (Brussaard et al., 2007; Cluzeau et al., 2012; Sylvan & Wall, 2011; Weil & Brady, 2017). Correlated with tillage is when the soil is left bare, erosion and loss of OM increases (Brussard et al., 2007; Cluzeau et al., 2012; Weil & Brady, 2017). Fertilization also affects soil structure. Research that is specifically relevant to the focus of this dissertation on switchgrass and N fertilization is that broadcast fertilization with NH_4+ fertilizer is associated with dispersion of soil particles, leading to erosion and reduced water infiltration (Fox et al., 1952).

Specific factors of disruption that have been researched include pesticides, fungicides, organic methods compared to conventional methods (Avio et al., 2013; Cao et al., 2011; Chang et al. 2013; Quist et al., 2016). One researcher was so convinced of farming being a driving negative force that he proposed that farming in the Netherlands led to predictable filtering of soil microarthropod diversity from high in old stand forests to low in high-input (including pesticides) grasslands (Siepel, 1996). Other factors are clearly at play besides farming based purely on the descriptive labels of old-stand forests versus grasslands. However, the fundamental idea that farming leads to low diversity in the soil community is still supported in current research (Bender et al., 2016).

Although switchgrass is a perennial crop that needs fewer major-disruption-farming-methods than food crops, it still is part of an agricultural production. At minimum, biomass is being removed from the system yearly. Since Wardle et al. (1999) found that permanent removal of living plant biomass from perennial grasslands resulted in a decline in the soil fauna population, and other research suggests plant detritus controls the soil community (Culman et al., 2010; Sabais et al., 2011; St. John et al., 2012), it seems that yearly removal of biomass could have a negative effect on the soil community. It even could be argued that the removal of biomass is actually a fundamental aspect of agricultural soil degradation through reduction of OM in the soil and the disruption of nutrient cycling (Cusack et al., 2018; Wang et al., 2017). Ecological communities exist in a state of balance, with nutrient cycling re-releasing nutrients from dead matter for use by living organisms (Hättenschwiler et al., 2005; van der Putten, 2005). Switchgrass, as a perennial grass, has a nutrient cycle fueled by the annual cycle of above-ground biomass senescence. How long can continuous, unfertilized switchgrass harvesting continue before soil degradation starts? While the timing of harvesting until after senescence of the leaves does preserve substantial nutrients in the plant roots, the loss of material contributing to the surface OM could be enough of a disturbance to disrupt the detrital food chain.

Alternatively, switchgrass is more similar to natural ecosystems than other agricultural crops, and thus may have a positive impact on the soil community in comparison to industrial farming. Many have the idea that farms can be looked at as agroecosystems—not totally a natural ecosystem, but something that has more self-sustaining features than a typical industrial monoculture farm (Bender et al., 2016; Brussaard et al., 2007). Although switchgrass will most likely be planted as a monoculture, research suggests that a grass polyculture is just as viable for energy production, but with much greater ecological positives

(Robertson et al., 2012). Nutrient management, water management, stress (both biotic and abiotic) mitigation are all considered ecosystem services provided by the soil community and are very relevant to farm management (Bender et al., 2016; Brussaard et al., 2007; de Groot et al., 2016; Hunt & Wall, 2002; Kibblewhite et al., 2008; Weil & Brady, 2017). The extensive root system of switchgrass may be enough to support a robust soil community capable of providing the necessary ecosystem services.

Species diversity of the soil community itself may be the key to supporting nutrient cycling services (Brussaard et al., 2007; Wardle et al., 2006), so loss of biodiversity in agricultural systems may be the main explanation for loss of ecological function. A number of research studies tie diversity itself—of plants, insects and soil community—to benefits in agricultural production systems that are similar to switchgrass (Robertson et al., 2012; Sabais et al., 2011; Tilman et al., 2012; Wedin & Tilman, 1996). Since most soil biota do not follow the intermediate disturbance theory, and maintain a high level of diversity until essentially catastrophic disturbance reduces the diversity severely (Bender et al., 2016; Wardle, 2006), when does that critical level of disturbance happen in agricultural systems? Is it merely the reduction of soil community biodiversity that represents the most obvious and potentially correctable problem in agricultural production? This connects to some fundamental questions: Does the soil community of switchgrass provide ecosystem services that lead to it being a sustainable biofuel crop? Or does the yearly removal of biomass disrupt the detrital food chain and create catastrophic disturbance to the soil community? Does N fertilization further disturb the system?

In conclusion, agricultural production methods are disruptive to soil communities. Some aspects of switchgrass production automatically reduce the most destructive agricultural practices such as tillage, however there is still evidence that switchgrass

production will be associated with reduced diversity in soil community, and that is relevant to its ability to not need exogenous nutrient input to maintain biomass yields. Thus, this proposal to look at the soil community as a way of understanding the sustainability of switchgrass production is reasonable. Will the reduction in tillage, pesticide, herbicide, and irrigation allow the soil community enough stability to function at close-to-normal, or will the annual fertilization and removal of biomass keep the soil community in a state of lower ecological functioning?

Questions: Nitrogen—nutrient cycling, and ecological impact

Though N application is a critical component of modern agriculture, as addressed in the previous section, excess N is associated with its own ecological risks; thus this section focuses on N and related ecological impacts.

It is a well-accepted idea that excess nutrients added to ecological communities threaten the balance of the communities, especially those typically low in available nutrients (Dighton et al., 2004; Siepel et al., 2018). It is also well established that humans have caused a significant increase in bio-available N, through particulate deposition from vehicular emissions and through direct application in farming practices (Du et al., 2014). The Green Revolution is called such because the invention of N fertilizer allowed for a significant increase in crop yields and stability. Plant biomass in general has a strong positive response to N additions (29% aboveground and 35.5% belowground) (Zhou et al., 2017). Other sources cite N fertilization as increasing crop yields 20% compared to organic methods (de Ponti et al., 2012) and an average of 26% in six major crops in the United States (Cao et al., 2018). Because of the critical need for stable agricultural production in our modern densely populated world, this potential threat to ecological systems through excess N loading is only increasing.

The N cycle is a critical part of life. The largest pool of N is atmospheric dinitrogen (N_2), which is not biologically available to organisms. Biological fixation of N_2 is performed by bacteria and archaea, producing ammonium (mineralization), NH_4+, which can undergo nitrification into nitrate, NO_3-. Plants can take up either form of N. The two forms act differently in soil: NH_4+ is relatively immobile compared to NO_3- which is mobile and thus more quickly lost/leached (Coskun et al., 2017). Coskun et al. (2017) estimate that the loss of N fertilizer to the local environment (not the target plants) or leached to waterways is 50-70%. Adding N fertilizer can result in an increase in total N in soil through being incorporated into microorganism bodies and plant litter that then is incorporated into the soil. Soil C can increase through the same mechanism, although usually any change is not to permanently stored C (Lu et al., 2011; Zhou et al., 2017). Higher N availability and soil C are associated with fertile soils and thus an increase in these factors is considered positive for soil quality. However, N fertilization is consistently associated with lowering the pH of the soil, leaching of base nutrients (calcium, magnesium, sodium and potassium) and the mobilization of aluminum, which are considered negative factors for nutrient availability and plant growth (Bowman et al., 2018; Weil & Brady, 2017).

Though on average, in ecological research, N addition is viewed as detrimental to biodiversity and thus negative, individual experiments do not always show consistent responses. The next few paragraphs briefly go into this research.

Multiple studies have shown that plant communities change in response to N deposition or N influx, and thus this statement is typically taken as fact. Hu et al. (2017) found that yearly fertilization at 300 kg/ha and 600 kg/ha for a 10 year period reduced plant species richness, reduced community biodiversity, and increased total plant biomass compared to unfertilized sites, and these changes to the plant community fundamentally

changed the nematode community. Bowman et al. (2018) found in an alpine ecosystem that nine years after simulated N-deposition plant species composition, abundance of fungi and bacteria, and soil nutrient pools were still impacted negatively by the N addition. Henning et al. (2018) found that N addition and fungicide application reduced both plant species richness and fungal richness in plots seeded with a prairie mix and sampled in four subsequent years.

Multiple studies have shown negative impacts of N addition on AMF. Ellis et al. (1992) found a reduction in AMF colonization over two years in soybean and grain sorghum due to N fertilization and a greater decrease due to the addition of manure, but found the greatest plant yield for the manure treatments, leading them to conclude that the manure treatment reduced the dependence of the plants on AMF. Treseder (2004) found in a meta-analysis that overall, N fertilization caused a mean decline of 15% to mycorrhizal abundance. However, 23% of the studies actually showed a positive response of AMF to N application, indicating great variety in results (Treseder, 2004). However, in studies that measured percent colonization, N fertilization reduced colonization by 5.8% on average (Treseder, 2004). Jumpponen et al. (2005) found that in a prairie system, N fertilization changed the AMF community, increased the intercellular coils, but did not affect total colonization, arbuscules or vesicles.

While some research has looked at the effect of N fertilization on other members of the soil community, generally questions of the effects of N fertilization on soil communities remain unanswered (Nijssen et al., 2017). Song et al. (2016) found in an old-field grassland in China that N addition reduced the generic richness of nematodes due to an increase in bacterivores, although overall nematode abundance was not affected. Cao et al. (2011) found over 11-years of winter wheat-summer maize rotation that chemical fertilization (a

combination of N and P) reduced the abundance and diversity of soil mites, especially four Oribatid mite species, which was correlated with an increase in available P and, they suggest, a suppression in fungi (fungi weren't measured directly).

Other studies have shown mixed effects of N application or deposition on the soil community. Emery et al. (2017) found in switchgrass biofuel systems an inconsistent response of AMF colonization and limited reduction in AMF diversity due to fertilization, however abundance of bacterivore and omnivore nematodes increased and fungivore nematodes decreased. Krumins et al. (2009) found in sandy scrub oak systems that ectomycorrhizal fungal biomass and community composition did not respond to N addition, although bacteria community changed. Zhang et al. (2016) showed that AMF helped to combat the negative influence of N addition on temperate meadow plant communities by increasing the plant biomass of species less able to take up N quickly, although there were some inconsistencies in their findings between the field and greenhouse experiments. Shao et al. (2018) concluded that plant presence mitigated the negative effect of N deposition on soil microbes because the response of soil invertebrate abundance to N deposition was impacted by the presence or absence of planted shrubs in an *Acacia auriculiformis* plantation. Some studies of soil enzyme activity did not show a change in activity levels in response to N fertilization (Jing et al., 2017; Jing et al., 2018), suggesting that the microbial community maintains its function despite changes in nutrient influx. Lemanski and Scheu (2014) found that fertilizer addition (N, P and K) shifted the diet of soil microarthropods in grasslands, but did not affect total abundance. Similarly, Gan et al. (2014) found that the trophic position of oribatid mires did not shift in N-enriched areas, although food resources did change (detritus, fungal biomass). Some studies have found an increase in microarthropod abundance in response to fertilizers (Benckiser, 1997; Chang et al., 2013), which could be

driven by species capable of quickly taking advantage of the nutrient influx (R- vs K-strategists).

The simplest explanation for the complexity in research results is that looking at N alone is the confounding factor (Eom et al., 1999; Johnson et al., 1998; Tischer et al., 2015; Treseder & Allen, 2002). Two major nutrients that are closely tied to N are C and P. N is linked to C through decomposition—the C:N ratio of detritus is a major predictor of the decomposition rate as well as the type of microbe (bacteria/fungi) that drives the decomposition, and relates to the availability of N in the soil solution (Bardgett et al., 1999; Weil & Brady, 2017). Growth limitation due to inadequate P-resources is a major factor across ecological (and agricultural) communities. P-limitation interacts with the N cycle through Liebig's law of the minimum, which states that plant growth will be limited by the necessary nutrient at lowest concentration.

Some clear examples of this phenomenon of interconnected nutrient limitation are found in other ecosystems. Johnson et al. (1998) found opposite responses of microbial biomass to N addition in N- versus P- limited sites in heathlands. Harrison et al. (1995) found leaching of nitrates in older forests where P and K limit tree growth, indicating that N cannot be used past the P- and K- limitation. Thus, if the alternate nutrient was not considered, research may be confounding and produce opposite conclusions from the same manipulations. However, since N fertilizer is so critical to crop yields, the singular focus on the question of the magnitude of the detrimental effect of N fertilizer application on soil community is still important and relevant, especially in agroecosystems (Jach-Smith & Jackson, 2018) where N-limitation on plant growth and crop production is a crucial management issue.

Thus, even though the N-cycle has been well studied, the specific effect of N on a system is not completely predictable. As Treseder (2004) concludes: "regardless of the mechanism, the significant variation in N responses among studies indicates that predictability of N deposition effects on mycorrhizal biomass for any given ecosystem is relatively low." Studying whether N fertilization leads to negative impact on the diversity of the soil community as a way of understanding the potential sustainability of switchgrass is worthwhile. Taken together, these data support the following assumptions: that N application can disrupt the plant-AMF relationship and reduce AMF colonization, N application can have direct effects on the soil community, and that there can be indirect effects on the soil community through the impact on AMF.

Does yearly fertilization have a negative and building effect on AMF in switchgrass production (most especially relevant to the expectation that a field can be harvested for up to 20 years)? And if so, does that effect get conferred to the whole soil community? Does N addition have a direct negative effect on the soil community? In summary, does N fertilization undermine the sustainability of switchgrass production by having a negative effect on AMF and the soil community?

Questions: Ecological context—Feedbacks, drivers, and passengers

Ecology is full of intricacies and connections. The fundamental question of "what-drives-what" has been asked many times (i.e. the driver versus the passenger theory of AMF communities [Zobel & Opik, 2013]). This opening line perfectly captures this: "Elucidating the factors that drive variation and change in the composition of natural plant communities, in space and time, is one of the longest-standing issues in ecology" (van der Putten, 2005).

From the largest limits of ecosystems to the small locality of a single plant there are feedback cycles between plants and soil. Plants influence soil through exudates and detritus

and, vice versa, soil influences plants through water holding capacity, nutrient availability, and the soil community (van der Putten, 2005). The environmental factors that contribute to and build soil, such as weather and the underlying parent material, also affect the plants capable of growing in a location, and then the plants impact the soil conditions further through their detritus. Grasslands typically occur in Mollisols, but the deep roots of perennial grasses are a significant factor in the development of the Mollisols (Moore et al., 2004). Alfisols and Spodosols exist in places that support forest growth, and the soil formation and characteristics of Alfisols and Spodosols is impacted by the recalcitrant leaves and wood that form the majority of the detritus (Moore et al., 2004). The same processes matter at local scales, with individual plants and plant species affecting the soil and soil community. Soil formation is closely linked to local plants and soil fauna through the deposition of dead plant material (i.e. leaf litter, root exudates) and the decomposition process driven by the soil fauna (Coleman et al., 2004). An estimated 90% of net primary production re-enters the soil from dead plant material in grasslands (Coleman et al., 2004).

Two undisputed facts are that different plant communities are associated with different soil communities and that changes in plant community composition impacts the soil community (Culman et al., 2010; Sabais et al., 2011; St. John et al., 2012; van der Putten, 2005; Wardle et al., 1999). Gastine et al. (2003) found that the below-ground food web was changed by a shift in the available leaf-litter. Wardle et al. (2004) conclude that populations of fungi- and microbe-feeding nematodes are regulated by plant communities because they are dependent on specific plant species. As previously mentioned (in the agriculture section), Wardle et al. (1999) found that permanent removal of living plant biomass from perennial grasslands resulted in a decline in the soil fauna population. There is a substantial body of

research that suggests that plant species and communities drive soil species and communities.

While there is less evidence of the soil community driving the plant community there is substantial evidence for AMF influencing plant communities. O'Connor et al. (2002) found significant influence of AMF on plant growth in greenhouse experiments and similar, but less strong, results in a co-designed field experiment where AMF seemed to promote evenness of the field community. Hartnett and Wilson (1999) found that when AMF were suppressed with benomyl in a tallgrass prairie, changes occurred in the plant community through a shift in the relative dominance of AMF-reliant versus non-reliant species. Püschel et al. (2007) found in a mesocosm experiment that a mix of three AMF species differentially affected growth of three plant species through suppression of the non-mycorrhizal *Atriplex sagittata*. Dhillion and Gardsjord (2004) find that suppression of AMF with benomyl resulted in a significant decrease in diversity in one of two boreal grasslands, in addition to a significant reduction in N and P concentrations in plant tissue.

There is some evidence for trophic cascades, which leads to the goal of measuring multiple members of the soil community. Hartley and Gange (2009) reviewed mechanisms of AMF and insect interactions with two relevant points to these questions: AMF can negatively impact insect herbivores through changes in the plant tissue, and AMF may also change the volatile emissions of plants that attract parasitoids to attack herbivores. Soil arthropods can also influence plant performance, most strongly through preferential feeding on pathogens, which suggests that they, too could have regulation capacity on plant communities. One specific example is that Mitschunas et al. (2006) found that collembola grazing on pathogens directly improved seed mortality rates in two of four plant species studied in a germination experiment.

Under either premise— if plants drive soil communities or vice versa— there is evidence that small changes can have larger impact on ecological communities and ecosystem services such as nutrient cycling (van der Putten, 2005). This connects to the idea that different choices for agricultural production of switchgrass can make a difference in GHG emissions as this process becomes commercial and widespread. The context of the ecological question of how N affects the plants and the soil community in switchgrass biofuel production is that in this example, the plants could be driving the soil community, the soil community could be driving the plant community, or the influence of agriculture could be the driver of both.

Conclusion

In summary, the hypotheses are: 1) that AMF and the soil community are reasons switchgrass needs less input than traditional crops (esp. corn), 2) that farming practices, even the reduced methods used for switchgrass, are disruptive to AMF and the soil community, 3) that the disruption of yearly N fertilization has a negative and building effect over time on AMF and the soil community, and 4) the impact of agricultural practice on AMF and the soil community will have negative impact on switchgrass biomass yields that is more critical over time given the expectation that switchgrass fields can be harvested for 15-20 years. These hypotheses lead to those in the research experiments describes in the upcoming chapters.

This research is centered in plant and soil ecology, but is connected to agricultural sustainability and the larger energy concerns of the present day. This research will contribute to the field of soil ecology by further refining our understanding of the link between plant growth and soil communities and the impact of excess N addition. The critical need for C neutral fuel heightens the need for research on the minute interactions occurring in

switchgrass fields that allow switchgrass to grow with minimal fertilizer additions so that best management practices consider these potentially energy-saving ecological interactions.

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Chapter 2: Soil community shifts in an established switchgrass (*Panicum virgatum*) field with annual nitrogen fertilization

Abstract

Switchgrass, a potential biofuel crop, supposedly may be harvested from the same field for up to 20 years. It is hypothesized that one reason switchgrass needs minimal fertilizer inputs is because of its mycorrhizal symbiosis. Regular additions of fertilizer may negatively affect mycorrhizal species through increasing available nutrients such that the plant no longer needs the mycorrhizal symbiosis. Since mycorrhizal fungi are a foundational part of the whole soil community, then a shift in the mycorrhizae-plant interactions due to fertilization could have cascading effects into the soil community that could undermine sustainable switchgrass production over the 20-year life of the field. A switchgrass field that was established in 2008 was studied for three years from 2013-2015. It was investigated whether yearly additions of nitrogen (N) fertilizer at 100 lb/ac (112.1 kg/ha), which is in the range of best-management practices, changed the soil community. A change in the soil community could indicate a reduction in long-term sustainability.

The results show minimal impact of N fertilizer on the soil community. Replicate plots planted with switchgrass were compared to adjacent unplanted farmland as a reference point to what the community may have been prior to switchgrass establishment and to plots planted with switchgrass and fertilized annually with N fertilizer. Soil arthropod communities were statistically different between fertilized and unfertilized plots on 3 of 6 sampling dates, whereas the planted areas combined differed from control areas on 4 of 6 dates. This suggests that the impact of fertilization is less than that of the change due only to plant establishment. Similarly, mycorrhizal structures were statistically different between planted and unplanted plots on 2 of 9 dates, whereas reference area was different from planted areas

on 5 of 9 dates. There were statistical differences in nematode abundance and microbial community function as measured by BIOLOG ecoplates. However, further testing showed the differences were in the comparison of the reference area to the planted plots rather than between the planted areas and due to N fertilization. When time was explicitly included in the analysis, there was evidence of a directional shift over time in the soil arthropod community and in the mycorrhizal structures, but these results need more investigation given the complicated nature of statistically analyzing repeat-measurements.

Soil extractable nutrients showed significantly higher amounts of NH_4 and NO_x and significantly lower amounts of PO_4 in fertilized plots. There was evidence that NO_x was increasing over time in the soil. Above-ground biomass yields as well as N content of stem biomass were different at the P = 0.1 level, with 75% higher biomass yields as well as 20% higher N content in the fertilized areas. Therefore, since the soil extractable nutrients are statistically different, and the plant measurements between fertilized and unfertilized areas are less statistically supported, soil changes do not directly translate into plant growth.

The results suggest that switchgrass is insensitive to manipulations of N levels, as the factors related to the response of the plant to fertilization were not significant at the P = 0.05 level. The results also show that the soil community is similarly insensitive to the changes due to fertilization because the soil community responded less often to fertilization than to the difference between planted and reference areas. These results confirm other research that has found that switchgrass has a limited response to N fertilizer, and supports the contention that switchgrass could indeed be a good biofuel crop requiring minimal inputs of fertilizer.

Introduction

Switchgrass*, Panicum virgatum*, is a promising cellulosic biofuel source because it requires low levels of tillage, irrigation, pesticide, herbicide, and perhaps most critically, fertilizer. Despite inconsistent biomass yield responses to N fertilization, it continues to be a best-practice recommendation to add low levels of N fertilizer to maximize switchgrass yields. It is hypothesized that switchgrass requires low levels of fertilization because of its mycorrhizal partnership. These two facts led us to wonder if annual N fertilization changes the fundamental symbiosis between mycorrhizae and switchgrass, whether there are other changes in the soil community members that interact with switchgrass roots and mycorrhizal hyphae in the soil, and whether these potential changes are magnified over time since switchgrass fields are projected to be harvestable without replanting for up to 20 years. If N fertilization leads to changes in the soil community, that may indicate that N fertilization undermines the sustainability of switchgrass as a biofuel crop and suggest that the best-management practices should be modified to reduce N fertilization recommendations.

Biofuel in most forms will require a multi-stage process before conversion into a final fuel product. Therefore, it is critical to reduce emissions throughout the entire process to ensure a net carbon dioxide (CO_2) absorption (e.g., Schmer et al., 2008; 2014). The focus here is on farming practices, as ecology can inform sustainable crop production. The practices where care should be taken about emissions include: fertilizer applications (since some fertilizers are created from petroleum sources and all application requires energy), the use of farm machinery for irrigation, tillage, harvesting, and transport, and the emissions from pesticide application (Schmer et al., 2008). In general, for effective biofuel production, less energy input leads to higher net energy gain (Jessup, 2009; Tilman et al., 2006).

Switchgrass has been identified as a potential energy crop since the 1980s. Much work has been done on breeding strains of switchgrass to align water requirements with natural water regimes, and a suite of strains are recommended for different areas of the country, therein promoting reduction in energy usage for irrigation (Bouton, 2007; Casler et al., 2018; Casler & Vogel, 2014; McLaughlin & Kszos, 2005). Since it is a perennial species, there is no need for tillage once the grass is established. There is a risk that switchgrass will not establish well enough in the first year to become productive. Thus, application of herbicide for broad-leaved weeds is recommended during the installation period of 1-2 years, but additional herbicide applications are usually not required (Mitchell et al., 2010; Sarath et al., 2008; Schmer et al., 2008). Best practice for timing of aboveground biomass harvest is in early to late fall, after the plant naturally stores its nutrients in roots; this promotes the sustainable yields of biomass for up to 20 years (Casler et al., 2018; Schmer et al., 2008). Thus, the main focus for further reducing energy requirements in the production of switchgrass is the need for yearly fertilization.

Though switchgrass does not require fertilizer to grow, N fertilizer seems to improve and stabilize biomass yields from year to year (McLaughlin & Kszos, 2005). The average fertilizer application rate is about 50 kg/ha, which is 44.6 lb/ac per year (McLaughlin & Kszos, 2005; Vogel et al., 2002). Switchgrass does not respond to applications of phosphorus (P), potassium (K), or calcium (Ca) (McLaughlin & Kszos, 2005), though recent research has suggested that P- and K-limited soils reduce biomass yields (Ashworth et al., 2019). However, the use of N fertilizer is still problematic as excess agricultural inputs change the N cycle, impact the global ecosystem, and is directly and indirectly associated with climate change (Baumgarten, 2020; Galloway et al., 2008). The current consensus is that

N fertilizer increases switchgrass yields (Ameen et al., 2018), thus this is the focus of the following experiment.

This experiment postulates that switchgrass is subject to plant-soil feedbacks through its reliance on AMF, and that fertilization may disrupt the AMF community. Multiple studies suggest that switchgrass has a limited response to fertilizer additions because of its mycorrhizal associations (Brejda et al., 1998; Vogel et al., 2002), which supports the idea that the switchgrass-AMF connection is important to plant growth. Similar evidence for the existence of positive feedback loops is found in a study where switchgrass root exudates enhanced AMF abundance (Mao et al., 2014). Other research shows that switchgrass has more AMF than other biofuel crops and this supports the contention that switchgrass will respond to a disruption in the AMF community to a greater extent than other biofuel species (i.e. Oates et al., 2016). A meta-analysis of N fertilization found that fertilization generally decreases mycorrhizal abundance (Treseder, 2004). Specific studies in agricultural settings found that N fertilization had a negative effect on AMF in other crops (Jach-Smith & Jackson, 2018; Miller & Jackson, 1998; Pietikäinen et al., 2009; Treseder & Allen, 2002). If annual N applications fundamentally change the relationship of switchgrass with arbuscular mycorrhizae, then the sustainability of a switchgrass field may be compromised over the projected lifetime of harvest—up to 20 years.

The soil community may also factor in maintaining sustainable production. Much research supports the important role of soil microarthropods in decomposing litter, which relates to nutrient cycling and ecosystem function (e.g., Carrillo et al., 2011; Soong et al., 2016; Wickings & Grandy, 2010). More specifically, two microcosm studies find that collembola increase N mineralization, the process of converting organic N into a plant-available form (Bardgett & Chan, 1999; Kaneda & Kaneko, 2008). Partsch et al. (2006)

directly show this in a greenhouse experiment; the combined presence of collembola and lumbricidae earthworms improved plant growth and increased the 15N uptake of plant biomass. In agriculture, Brusssard et al. (2007) identify soil biodiversity alone as a key factor in sustainable agriculture, through the role of soil meso- and microfauna in nutrient cycling, water use efficiency and indirect contributions to plant health through their influence on soil structure. Similarly, Bender et al. (2016) identify microorganisms as the key foundation for N cycling and soil diversity overall as critical for creating sustainable agriculture through ecological intensification of production. More relevantly, Culman et al. (2010) highlight an annually harvested, high-diversity perennial grass site that produced the same quality and quantity of biomass over 75 years with no exogenous fertilizer input, which was correlated with a diverse community of N-fixing bacteria.

Because the soil community is complex, it makes sense to measure multiple factors to get a more accurate picture of how fertilization might affect community composition and functioning. For instance, Sánchez-Moreno et al. (2011) measure food web components through three trophic pathways in order to understand soil community dynamics in a grassland and adjacent agricultural land, and they found association between food web connectance, soil N and agricultural intensification. Research supports the hypothesis that one biotic/trophic level may shift while another one stays the same in response to perturbation. One study of trophic cascades finds that while microbes often are what directly break down plant matter and change in response to manipulation, the animals that feed on the microbes, such as collembola, respond in measurable ways to the changing microbe communities (Milton & Kaspari, 2007). Larink (1997) cites multiple studies that suggest that microarthropods could be useful as bioindicators. Research consistently finds that nematodes are a reliable indicator of changes in the soil (i.e. Ferris, 2010; Liu et al., 2016;

Neher, 2010). Larink (1997) finds that fertilization can lead to an increase in soil arthropod abundance, which leads to the hypothesis that fertilization could result in a suppression of the AMF community in the switchgrass system. Similarly, research supports the idea that above- and belowground biodiversity are "somewhat uncoupled" (Hooper et al., 2000; Wardle, 2006; Wardle et al., 1999), which supports the need to measure multiple factors.

Finally, some research supports the idea that changes in the AMF will change the soil community. It is well accepted that microarthropods can influence AMF. Research most consistently shows that selective grazing by invertebrates can suppress dominant fungal species, although other research shows neutral effects (Wardle, 2006). Less direct interactions include collembola suppressing some AMF species through their movement breaking hyphae (Lussenhop, 1992). Two studies conclude that AMF drive changes in the soil community. Pietikäinen et al. (2009) found that AMF inoculation led to an increased abundance of fungal feeding nematodes. Chen et al. (2005) found that two different root stock of tomato, with one suppressing pathogenic fungi, had significantly different soil communities, including soil mites and nematodes. Ingham (1988) concluded that even though AMF and endoparasitic nematodes do not directly interact, they are "mutually inhibitory".

Ecological processes happen over longer scales than are usually measured in controlled agricultural field experiments. Koziol and Bever (2019) show significant effects of AMF on succession to show up in the second year of the experiment, that the second- and third-year plant composition is different. However, even their multi-year experiment may not properly represent longer-term changes (Koziol & Bever, 2019). Other studies of belowground community changes over successional gradients find divergence in the soil community (Castle et al., 2016; Roy-Bolduc et al., 2016), supporting the hypothesis that time

is an important factor in soil community structure. Jach-Smith and Jackson (2018) find a greater negative effect of N fertilization on AMF in a field with a 5-year history of fertilization compared to a field with no history of fertilization. Therefore, looking at the soil community over three years is an important feature of this study, as is the fact that switchgrass cultivation began five years prior to beginning the measurements.

The initial hypothesis of this experiment was that yearly N fertilization would increase the biomass yield of switchgrass, fundamentally change the soil nutrients, and alter the mycorrhizal relationship with switchgrass plants. Additionally, it was hypothesized that the predicted changes would have a cascading effect on the soil community. The final hypothesis was that the negative effects of fertilization would build over time which will undermine the sustainability of the cropping system. The predicted result of N fertilization changing the soil community was assessed by measuring mycorrhizal structures, soil arthropod morphospecies, nematode abundance, and microbial community function. Additionally, the condition of the soil and its response to fertilization was assessed by looking at soil extractable nutrients, plant biomass yields, and plant stem N content (as plant growth relies on soil nutrients).

Methods

Samples were collected in 2013, 2014, and 2015 from an established switchgrass biodiversity study at the Rutgers University Adelphia Extension Farm in Freehold, NJ (40.227053, -74.252517). Soil type is Freehold sandy loam described as well-drained and moderately permeable (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). The soil is rated as good for crop production by the NJ Soil Health Assessment and prime farmland by the Natural Resources Conservation Service (NRCS) farmland classification system.

The switchgrass biodiversity study was established in 2008 and maintained until 2018 by Dr. Stacy Bonos with the goal of studying biomass yields (with the last fertilizer application applied in 2016). The initial study design looked at thirteen plant combinations—switchgrass and three additional grass species planted alone and in combination with one of two legume species—and two fertilizer regimes— 0 lb/ac or 100 lb/ac (112.1 kg/ha) of N fertilizer (see Figure 1). Four of the thirteen biodiversity plot types were sampled for this experiment to capture the potential variability of the soil community in response to the addition of biodiversity and the additional N fixation from the legumes. Selected plot types were: switchgrass only (type 1), switchgrass planted with Panic grass and Niagara Big Blue (type 6), switchgrass planted with Panic grass, Niagara Big Blue, and trefoil (type 9), and switchgrass planted with Panic grass, Niagara Big Blue, and clover (type 12). Niagara Big Blue: *Andropogon gerardi*, Panic grass: *P. amarum*, Trefoil: *Desmodium canadense*, Clover: *Dalea purpurea*/*Petalostemon purpureum*. Each plot was 6 ft x 6 ft (1.83 m x 1.83 m), with a 3 ft (0.914 m) buffer between the randomized replicate rows (Figure 1). Each treatment combination was replicated three times. Additionally, unplanted farmland immediately adjacent to the experiment was sampled as a reference (N = 3)—representative of the field prior to switchgrass establishment. Note that there was very limited survival of Niagara Big Blue and that clover survived better than trefoil (in plot type 6, 9, and 12). Fields were fertilized yearly in May with YaraLiva Tropicote 15.5-0-0, derived from Ammonium Calcium Nitrate Double Salt. Altogether, each sample date resulted in 27 individual samples: three reference plus 12 fertilized and 12 unfertilized plot samples of the four biodiversity combination treatments (each replicated three times).

Soil cores were collected with a slam-bar on a 5 cm diameter split soil core to a depth of approximately 15 cm, within the plowed horizon. Since the plots were planned to be

sampled multiple times, X and Y coordinates within the plot were recorded for each sampling date, within a 10-inch (25 cm) buffer of the edge of the plot. The depth horizon of each sample was referenced to a datum "0 cm" mark started at the thin layer of litter. Soil arthropod samples were extracted from the 0 cm to 5 cm horizon. Roots for mycorrhizal colonization assessment were collected from the 5 cm to 15 cm horizon. Soil nutrients were analyzed from separate cores to a depth of 10 cm after removal of the leaf litter.

Soil nutrients were collected seven times over the three years (Table 1a) and were extracted for extractable N and P within 24 hours of field collection. Leaf litter was removed and the soil homogenized. 10 grams of soil was extracted in 25mL of Bray extract for P and KCl for N. Soil and extracts were shaken at 200 RPM for 60 minutes, and then vacuum filtered through #2 filter paper to separate soil from extract liquids. Extracts were frozen until they could be analyzed. Phosphate (PO_4) was assessed colormetrically using the ascorbic acid method. Nitrate/nitrites (NO_x) and ammonium (NH_4) were run on a Shimadzu TOC-Vcsh non-dispersive infrared gas analyzer and TNM1 chemiluminescence nitrogen monoxide analyzer (Kyoto, Japan).

Soil arthropods were collected six times over the three years (Table 1b) and were extracted by inverting the 5 cm cores in Berlese funnels. The cores were dried for seven days, with the heat/lights being gradually turned up to full strength. In this set up, the soil arthropods will crawl downwards away from the light and towards the attractive 70% methanol mixture with 10% glycerol, which both kills and preserves them. Arthropod morphospecies were assessed with a dissection microscope. To increase reliability of soil arthropod morphospecies identification, only one person did the identification, photos and notes from earlier results were reviewed and the data reclassified as needed, and peer reviewed works were used to guide identification. Collembola were fairly straightforward to

identify to family (using Christiansen & Bellinger, 1998). However, mite morphospecies may have been more heterogeneous. Mites were essentially identified to sub-order (using Dindal, 1990), and then further divisions to morphospecies were only loosely based on published keys.

Roots for mycorrhizal assessment were collected nine times over the three years (Table 1c) and were stored *in situ* in the refrigerator (4°C) for no more than three days before preparation. The roots were washed to remove all soil, then placed in 10% KOH to clear the cells. After seven days at room temperature, the roots were removed, washed three times in tap water, then placed in 1% HCl for 1 min to prepare the roots for staining. The roots were stained in 0.05% trypan blue in lactoglycerol for five days at room temperature. Trypan blue is a stain that selectively stains fungal tissue and not plant tissue (Brundrett et al., 1984). Finally, roots were stored in lactoglycerol until mycorrhizal colonization could be assessed with a modification of the magnified intersections method (Giovannetti & Mosse, 1980; McGonigle et al., 1990) on a compound microscope.

Plant biomass was clipped at ground level in December 2015 from 2 ft x 2 ft (0.61 m) subplots centered within the 6 ft x 6 ft (1.83 m) plots. Plant biomass was placed in Home Depot leaf bags and dried at 70°C to constant weight.

In 2017, plant biomass was subsampled for measuring total N content. Plant material was ground in commercial coffee grinders and 0.1g of ground material was digested in 5mL of Kjeldahl solution at approximately 370°C until the solution cleared entirely (average time: 12 hours). Digests were diluted with DI water to 20 mL then stored in 25 mL plastic bottles in a freezer until the liquids could be analyzed. To provide a check for the digest efficiency, a blank and NIST apple leaf 1515 were included in the digestion. N analysis was conducted using a Shimadzu TOC-Vcsh non-dispersive infrared gas analyzer and TNM1

chemiluminescence nitrogen monoxide analyzer (Kyoto, Japan), following high temperature combustion. A standard curve was run to check the machine efficiency before the samples were analyzed.

Nematodes were sampled in 2015 on a subset of plots, and in 2017, a more thorough sampling occurred. In 2015, about 5 g of homogenized soil from the 5 cm to 15 cm horizon were used. The samples were left for 18 hours in glass funnels with the soil, held on filter paper, in contact with the water for the entire extraction procedure. In 2017, a larger sample was used from the 0 cm to 10 cm horizon (not including the litter layer). Approximately 50 g of homogenized soil were placed on coffee filters, sitting on coated chicken wire in round plastic takeout containers, in contact with water for the duration of the collection time. This method collected more nematodes in the sampling period. Both of these methods work on the same principle, a modification of the Oostenbrink filter method (OEPP/EPPO, 2013). The nematodes move through the soil, enter the water portion of the setup and descend to the bottom of the container since they cannot swim against gravity. The liquid was collected into small 25 mL bottles in 2015 and 250 mL bottles in 2017 and counted immediately while the nematodes were still moving. For 2015, the force of the water descending upon release of the valve ensured that all nematodes that entered the solution made it into the plastic vial. In 2017, squeeze bottles were used to wash out any potential clinging nematodes, the liquid was brought up to 250 mL, stirred to homogenize, and 25 mL of water was counted for living nematodes. Extraction periods between 12 and 24 hours are the recommended period of time to ensure collection of slower moving nematodes, but the time is not too long to cause nematode death due to lack of oxygen diffusion nor to allow any resident eggs of fast breeding nematodes to hatch and skew the results (OEPP/EPPO, 2013).

In 2017, the samples used for nematode assessment were also used to evaluate enzymatic profile of the soil microbial community with BIOLOG ecoplates (Biolog; Hayward, CA, USA). This colorimetric method evaluates the ability of microbes to metabolize 31 known substrates. Measuring microbial community function through measurements of enzyme activity is a time-efficient way to address some ecological questions (Burns et al., 2013; Wang et al., 2015). A suspension of 1 g soil in 99 mL water, shaken for 20 min and allowed to sediment for 30 min in a fridge. A 150 μL aliquot of suspension was dispensed into each well of the BIOLOG ecoplate and incubated at room temperature. Plates were immediately read at 590 nm for background, and again after three and five days of incubation. Background absorption was subtracted from the final absorbance value for each cell and then corrected for the control. Pattern of absorbance values for all substrates was used in a multivariate analysis (PCA) to compare carbon source utilization between treatments.

Statistical analyses were performed in R version 3.5.2 (R Core Team, 2019) and RStudio version 1.1.463 (RStudio Team, 2020). Packages used include vegan (Oksanen et al., 2019), MASS (Venables & Ripley, 2002), tidyverse (Wickham et al., 2019), ggfortify (Horikoshi & Tang, 2018; Tang et al., 2016), Rmarkdown (Allaire et al., 2020; Xie et al., 2018), knitr (Xie, 2020; 2015; 2014), and lme4 (Bates et al., 2015). Data were evaluated with a linear model (LM) and two mixed-effect models where N fertilization was a fixed variable but one LM treated date as a random instead of fixed variable (ME 1) and the second LM treated biodiversity mixture as random instead of fixed variable (ME 2). Treating date or biodiversity mixture as a random variable allows that factor to be accounted for in the model but with fewer constraints. For instance, treating biodiversity mixture as a random variable allows for the possibility that the same pattern exists due to N fertilization between

biodiversity mixtures, but that the biodiversity mixtures might have slightly different mean values (which could throw off the results of the linear model if they were treated as completely independent). Similarly, since the same plots were samples across multiple dates, treating date as a random variable would allow for the replication to be accounted for but not miscounted as pseudoreplication. Akaike information criterion (AIC) was used to determine the best-fit model, because a lower AIC value is taken to mean the model is of better quality, although if AIC values are close ($|3|$), it means the models are essentially of equal quality (e.g., Gutierrez & Heming, 2018). Multivariate analysis of variance (MANOVA) and principle component analysis (PCA) were performed on combined data to better analyze and visualize the complex data.

Results

Three kinds of measurements were sampled multiple times over the 3-year period: extractable soil nutrients, soil arthropod morphospecies composition and mycorrhizal colonization. Individual results will be considered first, and then potential trends over time will be considered across the datasets.

Extractable soil nutrients

The best-fit model for the data for all three nutrients did not show biodiversity mixture to be a significant driver of the results. NH_4 was highest in the fertilized plots, followed by the unfertilized plots, and then the reference area ($P < 0.05$) (Figure 2, Table 2). NO_x was highest in the fertilized versus the unfertilized plots ($P < 0.05$), but the reference value was in-between the two ($P = 0.2$). PO_4 was highest in the unfertilized plots and lowest in the fertilized plots ($P < 0.05$) with the reference in-between the two ($P < 0.1$). Most dates for all three nutrients were significantly different as calculated with a t-test with Bonferroni correction.

Soil arthropod morphospecies

There were 62 soil mite morphospecies identified over all dates and six families of collembola. Abundance of other arthropods was also recorded (including ants, larvae, millipedes, flies, etc.). One morphospecies of mite was present in all samples (sm_white_mite: an indeterminable category), nine other morphospecies of mite were present in over 50% of all the samples. Five of six collembola families were each present in greater than 50% of the samples. Six mite morphospecies were recorded in only a single sample. There were 20 morphospecies of oribatid mites, 38 morphospecies of predatory mites and four mite morphospecies of undetermined category. Variation in morphospecies (across all categories) showed a significant effect of sampling date on the data (Figure 3, Table 3). The umbrella categories of soil arthropods (collembola, oribatid mite, predatory mite, and other mite) similarly showed that sampling date had a significant effect on the data, tested with a t-test and Bonferroni correction (Table 4). For each of the four groups of soil arthropod, the best-fit model included biodiversity mixture as a random effect. There was a lot of variation within the data. No consistent pattern existed across all dates between the reference, unfertilized biodiversity type 1 and fertilized biodiversity type 1 for any of the four groups of soil arthropods (Figure 4).

MANOVA and PCA were performed on a log transformation of the soil arthropod morphospecies data to analyze whether the soil communities differed between field treatments. Each date was analyzed separately to account for the inherent variation between sampling events. N fertilization was significant on the following dates: June 11, 2013 ($P = 0.039$), July 9, 2013 ($P = 0.001$), August 20, 2014 ($P = 0.010$), July 10, 2015 ($P = 0.001$), and October 14, 2015 ($P = 0.002$). Neither biodiversity mixture nor the interaction was significant on any date. Pairwise comparison shows that fertilization had a significant effect

(unfertilized vs. fertilized) on three of the dates, and the reference area was significantly different from planted areas (either unfertilized, fertilized, or both) on four dates (Table 5).

Grouping all the soil arthropod morphospecies data together is not statistically sound because different sampling dates have differing weight in the total dataset. However, grouping the data can show if there is any underlying pattern missed by looking at each date separately. The PCA shows no separation of treatments and MANOVA does not indicate any significant differences among treatments in the aggregated data set (Figure 5).

Mycorrhizal structures

The percent of views (of 50) with hyphae present ranged from 78% to 85%. Hyphae alone are not necessarily AMF. The sum of the three active structures which indicate AMF (coils, arbuscules, and paris) ranged from a mean total count of 43.5 to 55.2 (out of 150) per biodiversity plot over all dates (Figure 6, Table 6). Counts of four structures (spores, coils, arbuscules, and vesicles) were individually square-root transformed for analysis. The linear model was the best-fit model in all cases. Arbuscules were higher in fertilized plots ($P<0.05$) but spores were higher in unfertilized plots ($P<0.05$). Date was a significant factor for all four structures, although the number out of nine dates differed from two to nine (Figure 7).

PCA and MANOVA were run for untransformed mycorrhizal counts for each date separately. Counts of mycorrhizal structures were not normalized since the scale itself (out of 50 views) was consistent across all measurements. The MANOVA results showed fertilization to be significant on these dates: September 9, 2013 ($P = 0.001$), June 11, 2014 ($P = 0.001$), August 20, 2014 ($P = 0.085$), November 7, 2014 ($P = 0.052$), July 10, 2015 ($P = 0.002$), and October 14, 2015 ($P = 0.003$); biodiversity mixture to be significant on these dates: August 12, 2013 ($P = 0.005$), November 20, 2013 ($P = 0.054$), August 20, 2014 ($P = 0.054$) and July 10, 2015 ($P = 0.017$); and the interaction to not be significant on any date.

However, pairwise comparison shows that the significance in fertilization is due to differences between reference area and planted land on five dates, and only on two dates does N fertilization lead to significant difference between the unfertilized and the fertilized treatments (Table 7). Although it is not totally statistically sound, when all dates were combined together, fertilization was significant ($P = 0.003$), but pairwise comparison shows this difference is due to reference vs. planted (Figure 8).

Time factor

The three datasets that were measured over multiple years could have a component of directionality associated with time beyond the fact that different dates have different measurements. Linear modeling of soil extractable nutrients (Figure 9, Table 8) showed that only NO_x has a significant relationship (increasing) due to time. Additionally, the signal from lower values of PO_4 in fertilized plots is strong enough to be statistically significant in this analysis. Change over time in the mycorrhizal structures or the soil arthropod community could be expressed as narrower or wider divergence between the groupings in the PCA between the three years. Mycorrhizal structures by grouped by individual plot types (reference, biodiversity type 1 unfertilized and biodiversity type 1 fertilized) showed directional movement across the PCA plot for all three years and confirmed by MANOVA, however pairwise comparison showed no difference between years (Figure 10, Table 9). Soil arthropod morphospecies grouped by plot type (reference, unfertilized biodiversity type 1 and fertilized biodiversity type 1) similarly showed a pattern of directional movement across the PCA plot and a variation in group spread for all plot types (Figure 11). Pairwise comparison showed that 2015 differed from 2013 and 2014 in all biodiversity mixtures (Table 10).

Additional measurements

In 2015, biomass was collected from a subplot within each of the plots (Figure 12). Because this measurement necessarily required mature, unmown biomass, there was no comparable measure taken from the reference area of unplanted farmland. There was higher biomass in the fertilized plots (P = 0.070), but also a wider range of variation (Figure 12).

The biomass was subsampled in 2017 and digested for N content, but there was no pattern in the results (Figure 13). Additionally, there was no trend between biomass density in the plots and the biomass N content. Correlation between soil N (NH_4 and NO_x) and plant N was positive but not significant (Figure 14).

In November 2015 and July 2017 nematode samples were collected (Figure 15) from switchgrass-only plots (biodiversity type 1). The method in 2017 was slightly more reliable because more soil was used which makes it more likely that the results were not thrown off by patchy distributions. A linear model was used for each year separately. In 2015, the highest abundance of nematodes was in unfertilized plots, and the lowest in the reference plots (P = 0.40). In 2017, the highest nematode abundance was found in the fertilized plots (P = 0.20) and the lowest in the reference plots (P = 0.08).

In July 2017, samples were also taken to look at bacterial community using BIOLOG ecoplates from switchgrass only plots (biodiversity type 1). Data were transformed before use in PCA and MANOVA analyses (Figure 16). MANOVA results show significance due to treatment between reference, fertilized and unfertilized treatments (P = 0.04), and adjusted pairwise comparison shows that the difference was due to a difference between reference and unfertilized planted areas rather than due to N fertilization alone.

Discussion

The original experiment onto which this research was built was designed to manipulate plant biodiversity and fertilization. At the outset, it seemed logical to sample from plots with multiple plant species in them in addition to the switchgrass-only plots to capture a wider range of variation. However, upon review of the results, the biodiversity treatments added complexity to data that were already complex due to the very nature of soil ecological measurements. In almost all cases, biodiversity mixture (the factor that was not fully factorial) did not explain any more variation in the data than N fertilization or sampling date, and therefore it was legitimate to treat it as a random variable.

Extractable nutrients and plant biomass

Fertilization caused significant differences in extractable nutrients, which supports the hypothesis that fertilization is changing the soil. In the fertilized plots, NH_4 and NO_x were highest and PO_4 was lowest (Figure 2). Time was not a significant factor for NH_4 or PO_4, but there was a significant positive trend in NO_x (Figure 9), which supports the hypothesis that fertilization could have a building effect over time. However, accounting for the correlation in repeat-measurements is difficult, so this conclusion should be further tested with more statistical rigor. Additionally, by definition, the plant-available forms of N and P are not permanently stored in the soil. NO_x in particular is susceptible to leaching. Is it then reasonable to conclude that the soil has fundamentally shifted based on these temporary forms of nutrients?

There was an inverse relationship between mean values of extractable N and extractable P in fertilized versus unfertilized plots. However, individual results actually showed a statistically significant ($P = 0.004$) positive relationship between P and the

combined N measurements (NH_4 and NO_x). The relationship between extractable P and extractable N could be further explored, but is beyond the scope of this experiment.

Plant biomass yields and plant N content were not affected by fertilization (Figure 12-13), although there were higher biomass yields on average in fertilized plots ($P > 0.1$). The finding that the significant changes in soil extractable nutrients does not result in significant change in plant biomass could be related to limitation by other nutrients in the soil (Liebig's law of the minimum). The fact that P was lower in fertilized plots suggests that P regulation is a possible explanation. Some research suggests that the impact of fertilization may not be best measured by extractable N, but rather by base cation availability, such as magnesium (Mg^{2+}) and Ca^{2+} (i.e., Bowman et al., 2018). However, substantial foundational research, even on marginal land, shows that switchgrass does not respond to other fertilizers—including P, K, Mg, and Ca (McLaughlin & Kszos, 2005), though more recent research has shown limitation of P and K impacts biomass yields in two-crop production systems (Ashworth et al., 2019). If nutrient limitation explained the lower growth, that fact should be more present in the literature. Thus, it is not a complete explanation of what is happening for switchgrass biomass yields in this experiment.

The findings that soil N does not correlate with stem N (Figure 14) align with research that says that N deposition does not lead to increased plant N. A major theory to explain this is that the planetary system is past the point of N saturation (Bobbink et al., 2010; Bowman et al., 2018; Galloway et al., 2008). As just discussed, it could certainly be the case that the good farm soil at Adelphia has excess N availability.

Perhaps the disconnect between soil extractable nutrients and stem biomass can be explained by the theory that the soil is more sensitive than the switchgrass plants. Tian and Niu (2015) find in a meta-analysis that soils are very sensitive to N addition, although the

strongest effect was in response to NH_4NO_3 fertilizer, which was not used for this experiment. Yet, it is well proven that acidification of the soil due to N deposition is a strong factor in changes to the soil, plant community, and soil community (Bobbink et al., 2010; Bowman et al., 2018; Damgaard et al., 2011). Unfortunately, pH was not measured, which could have proven whether the conditions of the soil fundamentally shifted.

It was hypothesized that yearly fertilization was changing the soil chemistry, and in fertilized areas, extractable N was higher and extractable P was lower. It was predicted that fertilization would increase plant biomass yields, but although biomass yields were higher in fertilized plots, the difference was not statistically significant. Finally, it was predicted that soil nutrients would correlate with plant N content, and that does not hold up. Thus, while fertilization is having an effect on the soil, it is not consistently affecting the plants.

Mycorrhizae

MANOVA of mycorrhizal structures were statistically different due to fertilization on 2 of 9 dates, but were different between reference and either or both planted treatments on 5 of 9 dates. This suggests that fertilization is not having as strong an effect as the effect due to replacing mowed farmland with switchgrass. However, the individual mycorrhizal structures show a slightly different pattern. Arbuscules are higher in fertilized plots, spores are lowered by N treatment, and coils and vesicles are unaffected (Figure 7). Taken together, these data support the hypothesis that fertilization is changing the mycorrhizal community but not comprehensively. These results correlate with the findings of other research projects where AMF respond inconsistently to N. One paper finds the higher ambient N levels in the soil reduce spore abundance in the field, but in the greenhouse, there is no difference in colonization rates due to N fertilization (Miller & Jackson, 1998). Another paper finds that there is significant difference in the species composition due to N fertilization, but not in

total colonization (Jumpponen et al., 2005). Jach-Smith and Jackson (2018) hypothesized that there is a "curvilinear relationship between N availability and plant mycorrhizal growth response where mycorrhizal associations do not increase plant growth if both AMF and plant are N-limited."

There is no consensus of specific seasonal patterns in mycorrhizal colonization. However, some research suggests that spore and vesicle development happen after plant roots stop actively growing (Helgason & Fitter, 2009). The results of this experiment do not support this (Figure 7). For spores, the lowest abundance was on the sampling date in June, 2014, which supports this idea. However, the sampling dates in October, 2015 and November of all three years do not have consistently higher spore abundance than the dates in July, August, and September. Additionally, vesicles are lower in the three November sampling dates, which contradicts this theory. However, vesicles were also low in June, which does confirm this theory. As vesicles are storage units, the low abundance in the beginning and end of the season could make sense. There could be more short term turn over than previously described; vesicles may not develop until after the growing season is in full swing and the fungi have time to build extra resources, but then by November the fungi have needed to use up the extra resources. For arbuscules, the later dates in November are lower than earlier in the year, which supports the theory that structures relate to plant activity. This seasonal ebb and flow is worthy of more research.

PCA and MANOVA of individual plot types over time suggest that mycorrhizal colonization could be fundamentally changing due to fertilization. However, a shift in mycorrhizal colonization over the years would be somewhat confounded by seasonal patterns. Given that there is no consensus about seasonal variation, accounting for that fluctuation in order to understand larger patterns is challenging. Additionally challenging is

the conclusion that the response of AMF to fertilizer is not comprehensive in the first place. However, these results suggest that mycorrhizal colonization trends are worthy of further investigation.

It was hypothesized that N fertilization would reduce mycorrhizal colonization. N fertilization increased arbuscules and decreased spore occurrence with statistical significance. However, these shifts due to fertilization were not consistent enough to lead to a statistically significant pattern in a multivariate analysis across all 9 sampling dates. However, there is evidence that an underlying trend over time exists because multivariate analysis yielded statistically significant results due to N fertilization on 2 of 9 dates. Thus, the hypothesis that N fertilization is fundamentally changing the mycorrhizal community is partially upheld. However, the only concrete statement that can be made based on these data is that fertilizer is changing the community to a limited extent, and more research is needed.

Soil arthropods, nematodes and bacterial community

MANOVA of soil arthropod communities were statistically different due to fertilization 3 of 6 sampling dates, but were different between reference and either or both planted treatments on 4 of 6 dates. This indicates that fertilization is having an effect, but it is not consistent, and it is about as strong as the effect of planting tall, perennial grasses compared to mown field. This is corroborated by looking at the total morphospecies identified (Figure 3)—there was no statistical difference due to treatment, but there was marginal significance ($P < 0.1$) between reference and unfertilized plots. There were slightly higher numbers of morphospecies identified in the planted areas. The fact that reference was not different than planted areas in the PCA of every sampling date also shows that there is a lot of complexity and variation in the soil arthropod community.

The finding that there is not a consistent and clear difference between reference areas and the fertilized and unfertilized switchgrass areas is surprising because there was a layer of litter in both planted treatments but not in reference areas. Litter quality has been shown to strongly affect soil arthropod communities (e.g., Sauvadet et al., 2017). Because soil arthropods play an important role in litter decomposition (e.g., Carrillo et al., 2010; Soong et al., 2015), it would be more logical if every sampling date, there would have been differences between the reference areas, which often had bare areas intermixed with less than 0.2" of litter, and the planted areas, which had an average of 0.75" of litter.

One possible confounding factor to these data overall is that classification of soil arthropod morphospecies is not completely consistent across dates. However, care was taken in the data collection and review of the data to address this issue. Additionally, because of this probably inconsistency, individual morphospecies were only used in the PCA/MANOVA analysis as a snapshot of the community.

A second confounding factor is the patchy distribution of soil arthropods. One species of juvenile oribatid mites shows this very well; the morphospecies was only found in four plots on one date and when it was found, it had a high abundance. This is a well-known problem, and the transformation using log10 is a widely accepted way to account for this (Southwood & Henderson, 2000). However, although this method homogenizes the variance, it does not completely eliminate the spatial heterogeneity.

The patchy distribution of soil arthropods, which leads to the wide variation in species present due merely to sampling date, means that describing a fundamental shift in the soil arthropod community will be hard to describe statistically. However, as just described for mycorrhizal structures, PCA may show a change through either a directional shift in space or the development of greater separation between the communities with

different fertilization regimes over time (Figure 11). There are directional shifts between the years for soil arthropod communities, and MANOVA confirms that year is a significant factor. However, individual date also was a significant result in the MANOVA. As just described, variation between individual dates is a confounding factor to conclusions that can be made about these data. Though MANOVA took both year and date into account, it is possible that the variation in individual dates could be such a strong factor that it masked any true pattern due to year. Similar to the conclusion that was drawn for mycorrhizal structures, completely concrete conclusions cannot be made. Some of the sampling dates show significant differences due both to fertilization and a difference between reference and planted areas. Some evidence suggests that there are larger shifts over time due to fertilization. However, more research is needed to understand the mechanisms at play and to ensure that confounding factors are not leading to false conclusions.

Two studies delve into the complexity of soil arthropod communities in response to N perturbation. Gan et al. (2014) found that microarthropods are relatively "stable" in response to chronic N deposition. Shao et al. (2018) saw a significant decrease in microbial diversity and a non-significant positive increase in soil invertebrate diversity in the presence of shrubs in response to N deposition, but no change in the soil food web structure, most critically in there being stability of predator-prey interactions. They concluded that the presence of shrubs has a buffering effect of the N addition to the soil community and soil food web structure (Shao et al., 2018). These two studies showed that even when change is observed, understanding the mechanism is still hard.

Nematode abundance was lowest in reference areas and highest in fertilized plots, but unfertilized plots were not statistically different from either measure (Figure 15). Similarly, microbial community function was statistically different between reference and

planted areas, but fertilization alone did not lead to a difference in community function (Figure 16). These results were both sampled from switchgrass only plots in 2017 and the plots were not fertilized in 2017. However, the entire premise of this research is that lag effects from years of fertilization are important, thus the previous consistent fertilizing would be enough to leave a lingering effect if it existed. So, the fact that there was a significant difference between reference and planted areas but not between fertilized and unfertilized areas supports the idea that there is no accumulating effect over time due to fertilization.

It was hypothesized that the soil community would change over time in response to changes induced by fertilization and a shift in mycorrhizal function. Because only a few sampling dates had statistical differences in the mycorrhizal structures and soil arthropod community due to fertilization and there was no difference between microbial community function and nematode abundance due to fertilization, it is reasonable to conclude that the effect of N fertilization is not strong, consistent or increasing over time. However, using PCA and MANOVA to show trends over time on combined sampling dates of mycorrhizal structures and soil arthropods resulted in year being significant. These results are somewhat contradictory to the conclusion, and indicate that change over time could be happening. However, accounting for the confounding factor of repeated measurements through time is difficult. Therefore, this trend of change through time in the mycorrhizal structures and soil arthropod community should be investigated further. Other research in agricultural settings has found lag effects of fertilizer on nematode communities (Gruver et al., 2010), which further supports the conclusions made in this research that the disturbance of N fertilization is worth further investigation. It is crucial to point out that this was highly fertile farm soil, and N should not be a limiting factor. However, another unexpected finding is that organic

matter also was not a strong factor in shaping the soil arthropod community, because the reference was not consistently different from planted areas.

Conclusion

Fertilization is changing the soil extractable nutrients. Fertilization is changing some measures of mycorrhizal colonization and soil arthropod community but not in the strong, clear, consistent way that was hypothesized. Fertilization is changing neither nematode abundances nor microbial community function. Because a wide variety of data was measured, which by necessity limits the time that can be dedicated to each measurement, it is possible the resolution was not fine enough to capture changes. However, since the experimental measurements capture differences between reference and planted areas, the resolution of data should have been enough to capture differences if they existed. Substantial research supports the hypothesis that N fertilization has a negative impact on ecological communities. However, these results suggest that while fertilizer is having a measurable effect on the soil, it is not having a strong effect on plant growth, mycorrhizal structures, nor the soil community. These results neither support nor completely contradict the hypothesis that consistent yearly fertilization is having a building effect on the soil and soil community. If fertilization is changing the soil extractable nutrients, but not strongly affecting switchgrass, it is reasonable to expect to see cascading effects somewhere in the soil community in response to that fundamental change, but that was not found. This contradiction in the results shows that the interactions within a soil community are worthy of further investigation.

Nutrient balance, limitation, and nutrient cycling has been much researched, but these results did not align with commonly presented conclusions about the effects of N fertilization. This topic is worth further investigation with a continued interdisciplinary

focus. The clearest step for future research is to look into switchgrass growing on marginal land. This Freehold sandy loam is rated as good farm soil, and that could be limiting the necessity of N fertilization, since N is not significantly limited in the first place. Another direction for further study is to better understand seasonal changes in mycorrhizal structures and soil community composition to better understand changes to these systems that are due to perturbation versus natural shifts.

Tables

Table 1

Dates samples were collected for a) soil extractable nutrients b) soil arthropods and c) mycorrhizal colonization. Year is listed first corresponding to each row, followed by month/day in the subsequent cells.

a)

2013	7/27	10/28
2014	6/28	
2015	7/23	11/12

b)

2013	6/9	7/9
2014	6/11	8/20
2015	7/10	10/14

c)

2013	8/12	9/9	11/20
2014	6/11	8/20	11/7
2015	7/10	10/14	11/18

Table 2

Soil extractable nutrients were collected and analyzed for each individual treatment type. Biodiversity mixture was not statistically significant, and therefore was treated as a random variable in the best-fit mixed effect model. This table shows the p-values from the analysis of each individual mixed-effect model (ME Model 2) for NH_4, NO_x, and PO_4. "0.000" values indicate a p-value of less than 0.001. Multiple sampling dates were different with statistical significance. Fertilized areas were significantly higher for NH_4 and NO_x compared to unfertilized areas. PO_4 was significantly lower in fertilized than unfertilized areas. Graphs of these data can be found in Figure 2.

ME Model 2	NH_4	NO_x	PO_4
Fertilized	0.012	0.000	0.000
Reference	0.006	0.169	0.081
Oct 2013	0.000	0.067	0.000
Aug 2014	0.030	0.000	0.001
Jul 2015	0.100	0.000	0.344
Nov 2015	0.011	0.000	0.024

Table 3

Total soil arthropod morphospecies were identified by treatment type over all dates, however biodiversity mixture was not significant. Thus, a mixed effect model with biodiversity type as a random variable was used to analyze total soil arthropod morphospecies. Results showed that unfertilized, fertilized and control areas were not statistically different. However, the sampling dates were statistically different. "0.000" values indicate a p-value of less than 0.001. Graphs of these data can be found in Figure 3.

ME model	Pr(>|t|)
Fertilized	0.830
Reference	0.073
July '13	0.000
June '14	0.265
Aug '14	0.039
July '15	0.000
Oct '15	0.000

Table 4

Abundance within morphospecies of each umbrella category (Collembola, Oribatid mites, Predatory mites, Other mites) of soil arthropods was tested with a mixed effect model which included biodiversity mixture as a random variable. N fertilization did not cause statistically significant difference in the for any of the 4 groups. Collembola were statistically lower in reference areas compared to both fertilized and unfertilized areas. "0.000" values indicate a p-value of less than 0.001. Graphs of the data can be found in Figure 4.

ME Model 2	Collembola	Oribatid Mites	Predatory Mites	Other Mites
Fertilized	0.875	0.852	0.987	0.278
Reference	0.000	0.640	0.083	0.332
July '13	0.939	0.000	0.000	0.828
June '14	0.023	0.000	0.004	0.000
Aug '14	0.000	0.000	0.001	0.001
July '15	0.465	0.000	0.000	0.005
Oct '15	0.206	0.000	0.000	0.000

Table 5

Adjusted P-values calculated using nonparametric MANOVA of soil arthropod morphospecies by date and N type (UnF = unfertilized, F = fertilized, and R = reference). Unfertilized soil communities differed statistically significantly from fertilized soil communities on 3 of 6 dates, but did not achieve statistical significance when all dates were combined. Unfertilized soil communities differed statistically significantly from the reference soil communities on 4 of 6 dates, and was statistically significant when all dates were combined. Fertilized soil communities also differed statistically significantly from the reference soil communities on 4 of 6 dates, and when all dates were combined. Biodiversity mixture was included in the initial analysis, but was not significant, so only N fertilization was used in this follow-up analysis of the data. Each individual treatment had 3 replicates, hence the decision to use nonparametric pairwise comparison to identify individually significant sample dates. Principle component analysis (PCA) of these data can be found in Figure 5.

Fertilizer	Jun 2013	Jul 2013	Jun 2014	Aug 2014	Jul 2015	Oct 2015	All dates
UnF vs. F	0.012	0.003	1.000	0.378	0.093	0.021	1.000
UnF vs. R	0.261	0.012	0.531	0.036	0.009	0.012	0.036
F vs. R	0.195	0.015	0.303	0.042	0.018	0.021	0.072

Table 6

Linear model was the best-fit model for all four structures. This table shows the p-values that correlate with mycorrhizal structures over time for all treatment types and sampling dates. N fertilization was associated with significant differences between fertilized and unfertilized areas for arbuscules and spores, whereas the reference area was different from fertilized and unfertilized areas for coils and vesicles. Sampling date led to statistically significant differences in the measured results. "0.000" values indicate a p-value of less than 0.001. Graphs of these data can be found in Figure 7.

Linear Model	Coils	Arbuscules	Vesicles	Spores
Fertilized	0.871	0.000	0.388	0.002
Reference	0.000	0.880	0.001	0.040
Type 6	0.012	0.295	0.507	0.227
Type 9	0.066	0.941	0.600	0.841
Type 12	0.489	0.997	0.628	0.852
July '15	0.984	0.111	0.006	0.000
Aug '13	0.414	0.481	0.936	0.000
Aug '14	0.178	0.054	0.232	0.000
Aug '13	0.771	0.178	0.390	0.005
Aug '15	0.079	0.002	0.036	0.000
Nov '14	0.002	0.000	0.000	0.000
Nov '15	0.068	0.000	0.008	0.000
Nov '13	0.000	0.000	0.000	0.000

Table 7

Adjusted P-values calculated using nonparametric MANOVA for mycorrhizal structures. Table is organized by date and N type (UnF = unfertilized, F = fertilized, and R = reference). Unfertilized mycorrhizal structures differed statistically significantly from fertilized structures on 2 of 9 dates, but when all individual dates were combined, the p-value was not statistically significant. Unfertilized mycorrhizal structures differed statistically significantly from reference structures on 4 of 9 dates, and also when all dates were combined. Fertilized mycorrhizal structures differed statistically significantly from reference structures on 4 of 9 dates, and also when all dates were combined. Biodiversity mixture was included in the initial analysis, but was not significant, so only N fertilization was used in this follow-up analysis of the data. Each individual treatment had 3 replicates, hence the decision to use nonparametric pairwise comparison to identify individually significant sample dates. PCA of these data can be found in Figure 8.

Fertilizer	Aug 2013	Sep 2013	Nov 2013	Jun 2014	Aug 2014	Nov 2014	Jul 2015	Oct 2015	Nov 2015	All Dates
UnF vs. F	1.000	0.339	0.206	0.609	0.162	1.000	0.039	0.006	1.000	0.099
UnF vs. R	0.579	0.030	1.000	0.009	1.000	0.231	0.036	0.042	0.189	0.006
F vs. R	0.444	0.039	1.000	0.012	1.000	0.048	0.174	0.243	0.048	0.003

Table 8

Each extractable soil nutrients were analyzed with a linear model including time as a fixed variable. This table shows p-values are shown from the results. A trend of an increase in NO_x is the only relationship for nutrients over time that was significant (P = 0.007). This linear model also supports that PO_4 was significantly lower in fertilized areas compared to unfertilized areas. Graphs of these data can be found in Figure 9.

	NH_4	NO_x	PO_4
Time Proxy	0.885	0.007	0.632
Fertilized	0.928	0.043	0.024
Reference	0.024	0.508	0.259

Table 9

Adjusted P-values calculated using nonparametric MANOVA of mycorrhizal structures grouped by year and N fertilization. Results showed no statistically significant differences between N treatment and across all three years. Data used only for one biodiversity mixture: type 1, switchgrass-only. Each individual treatment had 3 replicates, hence the decision to use nonparametric pairwise comparison to identify individually significant differences. PCA graphs of these data can be found in Figure 10.

Year	Reference	Unfertilized	Fertilized
2014 vs. 2015	1	0.537	1.000
2014 vs. 2013	1	1.000	1.000
2015 vs. 2013	1	0.501	0.669

Table 10

Adjusted P-values calculated using nonparametric MANOVA of soil arthropod morphospecies grouped by year and N fertilization. Results showed that across all three N treatments, 2015 soil community was significantly different than in 2013 and 2014, but 2013 and 2014 are not significantly different from each other. Data used only for one biodiversity mixture: type 1, switchgrass-only. Each individual treatment had 3 replicates, hence the decision to use nonparametric pairwise comparison to identify individually significant differences. PCA graphs of tehse data can be found in Figure 11.

Year	Reference	Unfertilized	Fertilized
2013 vs. 2014	1.000	0.246	0.231
2013 vs. 2015	0.039	0.039	0.015
2014 vs. 2015	0.009	0.003	0.015

Figures

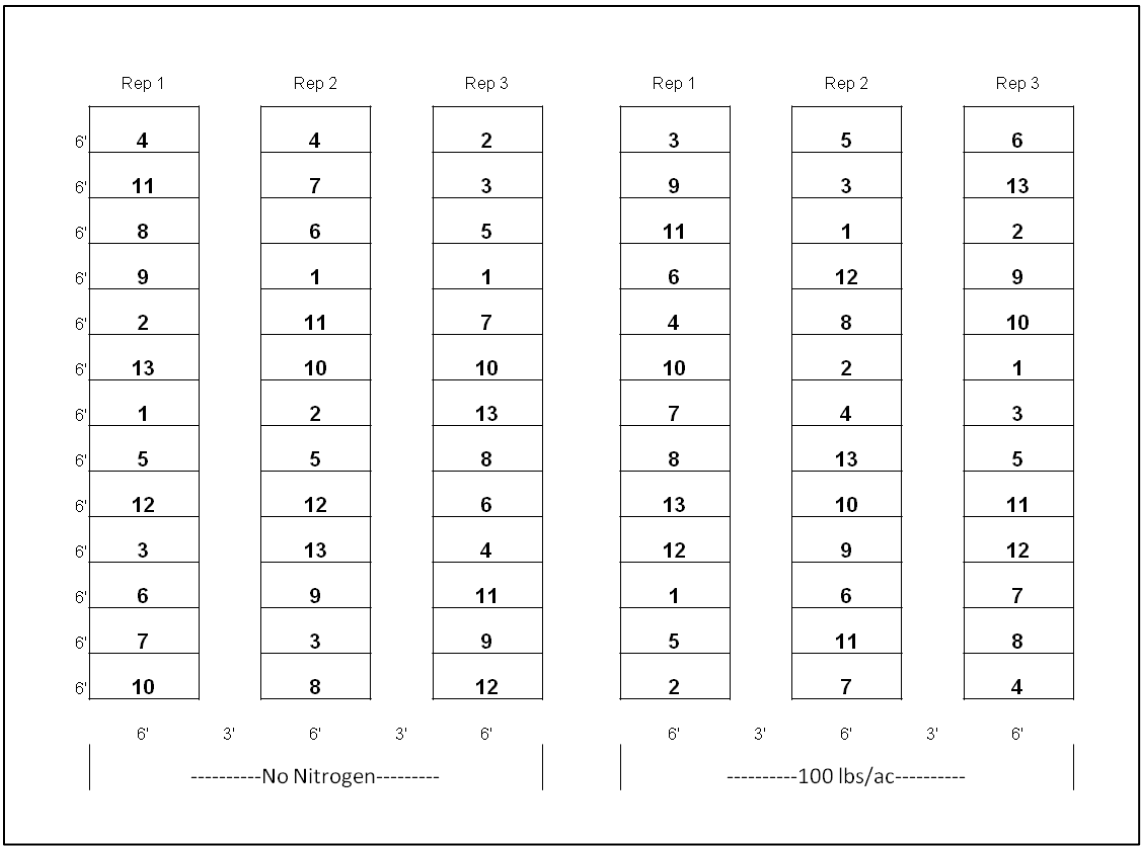
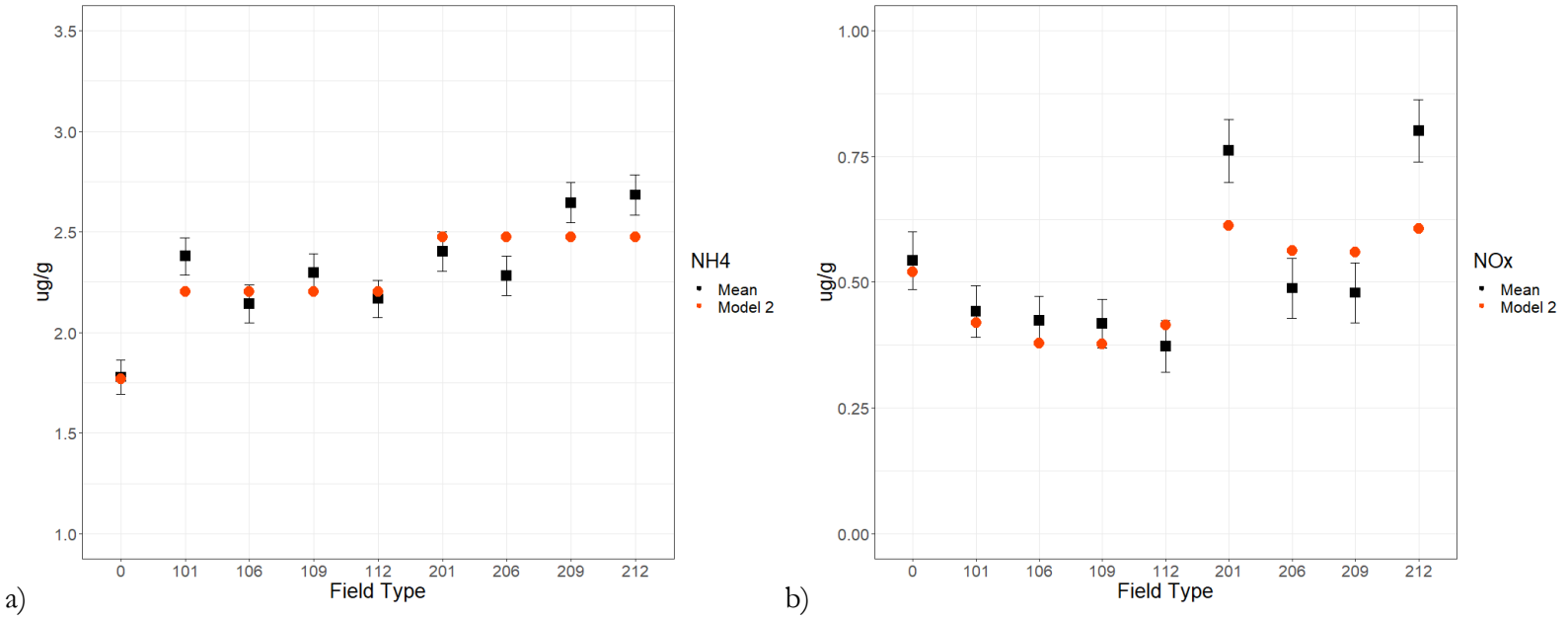


Figure 1

Layout of the field design. Type 1, 6, 9 and 12 were sampled. Location within the plots were recorded for each sampling date to ensure no direct overlap since these plots were sampled for 3 years. Scientific plant names are as follows: Niagara Big Blue: *Andropogon gerardi*, Prairie cordgrass: *Spartina pectinata*, Panic grass: *P. amarum*, Trefoil: *Desmodium canadense*, Clover: *Dalea purpurea* alternate name *Petalostemon purpureum*.

Type	Biodiversity ID
1	Cave-in-Rock (CIR)
2	Niagara Big Blue (BB)
3	Prairie cordgrass (PC)
4	Panic grass (PG)
5	CIR/BB/PC
6	CIR/BB/PG
7	CIR/PC/PG
8	CIR/BB/PC/trefoil
9	CIR/BB/PG/trefoil
10	CIR/PC/PG/trefoil
11	CIR/BB/PC/clover
12	CIR/BB/PG/clover
13	CUR/PC/PG/clover

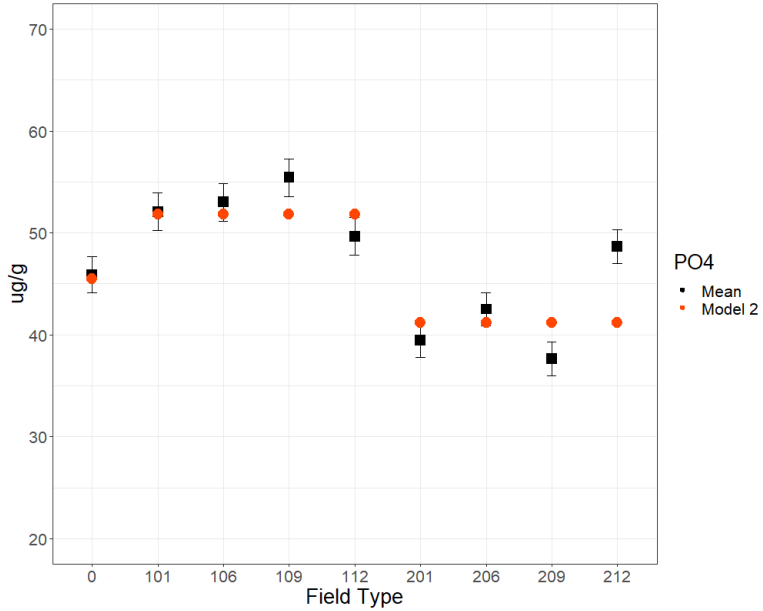


a) b)

Figure 2

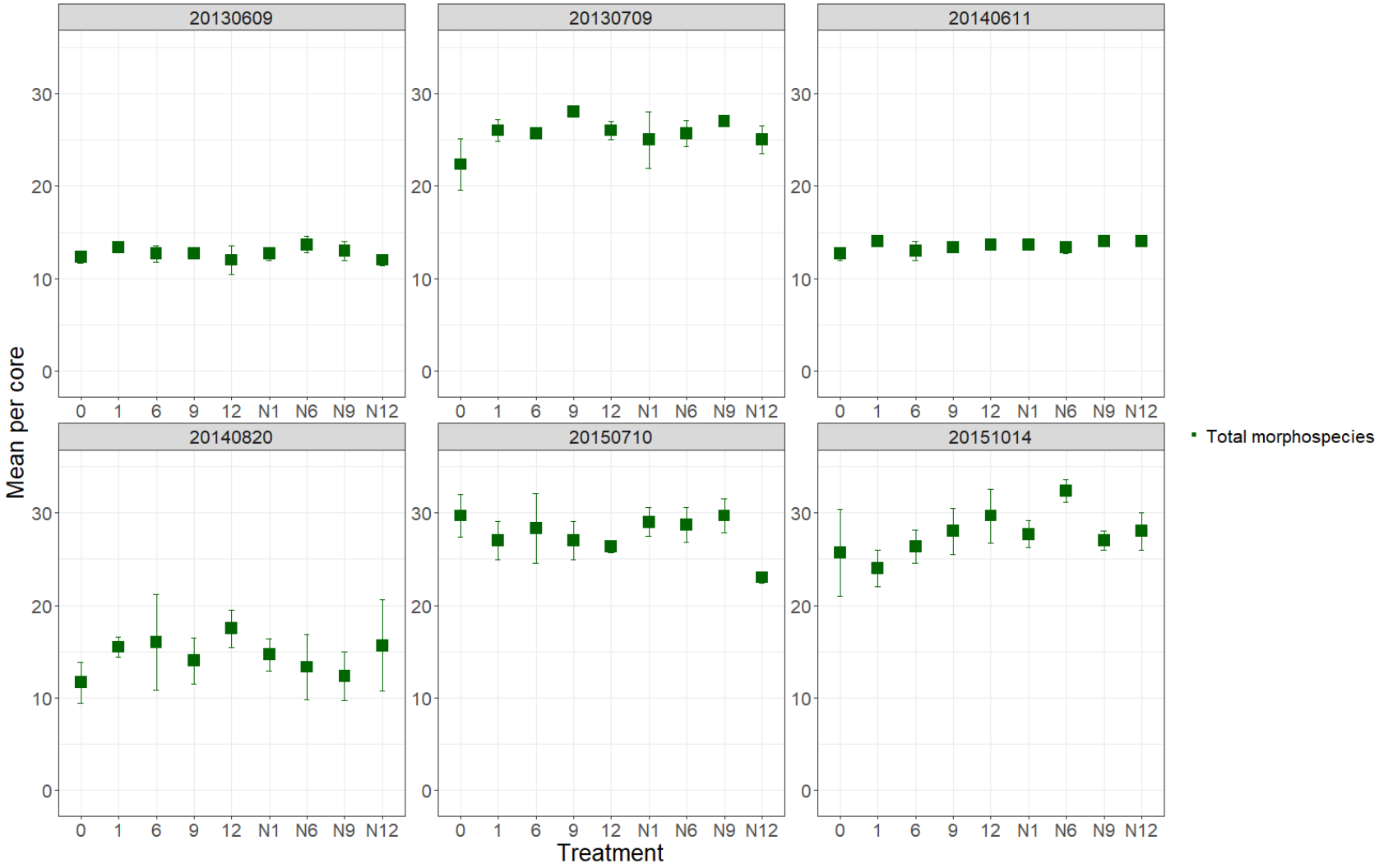
Mixed effect model (ME Model 2) with biodiversity mixture as a random effect shows that the fertilized plots are significantly different than the unfertilized plots for all three extractable nutrients—a) NH_4 b) NO_x and c) PO_4. P-values located in Table 2. Additionally, the reference differs from the unfertilized plots for NH_4. Mean values with standard error bars are identified by black squares. Orange dots identify the model predictions for each value, showing good correlation with the data. Field type labels identify biodiversity types ([#]01, [#]06, [#]09, [#]12), fertilized plots (2[# #]), unfertilized plots (1[# #]), and reference (0).

(Figure 2 continued)



c)

Figure 3


20130609
20130709
20140611
20140820
20150710
20151014
Mean per core
Treatment
0 1 6 9 12 N1 N6 N9 N12
0 10 20 30
Total morphospecies

(Figure 3 continued)

These graphs show total morphospecies, identified by individual treatment type, over all sampling dates, which are noted above each graph as YYYYMMDD. There is clearly an effect of sample date on the results since mean number of morphospecies varies; three dates group together with a higher number of morphospecies and three dates group with a lower number of morphospecies. A mixed effect model with biodiversity mixture as a random variable shows that unfertilized, fertilized and reference areas are not statistically significant, but the dates are, which correlates with the trend that can be seen here. P-values can be found in Table 3. Labels for the graph are as follows. Type 0 is the reference —unplanted farm soil adjacent to the field. Type 1, 6, 9, and 12 are the biodiversity mixture types and were sampled from unfertilized ([#]) and fertilized (N[#]) treatments (1 = switchgrass, 6 = grass mix, 9 = grass mix & trefoil, 12 = grass mix & clover)..

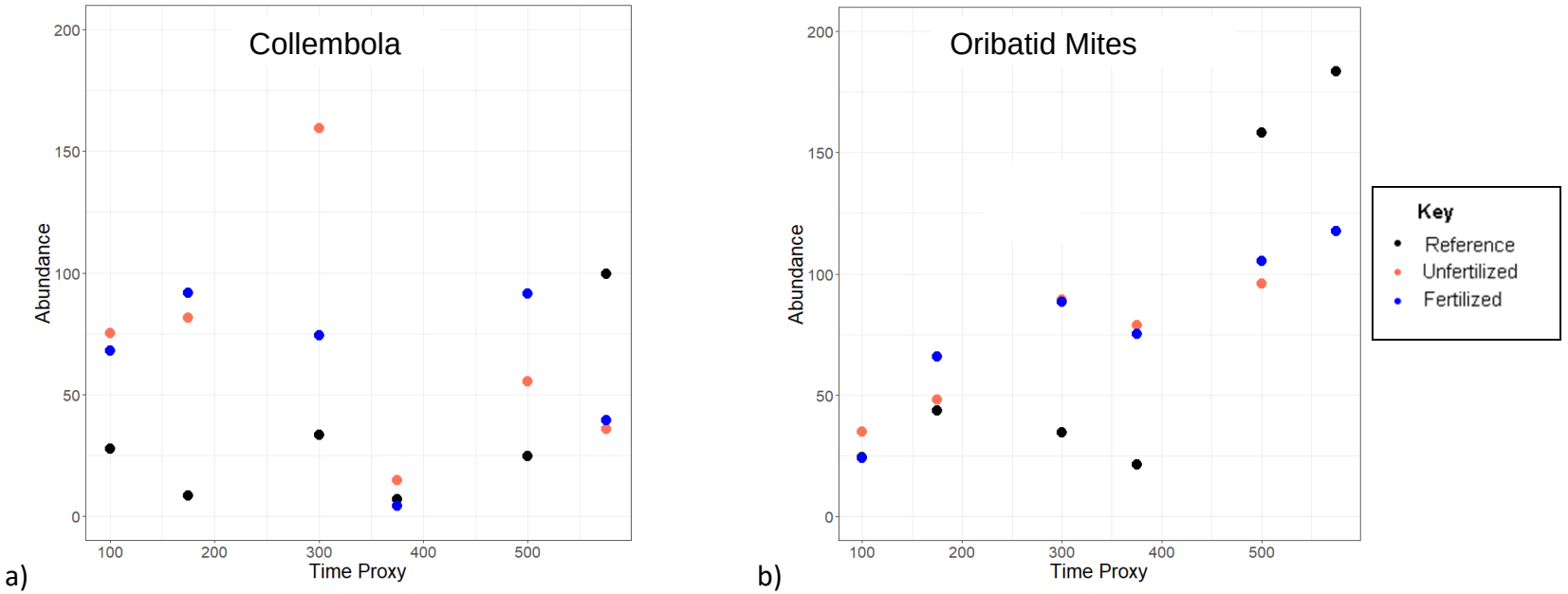
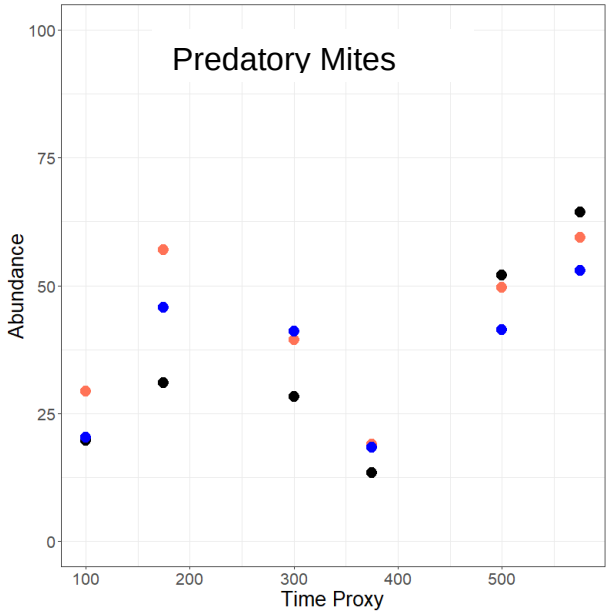


Figure 4

Switchgrass-only biodiversity mixtures are graphed here to display any trends for the four groups of soil arthropods (though all biodiversity mixtures were analyzed). Graphs show the umbrella groups of a) Collembola b) Oribatid mites c) Predatory mites d) Other mites. A "time proxy" instead of exact date is used to ease graphing; with 100/175 as the two dates in 2013; 300/375 as the two dates in 2014; and 500/575 as the two dates in 2015. Abundance within morphospecies of each umbrella category of soil arthropods was tested with a mixed effect model which included biodiversity mixture as a random variable. Sample date led to statistically significant differences in abundance.

(Figure 4 continued)

N fertilization did not lead to statistically significant difference in the abundance variation for any of the four groups. However, there is a statistically significant difference between reference and the planted areas for collembola, with lowest values in the reference areas. P-values from the analyses can be found in Table 4.



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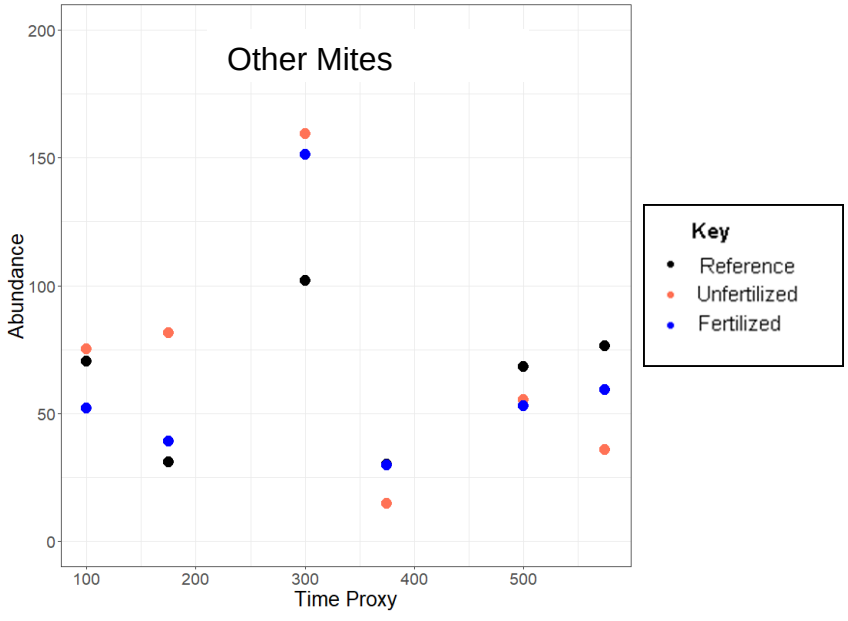

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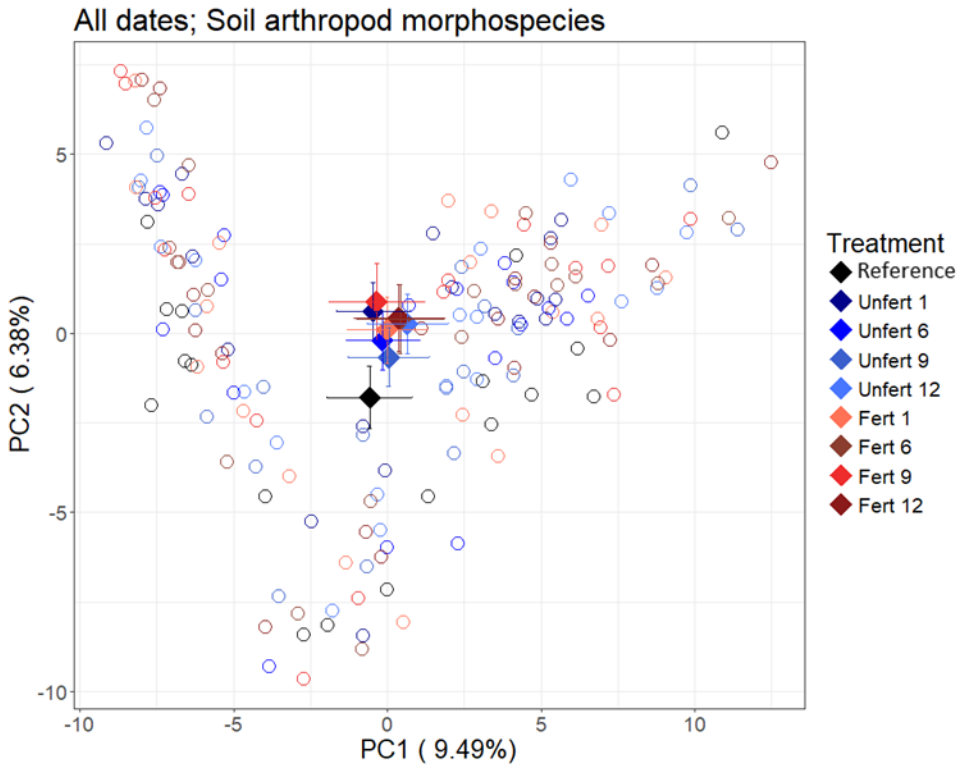


Figure 5

PCA of all dates grouped together of soil arthropod morphospecies data that has been log transformed. There was no additional adjustment made to the data to correct for variation in sample date, which means this graph may not fully account for the statistically significant variation between sampling dates. Even with that caveat, the results show some differentiation between reference, fertilized plots and unfertilized plots. However, MANOVA results show no statistical significance due to fertilization ($P = 0.068$), biodiversity mixture, or the interaction ($P > 0.1$). However, pairwise comparison shows significant difference between reference and unfertilized treatments (Table 5). Biodiversity IDs [#] are: 1 = switchgrass, 6 = grass mix, 9 = grass mix & trefoil, 12 = grass mix & clover. Reference treatment was the unplanted farm soil adjacent to the field.

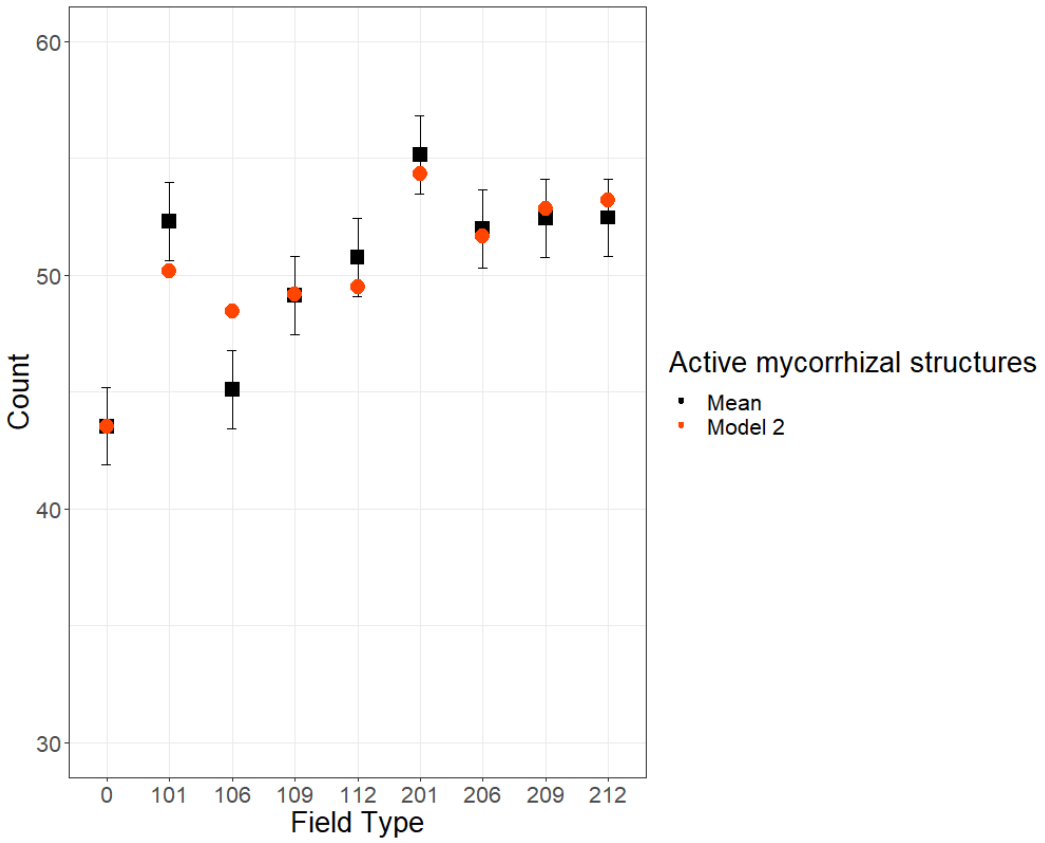


Figure 6

Mean count, in black squares with standard error, and ME model 2 predictions, in orange circles, of active structures shows good correlation between model predications and original data. The active structures are calculated as the sum of counts of three structures: coils, arbuscules, and paris structures. The highest values of these structures were found in the fertilized plots, followed by unfertilized plots, with lowest values in the reference area. However, the model predictions did not achieve statistical significance ($P<0.05$). Biodiversity types [##_] are: 1 = switchgrass, 6 = grass mix, 9 = grass mix & trefoil, 12 = grass mix & clover. Reference treatment (0) was the unplanted farm soil adjacent to the field. Fertilized treatments are labelled 2[##] and unfertilized are labelled 1[##].

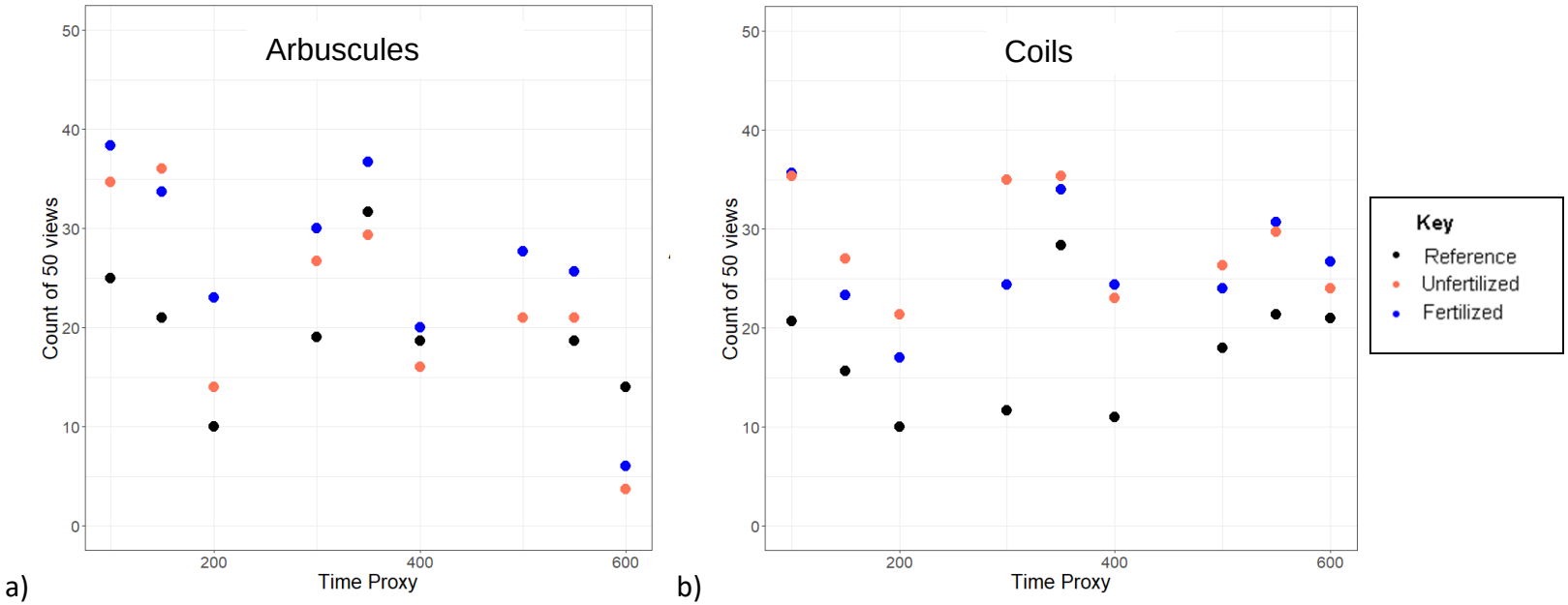
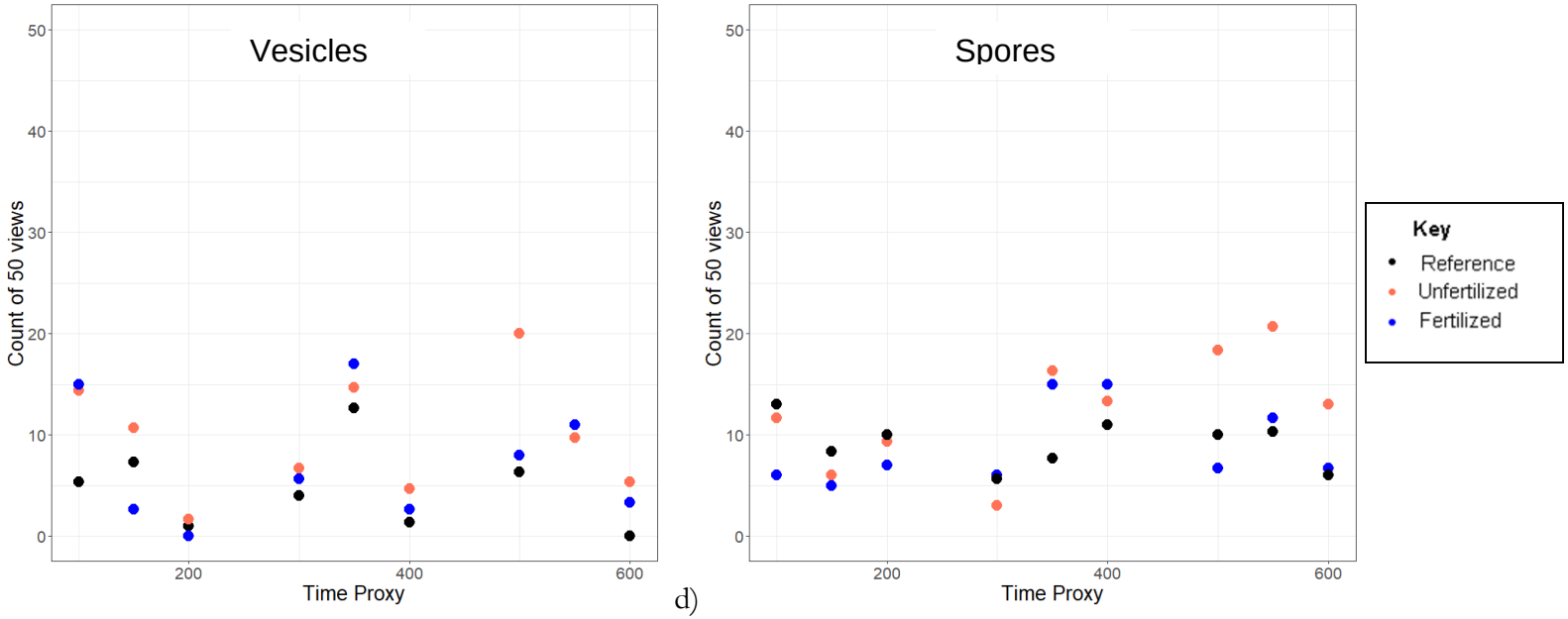


Figure 7

Graphs show mycorrhizal structures—a) arbuscules b) coils c) vesicles d) spores—over time for reference area, switchgrass-only unfertilized and switchgrass-only fertilized plots. While biodiversity mixtures 6, 9 and 12 were analyzed, they are not shown on the graphs for visual simplicity. Time proxy is used instead of exact date to ease graphing; 100,150,200 are the dates in 2013; 300, 350, 400 are the dates in 2014; 500, 550, 600 are the dates in 2015. Linear model was the best-fit model to the data for all four structures. N fertilization was correlated with statistically significant differences between the planted areas for arbuscules and spores, whereas the reference was

(Figure 7 continued)

statistically different from the two planted areas for coils and vesicles. Sampling date was a statistically significant factor, and the trend to have different abundance at different sampling events is visible on these graphs. P-values from the analyses can be located in Table 6.



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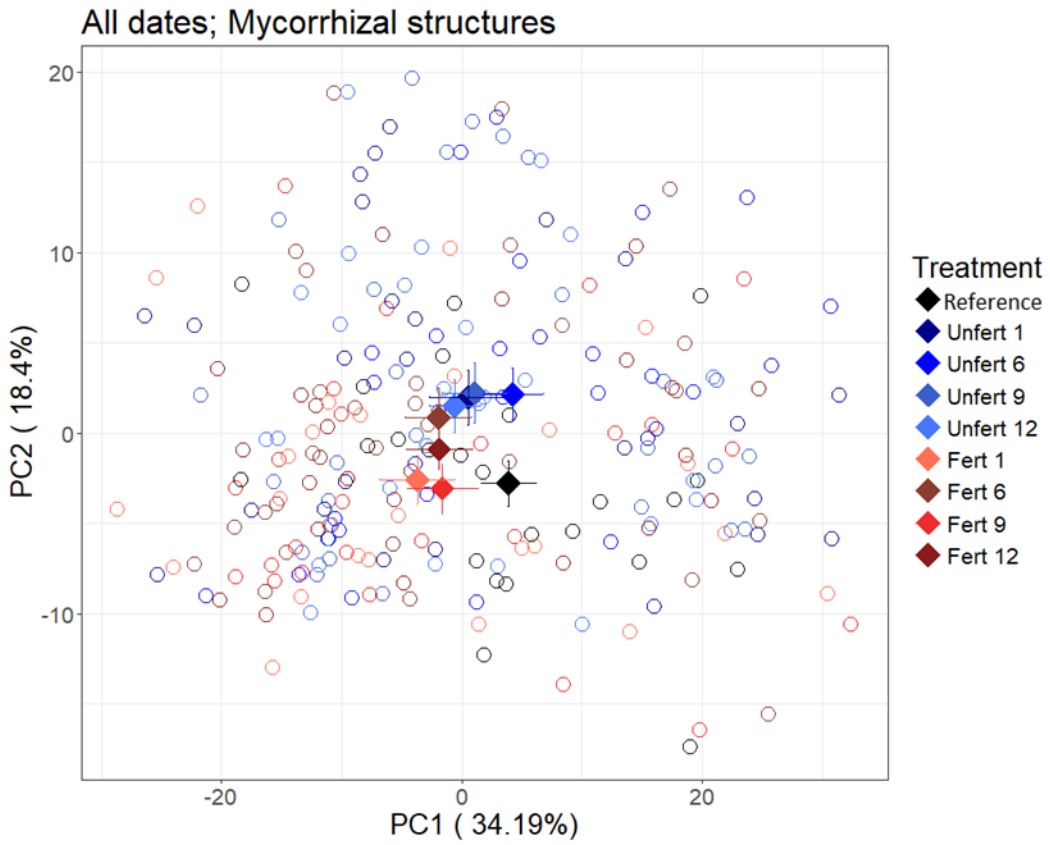
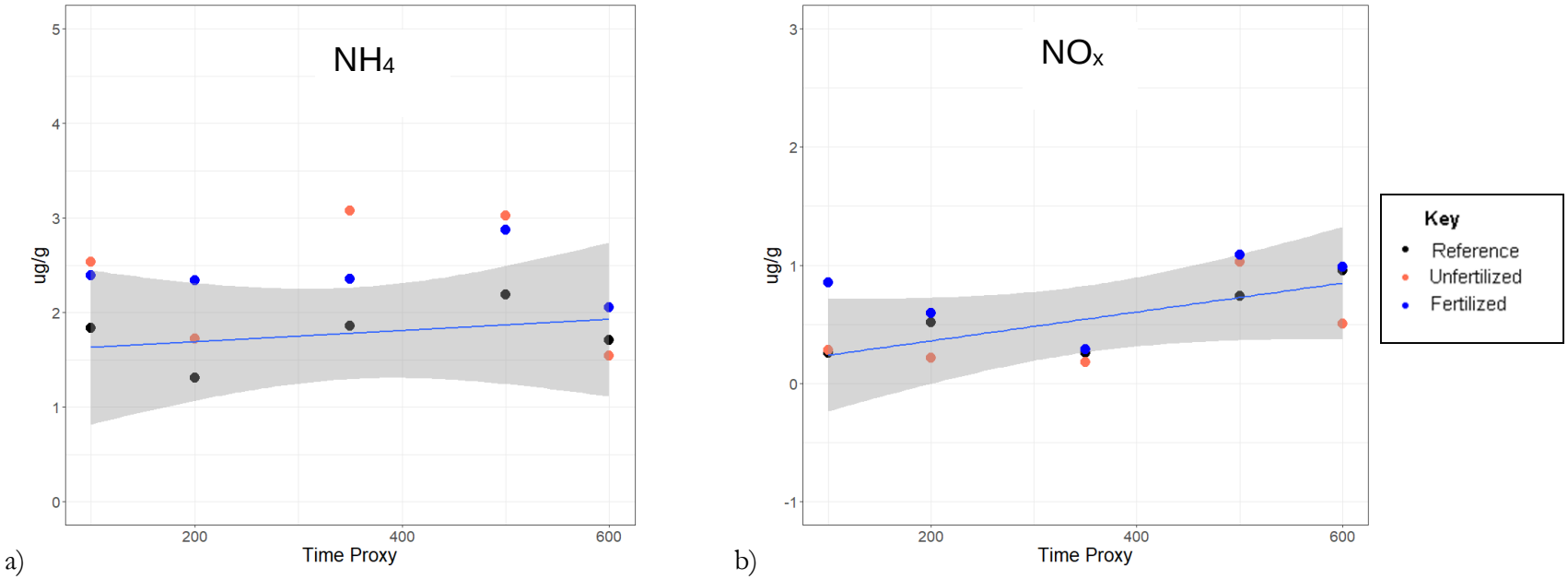


Figure 8

PCA of all dates grouped together. No additional adjustments were made to account for variation between dates, so the statistical significance between the dates is not fully accounted for. However, all data was on the same scale (out of 50 views). The fertilization treatments (fertilized, unfertilized, and reference) all are separating in the PCA space. MANOVA results support this conclusion, and show significance due to fertilization treatment (P = 0.002) but not biodiversity mixture nor the interaction. However, pairwise comparison (Table 7) shows that this difference is driven by reference vs. planted areas, not due to N fertilization between the plots. Biodiversity IDs [#] are: 1 = switchgrass, 6 = grass mix, 9 = grass mix & trefoil, 12 = grass mix & clover. Reference treatment was the unplanted farm soil adjacent to the field.

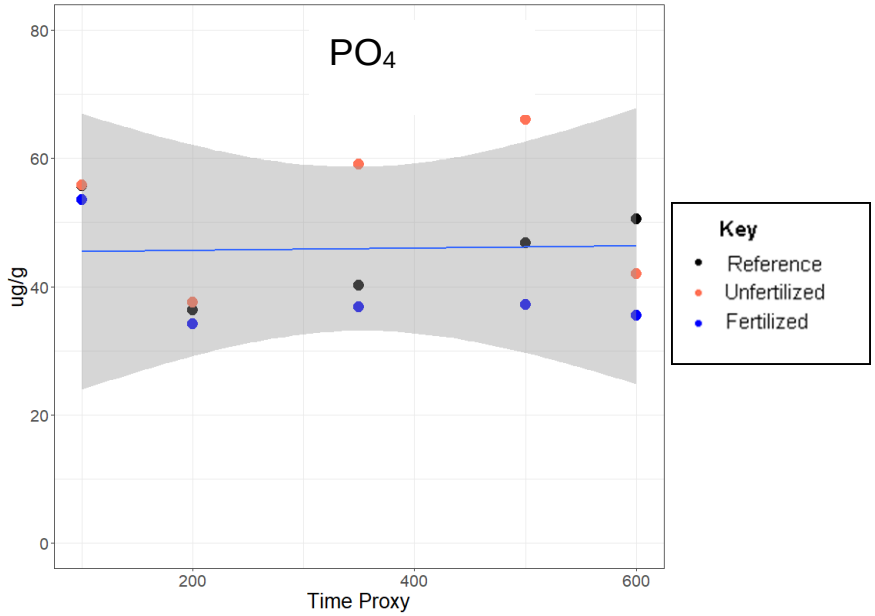


a) b)

Figure 9

Linear model for all soil extractable nutrients over time: a) NH_4 b) NO_x and c) PO_4. Time proxy was used instead of exact date to make linear modeling easier—100 and 200 representing the two dates in 2013, 350 representing the date in 2014, 500 and 600 representing the two dates in 2015. Linear model was the best-fit (vs. ME model) in all cases; based on AIC comparison. The increase in NO_x is the only statistically significant trend ($P = 0.007$). However, NH_4 also shows a positive linear trend over time. The best-fit model results for PO_4 indicate that fertilized areas have significantly lower values than unfertilized areas, which supports the findings of the analyses that did not include time as a factor. P-values from these analyses can be located in Table 8.

(Figure 9 continued)



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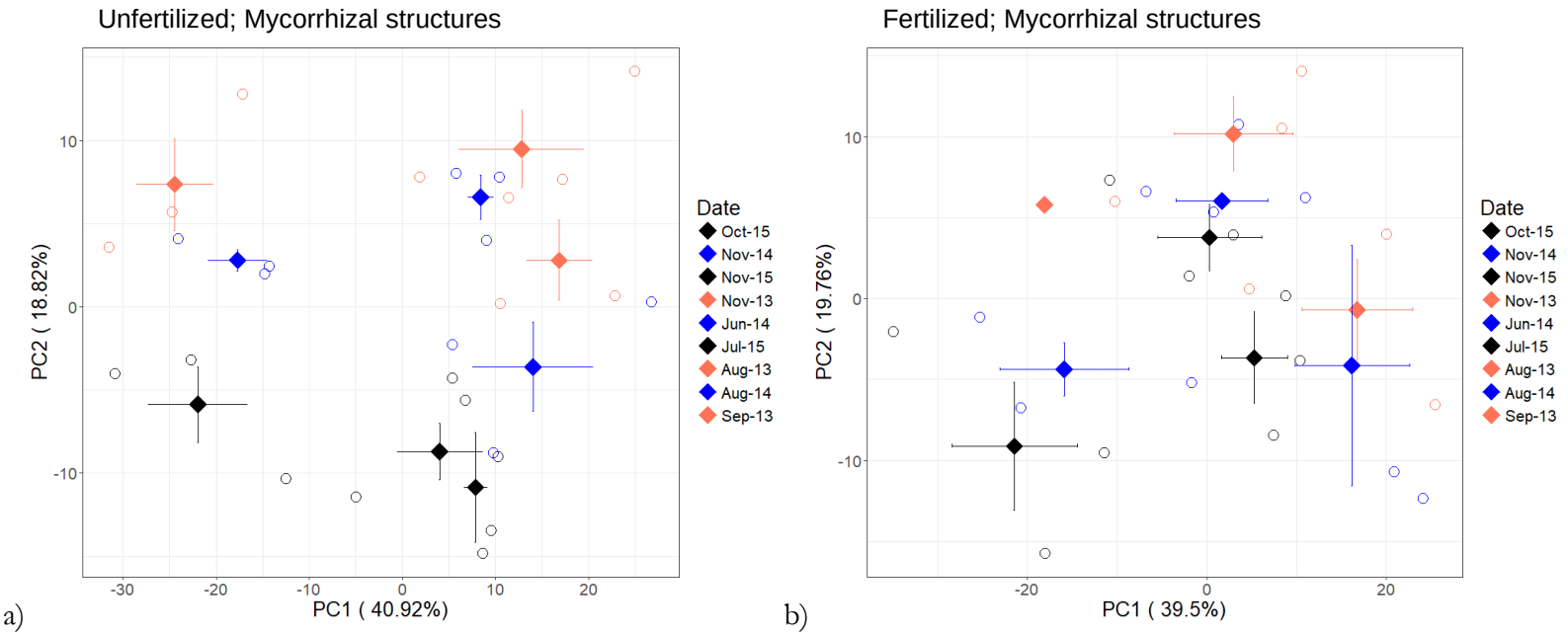


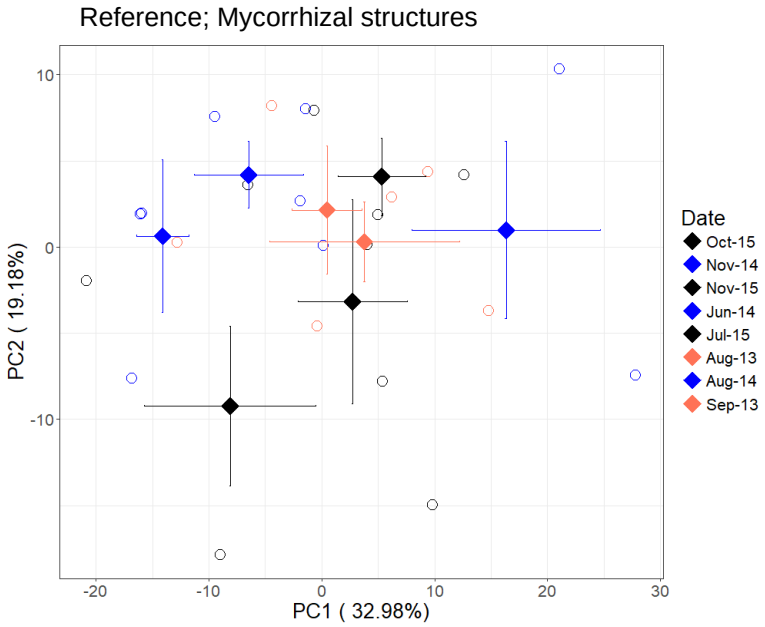
a) b)

Figure 10

PCA of mycorrhizal structures by date collected per biodiversity mixture. In each of these datasets shown by PCA, a MANOVA analysis shows individual sampling dates to be significant and year to be significant in (a) unfertilized biodiversity type 1 and (b) fertilized biodiversity type 1 but not in the (c) reference. However, in contrast, pairwise comparison (Table 9) shows that the adjusted p-value is not significant for year in any of the cases. Graphs shown for only the switchgrass-only biodiversity treatment. . Type 1 = switchgrass-only biodiversity plots.

(Figure 10 continued)

c)



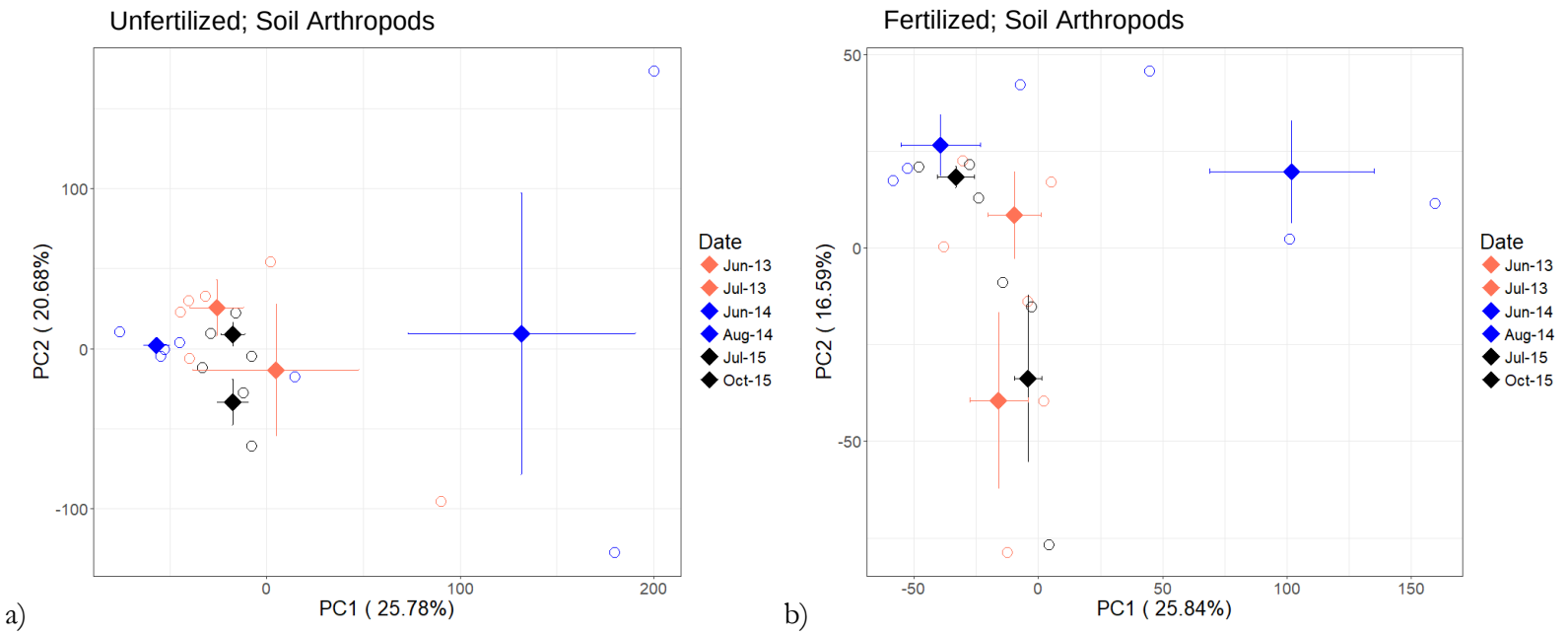
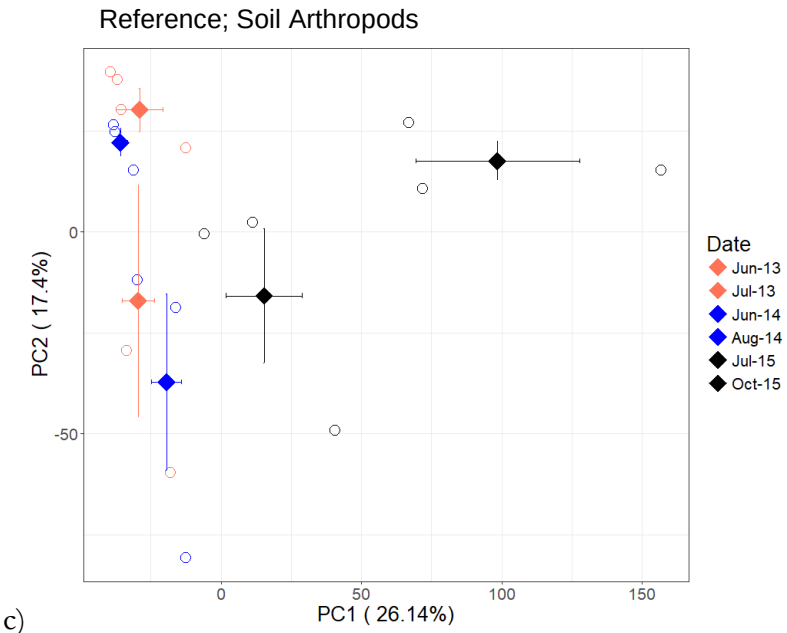


Figure 11

PCA of soil arthropod morphospecies (data was log transformed) by biodiversity mixture: a) unfertilized biodiversity type 1 b) fertilized biodiversity type 1 and c) reference. MANOVA results show that date and year is significant in all three cases. Pairwise comparison (Table 10) shows that year 2015 is significantly different from 2013 and 2014 in all cases. Type 1 = switchgrass-only biodiversity plots.

(Figure 11 continued)

c)



Biomass yields

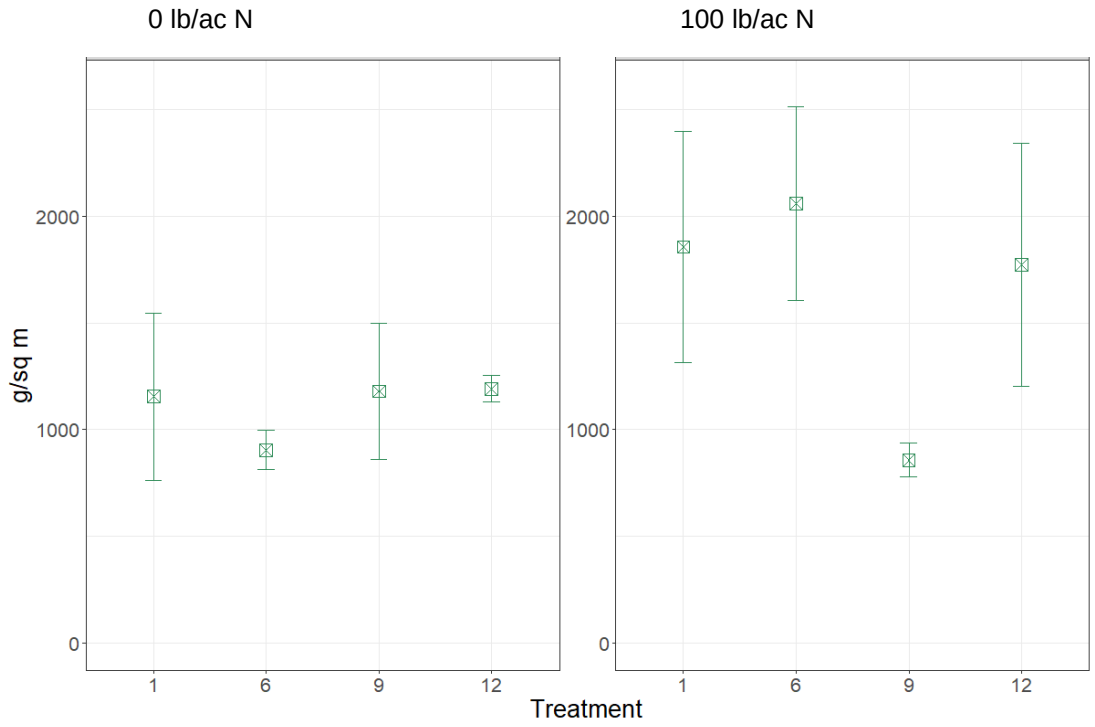


Figure 12

Biomass yield in g per m^2 collected on December 2015 from 2 x 2 ft (0.61 m) subplots located within each individual plot. A linear model shows no significance of fertilization treatment or biodiversity mixture, though the biomass yields in the fertilized areas are higher than in the unfertilized areas ($P < 0.1$). The yields from biodiversity mixture type 9 are low compared to the other fertilized plots. Biodiversity mixture IDs [#] are: 1 = switchgrass, 6 = grass mix, 9 = grass mix & trefoil, 12 = grass mix & clover. Reference treatment (unplanted farm soil) was not included because there essentially was no biomass yield.

Digested N in Biomass

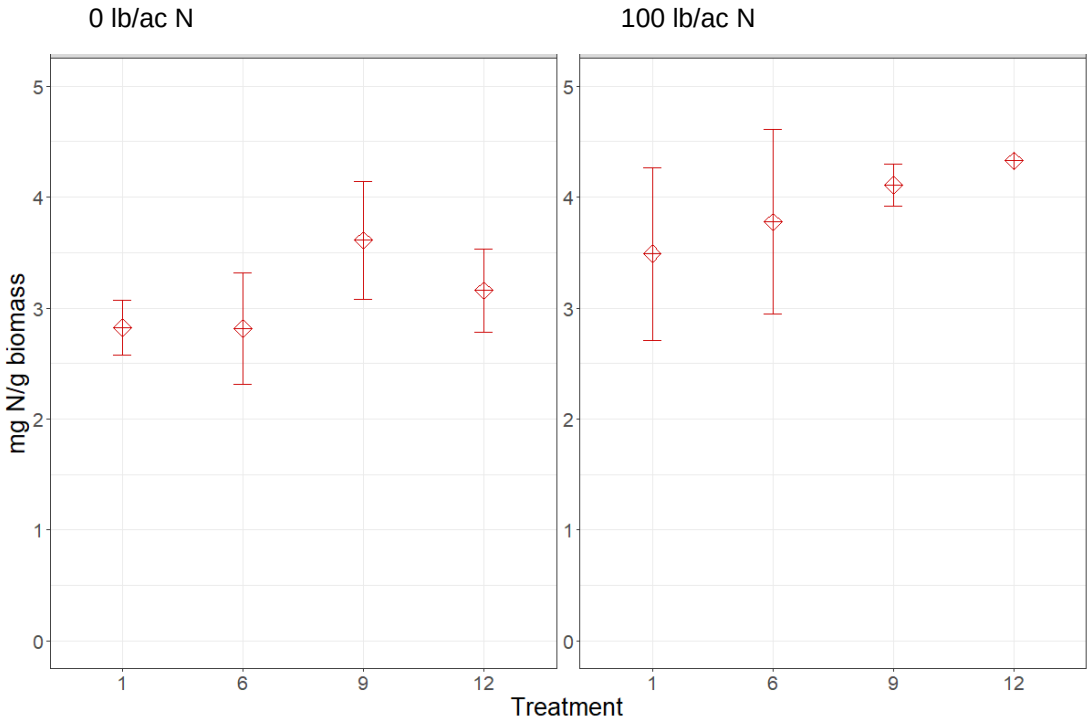


Figure 13

Each collection bag from the biomass yield subsamples was further subsampled in 2017 to digest plant biomass and asses the N content. Linear model showed no statistical significance for fertilization treatment or biodiversity mixture, though N content of fertilized areas is higher than that of unfertilized areas. Biodiversity mixture IDs [#] are: 1 = switchgrass, 6 = grass mix, 9 = grass mix & trefoil, 12 = grass mix & clover. Reference treatment (unplanted farm soil) was not included because there essentially was no biomass yield.

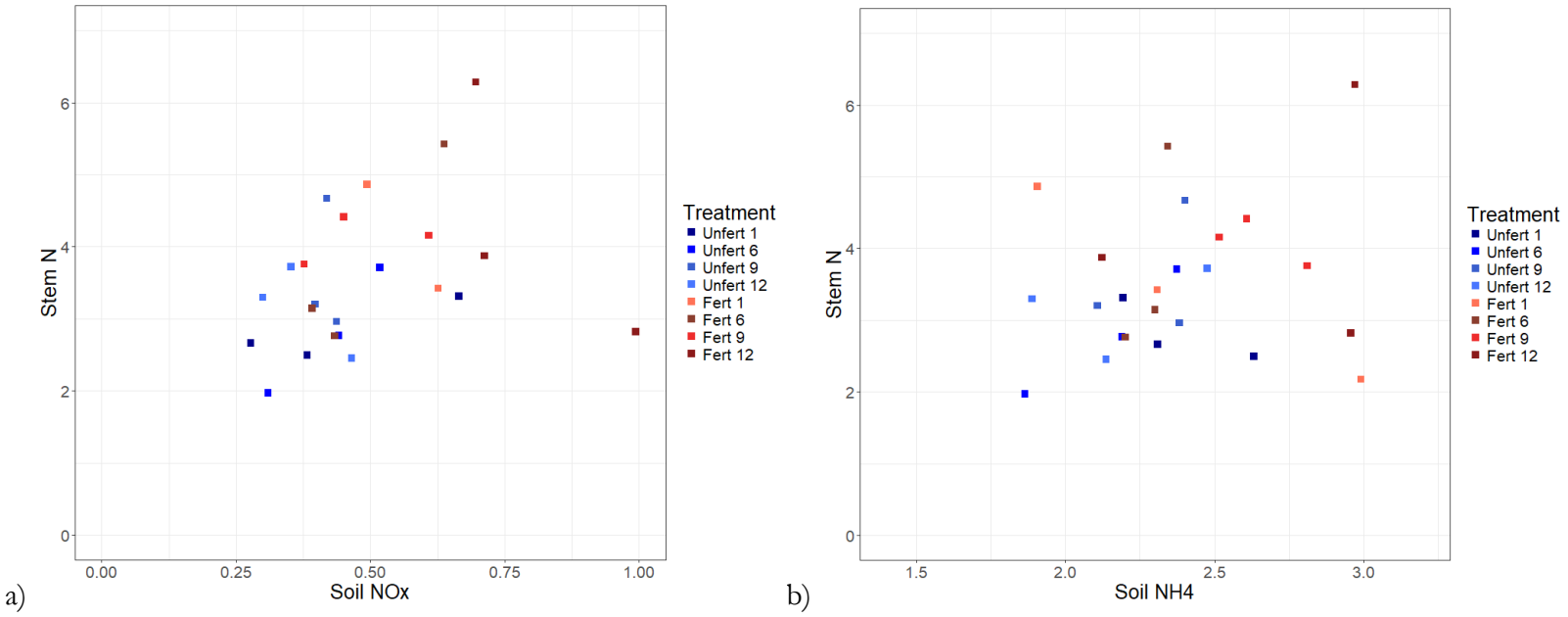


Figure 14

These graphs connect the plant N content to the soil N content. a) The individual values for the stem N and mean soil NO_x calculated for each individual plot (N = 5, one for each sampling date) have a positive correlation, but it is not statistically significant. b) The individual values for the stem N and mean soil NH_4 calculated for each individual plot (N = 5, one for each sampling date) have no clear trend nor statistical significance.

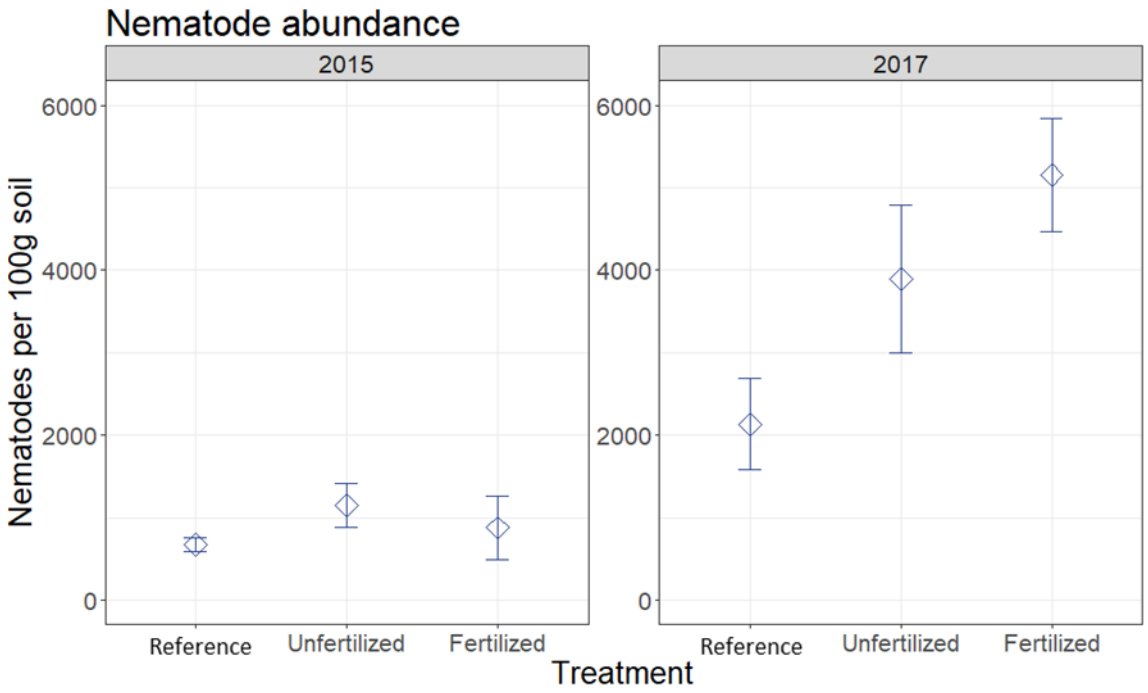


Figure 15

This graph shows nematode abundance scaled to 100g of dry soil. Both fertilized and unfertilized samples were collected from switchgrass-only plots at Adelphia farm and the reference was collected from adjacent unplanted farmland. Note that the big difference in estimated amount between the two sampling events is likely driven by the two different methods used to collect nematodes. Linear model results from 2015 show no statistical significance due to treatment; the highest abundance was found in unfertilized areas. Linear model of 2017 data showed that unfertilized samples are moderately different than reference samples ($P < 0.1$) and fertilized samples are significantly different than reference ($P < 0.05$), however the fertilized and unfertilized areas are not significantly different from each other. The highest abundance was found in the fertilized areas. In both years, the reference area had the lowest nematode abundance.

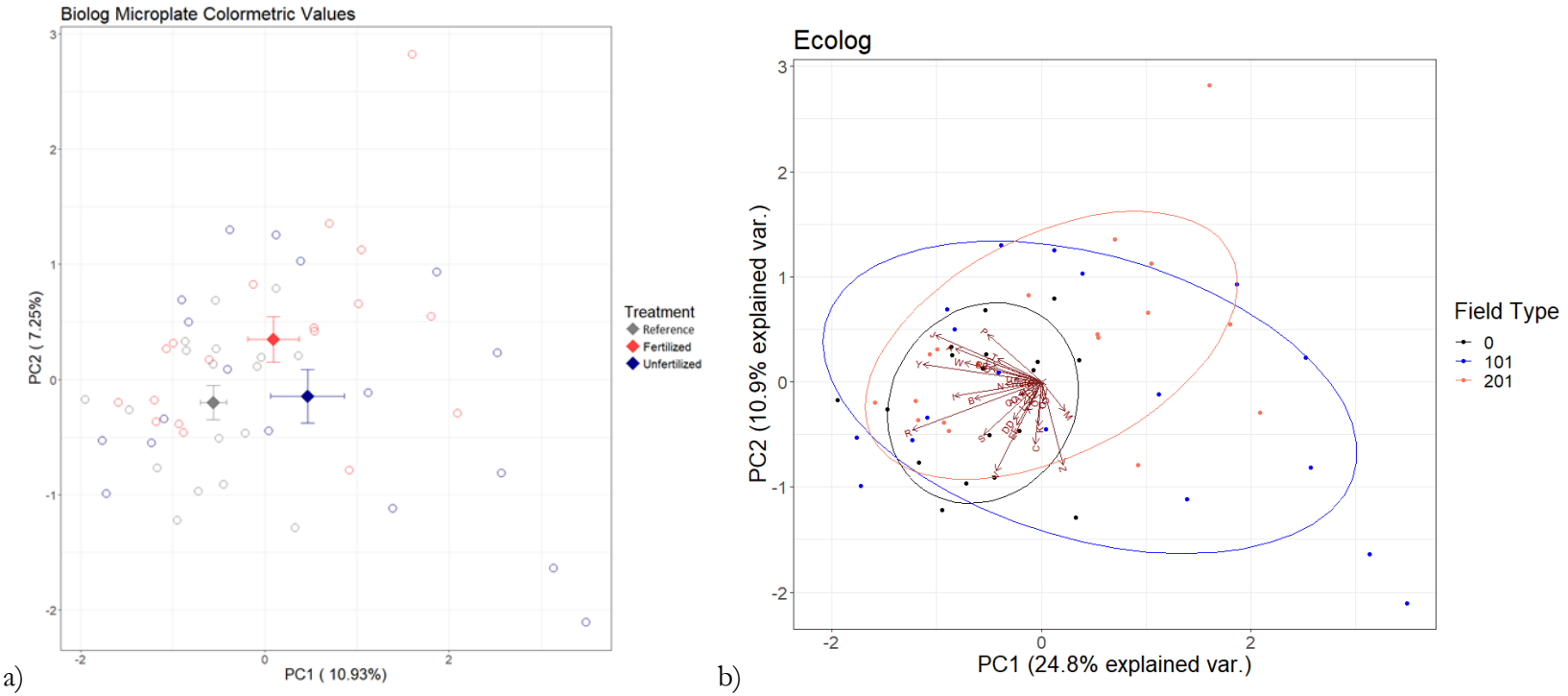


Figure 16

PCA of the bacterial community measured with BIOLOG ecoplates showing: a) center points with the calculated standard error overlaying the individual data points and b) ellipsoids drawn over the underlying data. Both fertilized and unfertilized samples were collected from switchgrass-only plots at Adelphia farm (biodiversity mixture 1), and the reference was collected from adjacent unplanted farmland. MANOVA of the normalized data showed significant difference between the bacterial communities of these three treatments, but pairwise comparison shows that difference is due to the reference versus unfertilized plots (0= reference; 101 = unfertilized; 201 = fertilized).

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Chapter 3: Limitations on plant-soil interactions in manipulated microcosms of the biofuel crop *Panicum virgatum*

Abstract

Switchgrass, a potential biofuel crop, often forms a relationship with arbuscular mycorrhizal fungi (AMF). This relationship might indicate a reliance on mycorrhizae for optimal growth and the existence of plant-soil feedbacks, especially in the more biodiversity-limited setting of agricultural production. The hypothesis that plant-soil interactions exist in agriculturally-produced switchgrass was tested with two related greenhouse experiments. The soil community and edaphic conditions were manipulated in a fully factorial design to see how commercial mycorrhizal inoculum, fungal feeding nematodes, and nitrogen (N) fertilizer impacted biomass yield of switchgrass during one growing season. A subset of the samples was overwintered and grown for a second season to measure any lag effects from the initial treatment, since switchgrass is a perennial crop.

Plant biomass and plant N content showed no statistically significant differences due to the experimental manipulations in the one-season or two-season experiment. There were no clear trends in the response of mycorrhizal colonization and structure formation based on experimental treatments in either experiment. The soil arthropod community changed due to N fertilization in the one-season experiment, but not in the two-season experiment. Nematode abundance showed no differences in response to experimental treatments in both experiments. When all soil response variables were combined in an NMDS analysis, none of the experimental treatments consistently affected the distribution of results for either experiment.

These results suggest that there are not any strong plant-soil feedbacks in this system. Although there was a limited response of the soil community to experimental

treatments, there was no response in the plants that could be attributed only to the experimental manipulations. These results contradict previous research that found impact on mycorrhizal communities from a N deposition gradient, but align with other research that suggests plant-soil feedbacks do not have strong effects in most plant-soil systems. These results show that the combined system of switchgrass plants and associated soil community does not have a linear response to nutrient addition, but is more complex. Additionally, since switchgrass did not respond strongly to fertilization, these results support the use of switchgrass as a biofuel crop since minimal fertilization will maximize the sustainability of the crop.

Introduction

Switchgrass, *Panicum virgatum*, is a potential biofuel crop that requires low levels of fertilizer, pesticide, irrigation, and tillage compared to other biofuel crops (Baumgarten, 2020a). Switchgrass is thought to have a limited response to fertilizer additions because of its AMF associations (Brejda et al., 1998; Vogel et al., 2002). This reliance on mycorrhizal associations may signal that the switchgrass-mycorrhizal system is subject to plant-soil feedbacks.

A yearly application of N fertilizer is recommended as best-practice for biofuel switchgrass (McLaughlin & Kszos, 2005; Vogel et al., 2002). However, managing agricultural systems to support soil communities with the goal of reducing fertilizer inputs may lead to different best-management practices. Most relevantly, a substantial body of research suggests that AMF are affected by N fertilization. A meta-analysis found a reduction in mycorrhizal abundance in response to N fertilization (Treseder, 2004). Other studies have found an impact on AMF communities due to agricultural practices in general (Bainard et al., 2015). Two studies have specifically found an effect of N applications on AMF communities in agricultural settings (Avio et al., 2013; Zhang et al., 2016). Finally, ecological studies have attributed decreases in AMF diversity to N deposition gradients (Egerton-Warburton & Allen, 2000; Egerton-Warburton et al., 2001). As previously noted by Baumgarten (2020a, b), the sustainability of a switchgrass field may be compromised over the projected period of harvest if annual N applications alter the relationship between switchgrass and AMF. The theory that switchgrass is subject to plant-soil interactions was tested with a 2-year greenhouse experiment in which edaphic (soil) conditions were directly manipulated.

Agricultural methods, especially applying fertilizer, can impact other soil community members as well. A meta-analysis of published research found that inorganic N additions

caused a shift in nematode communities, reducing species diversity and the community maturity index by causing an increase in bacterivores (Liu et al., 2016). For soil arthropods, there are mixed results. Cao et al. (2011) found a reduction in soil mite abundance and diversity in response to N-P (phosphorus) fertilization, which was driven by reductions in the Oribatid mites. Lemanski & Scheu (2014) found that fertilization with N-P-K (potassium) shifted soil arthropod diets, but not total abundance. A few studies, conversely, found higher soil arthropod abundance in response to soil nutrient fertilization (Eisenhauer et al., 2012; Hlava, 2015). In an assessment of changes that might be driven by an increase in climate change, Eisenhauer et al. (2012) found that applications of N led to an increase in some soil species—fungal feeding nematodes, astigmatic and prostigmatic mites—but reduced overall taxon richness in both communities (nematodes and microarthropods). Alternatively, one study found a strong decrease in collembola abundance due to agricultural planting via tillage, but an increase after the initial loss due to fertilization with N-P-K (Chang et al., 2013).

There is no debate that members of the soil community interact in complex ways (Wardle, 2006; Wardle et al., 2005). However, less research exists studying the interaction of AMF with other soil community members, and if these interactions in turn affect plant growth or communities. There is evidence that AMF interact with nematodes, and that fungal feeding nematodes can feed on AMF (Ingham, 1988). Helgason and Fitter (2009) hypothesize that grazing by collembola and parasitism by chytrids could have influenced the evolution of AMF, but do not cite any studies that researched these interactions directly. Collembola can affect plant performance through their feeding choice, although they usually prefer saprotrophic fungi over AMF. The clearest evidence of these interactions (plant-fungi-collembolan) are when collembolan species feed on plant pathogenic fungi

(Mitschunas et al., 2006; Tiunov & Scheu, 2005). These interactions can translate to aboveground herbivores as well—Hartley & Gange (2009) show that plants interacting with AMF have different effects on plant-feeding insects such as aphids and gall insects compared to plants without mycorrhizal colonization. Because the soil food web is complex, this research looks at multiple levels of the soil community to better understand how N fertilizer affects edaphic conditions.

Directly manipulating the soil community will reveal if there is an interaction between the soil community and plant growth. Previous research focused on plant-mycorrhizae interactions has supported the hypothesis that commercial production of switchgrass could be a system with plant-soil interactions, but other research suggests this system may not have strong plant-soil interactions. Instances of measurable plant-soil feedbacks, not just one-way influences, are not common (Ehrenfeld et al., 2005). When nematode communities are included in the calculation of plant-soil feedbacks, measured feedback interactions do not appear to be a strong force in shaping plant communities, although there was a strong effect of plant species on the nematode community (Viketoft et al., 2009). Smith et al. (2009) found that while some plants do respond strongly to mycorrhizal colonization, other plants show no response. A meta-analysis of plant response to mycorrhizal colonization suggests that plants with fibrous root systems, such as switchgrass, generally respond less strongly to mycorrhizal colonization (Yan et al., 2015). Additionally, Helgason & Fitter (2009) suggest that AMF evolved in response to soil conditions rather than in response to host plants, which supports the contradictory findings that even though AMF can be vital for plant health, the response is not consistent across all plant species. So despite the theory that the AMF-switchgrass interaction allows switchgrass

to grow with minimal management, much previous research suggests that this interaction may not be strong enough to function as a feedback loop.

This experiment will measure change in switchgrass biomass yields, and can attribute measured changes to specific soil community members: AMF or fungal feeding nematodes. If this experiment shows that these interactions between plant and soil community are modified by the addition of N fertilizer, then over the projected life of a field, consistent N fertilization is likely to undermine the soil community that positively impacts switchgrass growth.

Results from the combined edaphic manipulations could be positive or negative. Previous research has shown plant biomass responds to plant-soil feedbacks, such as in the classic experiment in which seedlings grow better in soil primed with their same species than in soil primed with a competitor neighbor species (Kulmatiski et al., 2017). Thus, plant biomass yields will be the primary measure of whether these interactions exist. If present, the plant-soil feedbacks could be positive and lead to increased plant biomass, or they could be negative and decrease plant biomass. The types of treatment could interact, leading to unpredictable results. The commercial inoculum could help switchgrass acquire nutrients, leading to an increase in plant growth. The inoculum could also act as a carbon sink from the plant, leading to a decrease in plant growth. The mycophagous nematodes could cause an increase in plant growth by releasing more nutrients into the soil through their actions, but it also may have no effect, or may change conditions in the soil and have an indirect negative effect. The interaction of the AMF and the nematodes could go both ways—the nematodes, through feeding choice on the AMF, could increase plant growth if the AMF are a carbon drain on the plant, but decrease plant growth if the AMF are increasing plant growth.

This research includes measurements of the soil community as outcomes, which is not always the case in classic plant-soil feedback studies. An additional divergence from classic research is that in this system, an unnatural addition of N fertilizer is the hypothesized driver of any feedbacks. However, this research asks how changes in the soil may change plant growth in unexpected ways, which aligns with the plant-soil-feedback structure. Since the soil community was manipulated directly, a specific part of the soil community that leads to a change in plant biomass can be pinpointed.

The hypothesis is that direct manipulation of edaphic conditions will result in a change in the amount of biomass produced by each plant if plant-soil feedbacks exist. In the plants grown for one year, it is hypothesized that all treatments will be additive in benefiting plant biomass production. In other words, within the same N fertilization level, it is predicted that there is a constant addition of plant growth associated each treatment: an increase from nematodes, a larger increase from AMF, and the largest increase with both. Additionally, it is predicted that this pattern will be a similar magnitude at each level of fertilization, but the fertilizer will increase plant growth linearly with 50 lb/ac and 100 lb/ac (22.7 kg/ac and 45.4 kg/ac). Finally, since the pots are placed in a greenhouse that is not maintained as a sterilized environment, the pots will act as "sinks" that attract soil microarthropods, and it is predicted that different microarthropod communities will be found in different treatments. It is hypothesized that a greater complexity of soil community will develop in soil treated with both mycorrhizal inoculum and mycophagous nematodes. The hypothesis for the pots overwintered and grown for two years is that the plants will show the responses seen in the single year experiment, but with greater magnitude. The second year of growth may reflect more accurately the plant response to treatments, as the

switchgrass is a perennial, and the second year of growth may allow the response to the treatments to more fully develop.

Methods

Soil was collected from freshly tilled fields at Adelphia Farm in Freehold, NJ (40.227053, -74.252517), where previous research was conducted on a biodiversity x N switchgrass biomass experiment (Baumgarten, 2020b). Soil type is Freehold sandy loam described as well-drained and moderately permeable (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). Collected soil was manually homogenized with shovels. Soil was sterilized in 3 L batches, running for 120 seconds on high in a commercial microwave (calculated based on Ferriss, 1984). Then soil was placed into "Tall One Treepots" by Hummert International, total size 2.84 L and 4" x 14" dimension, covered with foil and allowed to settle. Once the pots were filled evenly by volume, they were taken to the greenhouse and left covered with foil until the seedlings were transferred.

Seeds were germinated in sterilized soil in Hummert GT73SR growing trays with underlying sub-irrigation tray with no holes. Three seeds were planted in each individual plug in the tray. Soil was kept moist by ensuring water was consistently in the sub-irrigation tray. Seedlings were transplanted when they were between 5 cm and 15 cm tall. One entire plug was transferred from the growing tray to one pot of sterilized soil. Seedlings were allowed to grow for one week to ensure survival, and failing seedlings ($N < 10$) were replaced two days after the initial transfer. After a week, seedlings were thinned to the single most healthy-looking plant per pot and the height was recorded.

Mycorrhizae treatments were composed of a powdered inoculum that contained 7 *Glomus* species; brand Diehard Endo Drench. Mycorrhizal inoculum was added at a rate of

10 g per pot, and the same weight of sterilized inoculum was added to the null treatments. Inoculum was sterilized in a 3 L batch for 120 sec in a high-power commercial microwave (Ferriss, 1984). Both regular and sterilized inoculum treatments were dissolved into water in 25 mL plastic bottles to allow the treatment to be evenly applied.

Nematodes of the fungal feeding species *Aphelenchus avenae* were bought from a lab at Clemson University and arrived in culture on non-mycorrhizal fungi. Small sections of these cultures (cut from areas full of nematodes) were transferred to jars of wheat berries prepared according to the Evans (1970) method (*pers. comm.* recommended by Dr. Amy Treonis). After about two weeks, in which time the nematodes propagated exponentially, nematodes were extracted from the wheat berries using a screen, water, and gravity; a modification of the Oostenbrink filter method (OEPP/EPPO, 2013). It was assumeded that this liquid acquired some nutrients from the wheat berries; thus, half of the liquid was sterilized for the no-nematode treatments. This sterilized portion was placed in a microwave on high power in one-minute intervals until it was confirmed with microscopic visualization that no living nematodes remained. The two batches of nematode liquids (sterilized and live culture) were divided evenly into the number of treatments (168 in total). This resulted in approximately 45 mL liquid per pot. This nematode treatment was applied to the pots one week after the mycorrhizal treatment was added. Nematode counts in the live culture were estimated at 750 nematodes in 10 mL; approximately 30,000 nematodes total.

Two weeks later, pots were fertilized at a rate of 0 lb/ac, 50 lb/ac, or 100 lb/ac. N fertilizer was dissolved in 25 mL bottles of water. Unfertilized pots received pure water. Fertilizer was YaraLiva Tropicote 15.5-0-0, derived from Ammonium Calcium Nitrate Double Salt. Throughout the remainder of the growing season, all pots were watered evenly.

After the first growing season, in November, all pots had their above-ground biomass clipped on the same day. Then the 1-year pots (N = 120) were taken into the lab in batches of 24 for further processing of the soil. The 2-year pots (N = 48) were left in the unheated greenhouse to overwinter. In April of the second year, the 2-year pots were placed on top of pots of screened and homogenized sand to allow for root expansion. They were then watered evenly throughout the growing season.

The 1-year pots were brought from the cold greenhouse into the lab in batches of 24 for soil sampling. This batch size was determined based on the required tests that were time-sensitive after initial soil disturbance (nematode counts, soil arthropod extractions, root staining for mycorrhizae). Pots were watered and left to sit for 24 hours before sampling to ease the process. The bottom 5 cm of the pots were sub-sampled for mycorrhizal colonization and for nematodes. Soil cores (5 cm) centered on the plant base were used to sample for soil arthropods. Roots were weighed and sub-sampled for mycorrhizal staining and for moisture content to be used to calculate total root biomass. Soil was then air dried and stored in the lab until the remaining root biomass could be separated from the soil, using 1 mm sieves.

Soil arthropod sample cores were inverted and placed in Berlese funnels for five days, with the light intensity turned up at regular intervals. Soil arthropods crawled downwards away from the light and towards the attractive 70% methanol mixture with 10% glycerol, which killed and preserved them. Arthropod morphospecies were assessed with a dissection microscope. To increase reliability of soil arthropod morphospecies identification, only one person did the identification, photos and notes from earlier results were reviewed and the data reclassified as needed, and peer reviewed works were used to guide identification. Collembola were fairly straightforward to identify to family (using

Christiansen & Bellinger, 1998). However, mite morphospecies may have been more heterogeneous. Mites were essentially identified to sub-order (using Dindal, 1990), and then further divisions to morphospecies were only loosely based on published keys. This method was the same as that of Baumgarten (2020b).

Soil nematodes were extracted from approximately 5 g of homogenized soil collected from the bottom 5 cm of the pots. These were left for 18 hours in glass funnels with the soil, held on filter paper, was in contact with the water for the entire extraction procedure, a modification of the Oostenbrink filter method (OEPP/EPPO, 2013). The nematodes move through the soil, enter the water portion of the funnel and descend to the bottom of the glass filter stem since they cannot swim against gravity. The liquid was collected into small 25 mL bottles and counted immediately while the nematodes were still moving. The force of the water descending upon release of the valve ensured that all nematodes that entered the solution made it into the plastic vial. Extracting for between 12 and 24 hours is the recommended period of time to ensure collection of slower moving nematodes, but the time is not too long to cause nematode death due to lack of oxygen diffusion nor to allow any resident eggs of fast breeding nematodes to hatch and skew the results (OEPP/EPPO, 2013). This method was the same as that of Baumgarten (2020b).

Roots were collected for mycorrhizal assessment from the bottom 5 cm of the pot. The roots were removed from the soil that was then homogenized for nematode counts and stored briefly in the refrigerator (for no more than seven days) until the staining process could be initiated. The roots were washed to remove all soil, then placed in 10% KOH to clear the cells. After seven days, the roots were removed and washed three times in tap water. The roots were placed in 1% HCl for one minute to prepare the roots for the stain. Then the roots were placed in 0.05% trypan blue in lactoglycerol for five days. Trypan blue

selectively stained fungal tissue and not plant tissue (Brundrett et al., 1984). Finally, roots were stored in lactoglycerol until mycorrhizal colonization could be assessed with a modification of the magnified intersections method on a compound microscope (Giovannetti & Mosse, 1980; McGonigle et al., 1990). This method was the same as that of Baumgarten (2020b).

In the second year, the above-ground biomass of the 2-year pots was collected in December. The processing for the soil measures were the same as for the 1-year pots, with the addition of the top pot being clipped free of the bottom pot before harvesting. The soil processing steps were run in two batches of 24 pots each. Additionally, the roots from the lower sand pots were screened out of the soil using a 0.5 cm sieve.

Root biomass was collected from air-dried soil using 1 mm sieves. A consistent amount of time was spent for each sample to ensure that a similar percent of the root mass was recovered. Roots were placed in a paper bag, and then left at room temperature until a later date when the remainder of the soil clinging to the roots could be gently washed off. At that point, the cleaned roots were placed back into the paper bag (which was shaken vigorously before placing roots back in it to remove any soil dust), and placed immediately into an oven to dry. Similarly, after the soil arthropod collection, the soil cores (which were at that point air dry) were stored in zipper-sealed plastic bags until the roots were able to be gently washed clean of the soil and placed in a paper bag to be oven dried and weighed. For the 2-year pots, the roots from the lower pot were washed and dried and added to the total. Weight for the roots stained for mycorrhizal colonization was corrected for water content and added back to the total root biomass.

All weights were calculated based on oven-dried material. If the samples were in a paper bag, the dried bag was first weighed in total. The plant mass was then removed and

the empty bag weighed in order to ensure that any plant biomass lost in the transition process did not result in an inaccurate weight. The plant biomass was left stored in the paper bag at room temperature until it was acid digested for N content.

Total N was calculated by digesting 0.1 g of ground and homogenized—with coffee grinders—plant biomass (roots and shoots separately) in 5 mL of Kjeldahl solution. The samples were heated at approximately 370°C until the solution cleared entirely (average run time: 12 hours). These liquids were diluted with DI water to 20 mL, then stored in 25 mL plastic bottles in a freezer until the liquids could be analyzed. One switchgrass sample (collected from Adelphia Farm experiment [Baumgarten, 2020b]) was included in each digest run, in addition to a blank. Additionally, NIST apple leaf 1515 was digested in three of the digestion runs to get a clearer picture of digest precision. These standards were used to standardize all of the digest runs to each other. N analysis was conducted using a Shimadzu C:N analyzer. A standard curve was run on each day that a batch of samples was analyzed in order to check machine efficiency.

In summary, there were 12 treatments in total for the 1-year plants, in a 2x2x3 factorial design, with either fresh or sterilized mycorrhizal inoculum, living or dead nematodes, and 0 lb/ac, 50 lb/ac or 100 lb/ac of N fertilizer (0 kg/ac, 22.7 kg/ac and 45.4kg/ac, respectively). This treatment block was repeated 10 times for 120 pots total (Table 1). For the 2-year pots, the design was reduced to a 2x2 factorial design—either living or sterilized soil community additions were added (of both nematodes and mycorrhizal inoculum), crossed with either no fertilizer or 100 lb/ac (45.4 kg/ac) (Table 2). This allowed for 12 replicates of each treatment (48 total), which would still yield a fair number of complete replicates in case plant death was an issue with the over-wintered pots. Measured variables for both years include: above- and below-ground biomass, shoot and root total N

content, soil arthropod community, mycorrhizal colonization, and total nematode abundance.

Statistical analyses were performed in R version 3.4.2 (R Core Team, 2019) and RStudio version 1.1.383 (RStudio Team, 2020). Packages used include vegan (Oksanen et al., 2019), MASS (Venables & Ripley, 2002), tidyverse (Wickham et al., 2019), ggfortify (Horikoshi & Tang, 2018; Tang et al., 2016), Rmarkdown (Allaire et al., 2020; Xie et al., 2018), knitr (Xie, 2020; 2015; 2014), plyr (Wickham, 2011), and ResourceSelection (Lele et al., 2019). Multivariate analysis of variance (MANOVA) was calculated using the adonis function in the vegan package (Oksanen et al., 2019). Model analysis for 2014 included N, mycorrhizae and nematodes as factors. Model analysis for 2015 included N and soil community (since mycorrhizae and nematodes were added concurrently) as factors. Finally, as discussed earlier, it is possible that the species within the soil community are interacting and affecting each other. Non-metric multidimensional scaling (NMDS) analyses was performed on the combined results of mycorrhizal structures, soil arthropod morphospecies, and nematode abundance. For soil arthropod morphospecies, only species that were present in more than 25% of the samples were included in the analysis. This translated to presence in more than 30 samples in 2014 (N = 6), and in more than 12 samples in 2015 (N = 8). To reduce the chance that one category would skew the results, each individual column was scaled to itself to a value between 0 and 1 (using decostand in the vegan package [Oksanen et al., 2019]), however the conclusion did not change if the data were scaled only as they were for the respective initial analysis. MetaMDS in vegan was used to calculate the values for the location of the points on the 2D graph (Oksanen et al., 2019).

Results

Biomass yields were evaluated as multivariate data, including both aboveground biomass and belowground biomass. Biomass yields in 2014 (Figure 1) were not predicted by mycorrhizal inoculum treatment, nor fertilization, but nematode treatments were weakly significant at the $P = 0.1$ level (Table 3a). Four plants died before the end of the experiment in 2015. Biomass yields in 2015 (Figure 2) were not significantly affected by treatments (Table 3b). The shoot-to-root ratio in 2014 (Figure 3) was positive, but was flat in 2015 (Figure 4), which makes sense given the perennial nature of the plants. Root mass should build over time, since year-to-year, nutrients are stored in the roots. Finally, the stem biomass for the 2-year pots was collected in the first year. The yields from the 2-year pots in the first year had a slight positive, non-significant correlation with the yields produced in the second year (Figure 5).

Mycorrhizal colonization was evaluated as multivariate data for spores/vesicles, coils, and arbuscules as count of 50 views. Hyphae presence was recorded, but was excluded from analysis since hyphae can exist that are not due to mycorrhizal colonization (Brundrett, 2009). For both years (Figure 6 and Figure 7), none of the explanatory variables were statistically significant (Table 3).

Soil arthropod morphospecies were evaluated as multivariate data. Overall, 119 out of 120 samples were able to be collected for analysis (one sample was lost due to soil crumbling). In 2014, N additions caused a shift in the soil arthropod morphospecies community ($P < 0.05$), but the other explanatory variables and interactions did not (Table 3). Mean counts of three morphospecies that commonly occurred are shown in Figure 8: "Isotomidae" were in 47 of 119 samples, "Oribatid, 4-dot" were in 115 of 119 samples, and "Mesostigmatic, setae" were in 94 of 119 samples. In 2015, neither explanatory variable, nor

the interaction, were significant (Table 3). Mean counts of three morphospecies that commonly occurred are shown in Figure 9: "Oribatid, 4-dot" were in 44 of 44 samples, "Mesostigmatic, setae" were in 40 of 44, and "Mesostigmatic, x-legs" were in 22 of 44. The samples from the four dead plants were not included in this analysis.

Nematode abundance was evaluated using a linear model and the square root of the abundance data. The abundance data did not follow a normal distribution nor a Poison distribution. For both years (Figure 10 and Figure 11), none of the explanatory variables were significant (Table 3).

N content of stem and root tissue was evaluated together as multivariate data. Total N content in 2014 (Figure 12) was not significant due to any treatment variables (Table 3). Total N content in 2015 (Figure 13) was not significant due to soil community nor fertilizer, but the interaction of the two was significant with $P = 0.046$ (Table 3). This interaction effect is visible in Figure 13; in 0 lb/ac N, the treatment with "both" soil ecology additions has lower stem N and higher root N compared to the treatment with "none", but in 100 lb/ac, the "both" treatment has higher stem N and lower root N compared to the "none".

Two results were recorded in 2015 that may have been attributable to experimental treatments, but were not initially considered as outcome variables: plants that died, and plants that produced seeds. These two results were analyzed as binomial distributions using generalized linear models.

In the 2-year portion of the experiment in 2015, four plants died (Figure 14)—zero plants died in 2014. Plant death was defined to be plants that produced less than 2 g of stem biomass in 2015. Living plants all produced greater than 6.3 g of stem biomass. One plant produced 1.9 g of stem biomass; the other three plants produced less than 0.5 g of stem

biomass. Analysis with generalized linear models and a binomial distribution concluded that these plant deaths could not be differentiated from random chance.

In the 2-year portion of the experiment in 2015, 22 of the 44 living plants produced seeds (Figure 15). In contrast, 1 of 168 plants produced seeds in 2014. Analysis with generalized linear models and a binomial distribution showed that the production of seeds was not due to experimental treatments, nor was it due merely to stem biomass production. Power analysis of the model where seed production was predicted by fertilizer application showed that if a trend at the measured magnitude of the mean for each N treatment were significant (fertilized produced seeds 54.5%, unfertilized produced seeds 45.5% of the time), the number of replicates in the experiment would produce significant results 100% of the time. This power analysis concluded that any trend in the seed production cannot be attributed to experimental factors over random chance.

When all the soil community data were combined, NMDS results from 2014 (Figure 16) overlap completely. This supported the previous conclusions for the data analyzed separately that the treatments did not affect the soil community. NMDS results from 2015 (Figure 17) showed the same pattern of complete overlap.

Discussion

The overall story is that there is no consistent response in soil community nor in the switchgrass plants to these direct manipulations of edaphic conditions.

Neither plant biomass nor total N content of plant biomass was consistently and significantly affected by the addition of N fertilizer, mycorrhizal inoculation, nor fungal feeding nematodes. Perhaps most surprising is that the plants did not respond to fertilization. There was a potential effect of the nematode additions on the plant biomass yields ($P < 0.1$), however given that there were ten replicates in the study, it is not a strong

statement. These findings correlate with the general concept that switchgrass tolerates a wide range of conditions (Sanderson et al. 2006), as tolerance suggests that small changes in environmental conditions should not have a strong effect on plant health.

Other research has found a complicated relationship between N fertilizer and switchgrass biomass growth. One study found that the response of switchgrass to fertilizer depended on the location—only 1 of 3 locations responded positively to fertilization (Jung & Lal, 2011). Another study found that fertilization led to only small, non-significant increases in biomass yield (Duran et al., 2016). One study found that N fertilization increased the amount of leaf biomass, but it fell to the ground before harvest (Miesel et al., 2017). Thus, the amount of harvested biomass was unchanged despite the increase in overall plant biomass (Miesel et al., 2017). However, the general consensus of the research is that N fertilizer positively affects switchgrass yields (Ameen et al., 2018; Heaton et al., 2004; Waramit et al., 2011), and the recommendation continues to be to add fertilizer to maintain biomass yields (e.g., Emery et al. 2017; Miesel et al., 2017). The findings in this study do not align with this consensus.

The soil community was mostly unaffected by the three treatments. Mycorrhizal structures and nematode abundance were not affected by treatment. Soil arthropod morphospecies were weakly significant in response to N fertilizer.

Mycorrhizal colonization was not affected by treatment in 2014 or 2015. These results contrast with other research that finds an effect of N on AMF communities and abundance. A study on switchgrass and miscanthus looking at fertilizer effects on arbuscular mycorrhizae found a weak-but-significant effect of fertilizer on AMF operational taxonomic units, and a non-significant increase in extra-radical hyphae in unfertilized plots (Emery et al., 2017). A meta-analysis found an overall negative impact of N on mycorrhizae (Treseder,

2004), although there was variation in the results of the studies used. A potential explanation on why the results of this paper diverge from other research lies in the findings of Jumpponen et al. (2005). They found that N fertilization changed the mycorrhizal species composition, but did not change the rate of colonization nor the development of different mycorrhizal structures in the roots. Molecular identification of the arbuscular mycorrhizal community was not possible for this research due to financial constraints. The evidence that N does affect AMF and the findings of Jumpponen et al. (2005) suggests that species-specific interactions might have occurred in this experiment which were not captured by looking only at mycorrhizal colonization.

Nematode abundance was not affected by treatment in 2014 or 2015. A similar study to ours with a focus on multiple components of the soil community found an effect of N fertilizer on nematode community structure (Emery et al., 2017). Other research on the impact of fertilizer on nematodes has found shifts in feeding guild or families, but not always in the total nematode abundance (Emery et al., 2017; Liu et al., 2016; Song et al., 2016). Similar to the findings for mycorrhizal colonization, changes in the nematode community may have been missed because of the broad scope of measurement; total nematode abundance rather than guild groups or species.

Soil arthropod morphospecies showed a small response to N fertilization in 2014. However, in 2015 the effect disappeared. These results suggest that any effect from the fertilizer is neither having a compounding nor lag effect on the community. These findings do not correlate with the more common finding that N fertilization changes soil microarthropod abundance, both positively and negatively (Cao et al., 2011; Eisenhauer et al.,2012; Hlava, 2015). This contradiction of previous research is surprising, because this research had a similar level of detail (morphospecies) as the other studies. However, this

research was on sterilized soils, and on recently developed soil arthropod communities. The soil communities were likely composed of highly mobile species, whereas most research that has found an impact of fertilization on soil microarthropods have looked at the impact on well-developed soil communities.

Because none of the results that were measured were statistically significant due to the experimental treatments, it is possible that the initial goal of changing the edaphic conditions did not actually occur. If that were the case, it would understandably lead to inconclusive results. However, this experiment was conducted on good farm soil, which could contribute to the minimal responses of both plants and soil community members. The available nutrients inherent in the soil could have dampened any potential effect of the treatments on the soil community and plant performance. Additionally, it is possible that any changes that were caused by the treatment applications were naturally mitigated before the end of the growing season when the results were collected. Further research into these manipulations should include sampling at times closer to the treatment application as well as use soil from marginal farmland (the ideal growing location of this biofuel crop to prevent competition with food crops).

It is surprising that mycorrhizal colonization was not predicted by experimental treatments. The mycorrhizal treatment application rate was guided by commercial recommendations. Perhaps the sterilization treatment was not effective. Inoculum was sterilized in a commercial microwave (Ferriss, 1984), and the sterilized inoculum got very hot. However, the inoculum was not tested to ensure sterilization. Additionally, the sterilized soil also was not tested to ensure its sterile state. If the native AMF community was not completely eradicated with the microwave sterilization that could lead to the findings that plants in all treatments had similar levels of mycorrhizal colonization.

The nematode treatment may or may not have worked. The nematode treatment was a newly developed technique for the lab. For this application, the flaw was not in the sterilized application, because the death of living nematodes was verified under a dissecting microscope. The living nematode application had an estimated count of 30,000 nematodes. This may have been an unstable addition that did not turn into a breeding population. Alternatively, given the short life cycle of nematodes, any changes that were induced in the nematode community may not have lasted through the growing season. Additionally, the nematode treatment almost certainly included a bacterial community component. The bacterial component was not monitored initially. Similarly, there was no procedure established for analyzing the bacterial community at the end of the growing season. The technique of manipulating the nematode community is valuable to perfect for future studies. Manipulation of the mycophagous nematode community as a way to investigate changes in the soil community and plant performance could provide better understanding of how soil communities and plants interact if a method for direct manipulation could be perfected.

Soil sterilization methods in general can impact experimental results. Some studies have found increased plant growth in sterilized soils due to changes to the soil itself caused by sterilization (Mahmood et al., 2014), which can lead to false conclusions about the significance of plant-soil feedbacks. Other research suggests that soil sterilization combined with the general limitation of greenhouse experiments can affect conclusions about plant-soil feedbacks (Kulmatiski et al., 2017). However, it is unliely that soil sterilization effects were an issue in this experiment. First, microwave sterilization is less invasive than other methods (Ferriss, 1984). Second, sterilized soil was not compared to unsterilized soil, which is where increased plant growth in sterilized soil would be especially confounding to experimental conclusions. Since all the soils were treated in the same manner, even if the soils were not

completely sterilized, it is safe to assume that any effects were because of the applied treatments.

A final limitation in the experiment was the fact that the 2-year plants were not set up as a full-factorial design, because of the hypothesis that there would be an additive effect of the two soil community treatments (commercial mycorrhizal inoculum and mycophagous nematodes). However, the hypothesis did not prove true in the 1-year portion of the experiment. Since Baumgarten (2020c) shows that some soil community changes can in fact build over time, it would be important in future research to maintain separate nematode and inoculum treatments. Conversely, it is possible that the manipulations to the edaphic conditions did not even last through a single season of growth, so the results from this hypothetical second season would not have changed the overall conclusion that this system is resilient to these edaphic manipulations.

However, despite all the potential limitation, the main treatment under investigation was the effect of N fertilizer on plant growth and soil community structure. The fertilizer application had little potential for mishaps. The fertilizer was applied at the same time of year as would be applied in a commercial setting. Thus, the conclusions that the fertilization did not affect the soil community, nor the plant biomass production, is a significant finding.

Conclusion

These results show that there is no consistent difference in the measured outcomes due to the applied edaphic manipulations. However, there is the potential that further detailed study would reveal changes that occur at finer scales than were used in these methods. This research supports the argument that switchgrass does not need fertilizer because yields were not affected, even with the caveat that these experiments were conducted in a greenhouse for no more than 2 years. Additionally, this research shows that

the soil community is robust to N fertilization, as well as the perturbation to the system of adding commercial inoculum and a pulse application of fungal feeding nematodes. Finally, in answer to the initial question, these results suggest that there are no plant-soil feedbacks at the scale of the perturbation applied in this experiment. This does not invalidate research that suggests the arbuscular mycorrhizal association is important to switchgrass growth. Rather, it shows that the interaction is not linear, and that the perturbation of farming that comes from N fertilization alone does not appear to shift this foundational and important association between mycorrhizae and switchgrass. Since switchgrass did not respond strongly to fertilization, these results support the use of switchgrass as a biofuel crop since minimal fertilization will maximize the sustainability of the crop.

Tables

Table 1

Treatment number and applications for the 1-year (2014) pots. Full-factorial design.

Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Nematodes
3	0	Mycorrhizae
4	0	Both
5	50	None
6	50	Nematodes
7	50	Mycorrhizae
8	50	Both
9	100	None
10	100	Nematodes
11	100	Mycorrhizae
12	100	Both

Table 2

Treatment number and applications for the 2-year (2015) pots. Partial-factorial design.

Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Both
3	100	None
4	100	Both

Table 3

P-values calculated using nonparametric MANOVA for all result variable except nematode abundance, which was evaluated with a linear model. Bold and italics numbers are P-values less than 0.1. a) 2014 results. b) 2015 results

a)

2014	Fertilizer	Mycorrhizal Inoculum	Nematodes	F x M	Fx N	M x N	F x M x N
Biomass yield	0.289	0.45	***0.063***	0.305	0.795	0.619	0.239
Biomass nitrogen content	0.81	0.642	0.663	0.891	0.816	0.965	0.594
Soil arthropod morphospecies	***0.044***	0.676	0.364	0.772	0.403	0.997	0.424
Mycorrhizal structures	0.26	0.636	0.25	0.211	0.462	0.927	0.173
Nematode abundance	0.515	0.766	0.961	0.96	0.634	0.987	0.774

b)

2015	Fertilizer	Soil ecology addition	Fert x S.E.
Biomass yield	0.486	0.542	0.988
Biomass nitrogen content	0.871	0.701	***0.046***
Soil arthropod morphospecies	0.942	0.233	0.803
Mycorrhizal structures	0.936	0.889	0.771
Nematode abundance	0.841	0.873	0.229

Figures

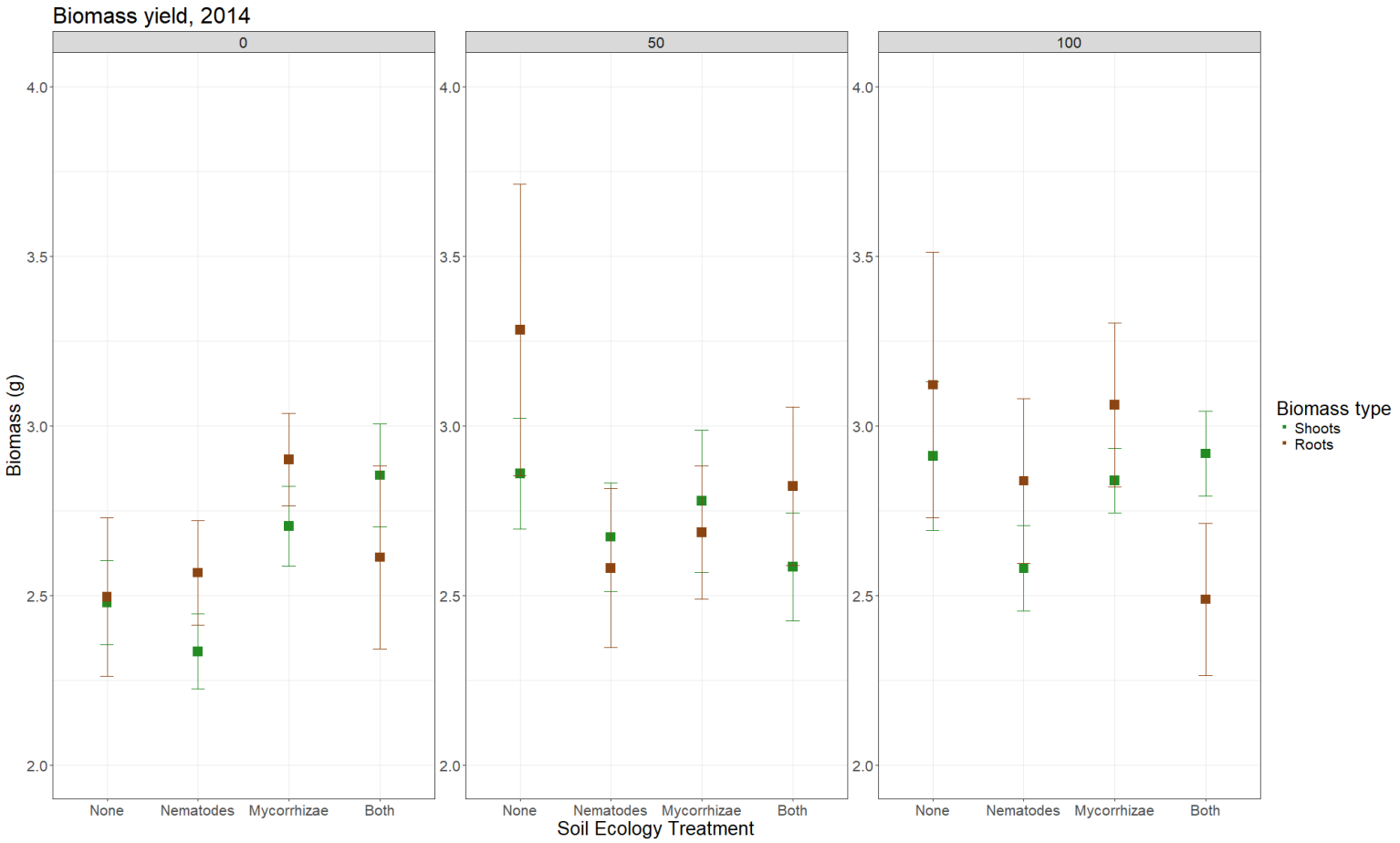

Biomass yield, 2014
0
50
100
4.0
3.5
3.0
2.5
2.0
Biomass (g)
None
Nematodes
Mycorrhizae
Both
Soil Ecology Treatment
Biomass type
Shoots
Roots

Figure 1

(Figure 1 continued)

Mean biomass yields of the 1-year plants with standard error bars (values not added together). The graph boxes are divided by amount of N fertilizer (0 lb/ac, 50 lb/ac or 100 lb/ac). There were no statistically significant differences due to experimental treatments, but the lowest biomass yields were in the unfertilized pots, and mycorrhizal inoculation tended to increase biomass productions whereas nematode addition tended to decrease biomass production. Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

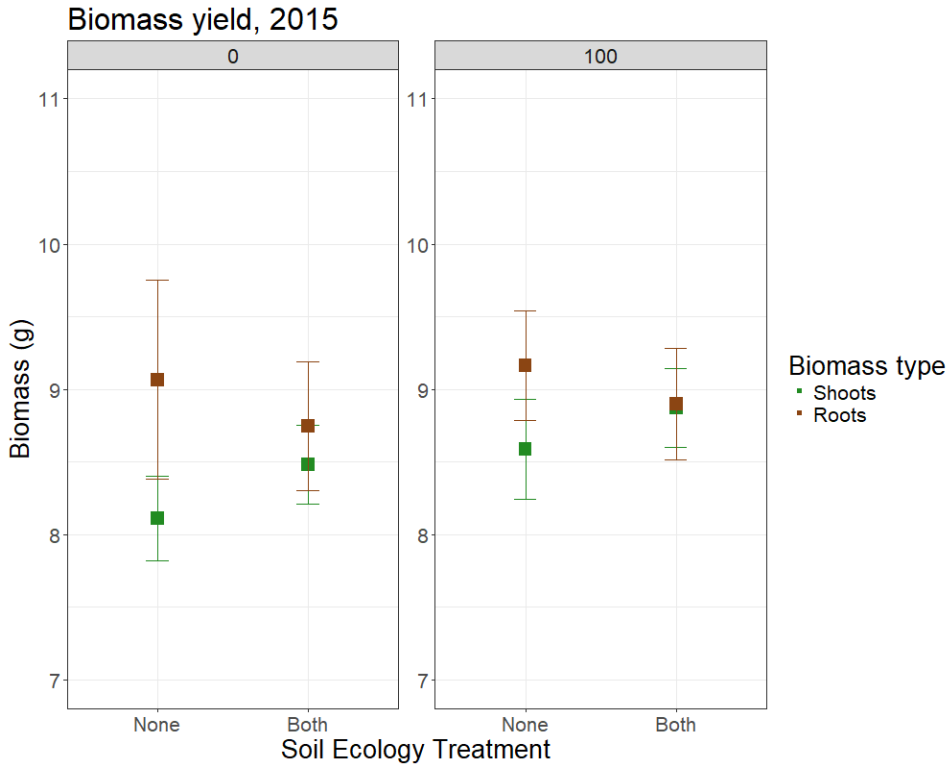
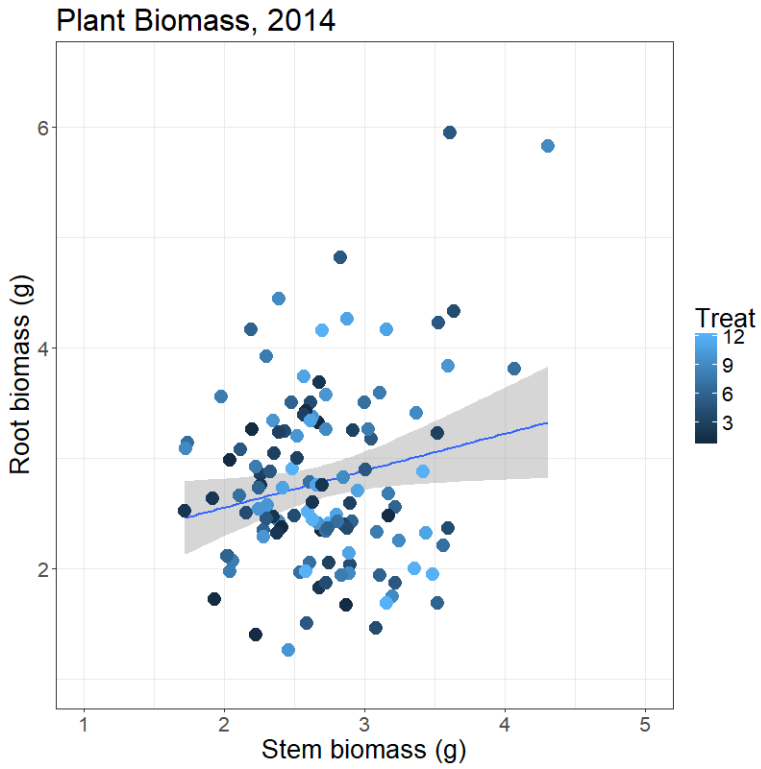


Figure 2

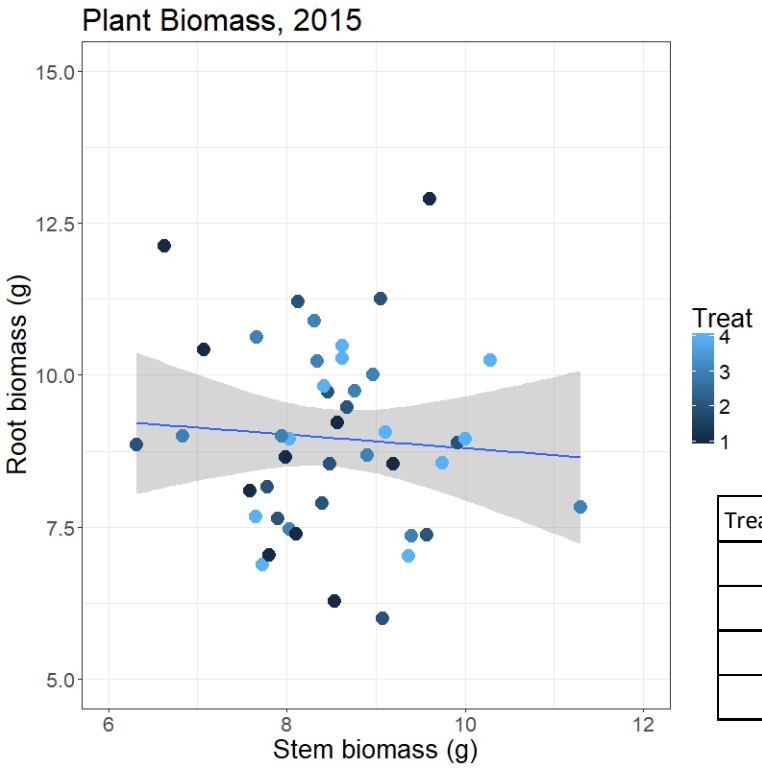
Mean biomass yields for the second year of growth in the 2-year pots with standard error bars (values not added together). Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). There were no statistically significant differences due to experimental treatments. However, the addition of soil community tended to increase stem biomass production and decrease root biomass production. Results were analyzed together using nonparametric MANOVA; p-values can be found in Table 3.



Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Nematodes
3	0	Mycorrhizae
4	0	Both
5	50	None
6	50	Nematodes
7	50	Mycorrhizae
8	50	Both
9	100	None
10	100	Nematodes
11	100	Mycorrhizae
12	100	Both

Figure 3

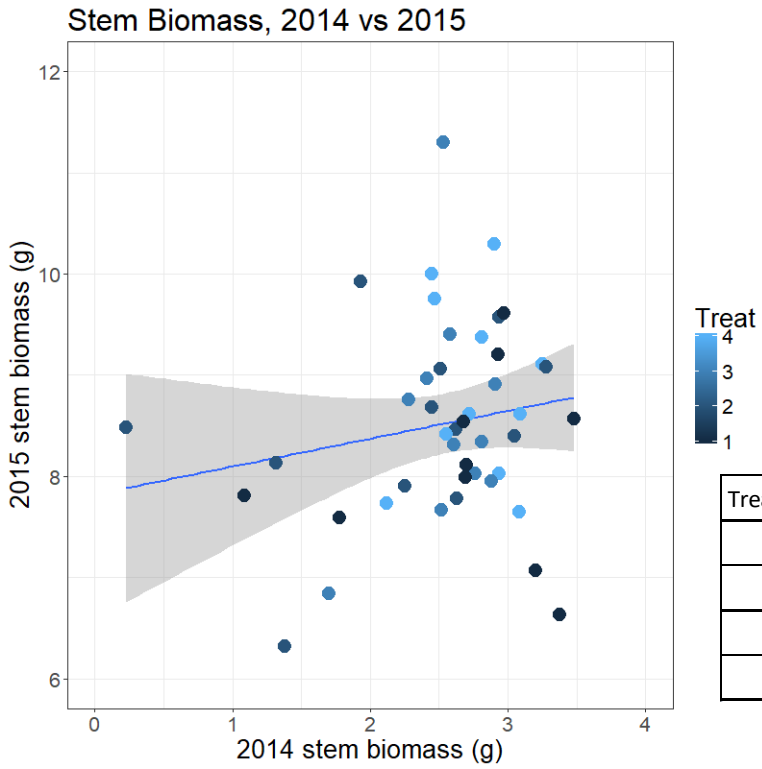
Stem biomass plotted against root biomass for the 1-year pots (2014). There is a positive slope to the relationship, but it is not significant, and the experimental treatments do not explain much variation ($P > 0.05$). The colors for all twelve treatments are hard to differentiate, but the overall point of this graph can be interpreted from the extreme colors: the biomass production within each treatment type varied greatly, and can be seen by the lighter and darker colors being scattered all over. Results were analyzed using a linear model.



Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Both
3	100	None
4	100	Both

Figure 4

Stem biomass plotted against root biomass for the two-year treatments (2015). There is a non-significant negative slope ($P > 0.05$). This negative trend matches the expected trajectory of root growth for perennial grasses—the second year of growth should have more root biomass compared to stem biomass because the roots are a storage organ for the plant. Results were analyzed with a linear model.



Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Both
3	100	None
4	100	Both

Figure 5

Stem biomass for 2014 biomass yields versus the 2015 biomass yields (linear model adjusted $R^2 = 0.009$, $P > 0.05$) shows little predictive power of the biomass yields in 2014 to predict the yields in 2015. Results were analyzed with a linear model.

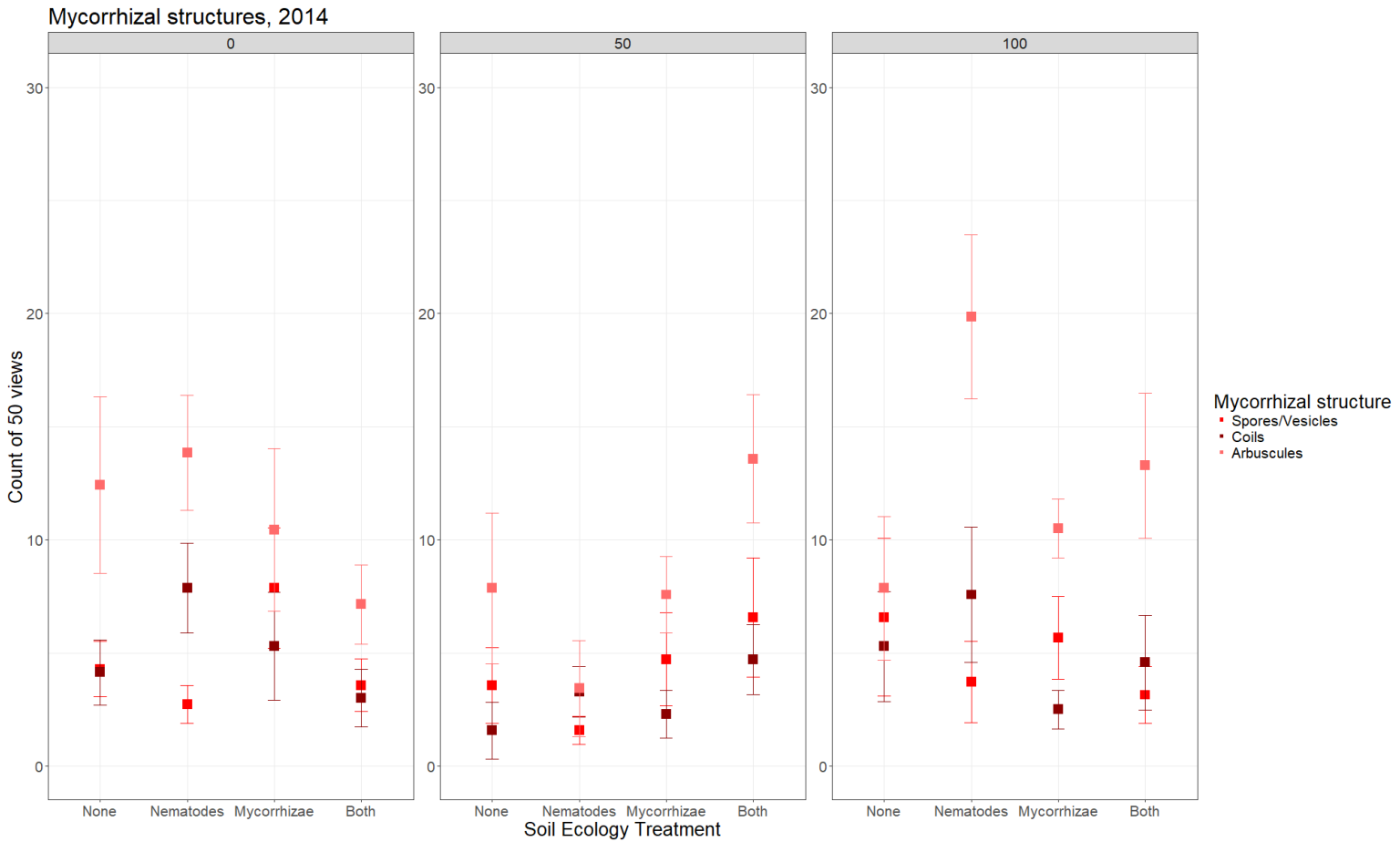

Mycorrhizal structures, 2014
0
50
100
Count of 50 views
30
20
10
0
None
Nematodes
Mycorrhizae
Both
Soil Ecology Treatment
Mycorrhizal structure
Spores/Vesicles
Coils
Arbuscules

Figure 6

(Figure 6 continued)

Mean mycorrhizal structures measured out of 50 views in the 1-year pots with standard error bars. There were no statistically significant differences due to experimental treatments. Soil ecology treatments are no additions, mycophagous nematodes, mycorrhizal inoculum, or both nematodes and mycorrhizal inoculum. The graph boxes are divided by amount of N fertilizer (0 lb/ac, 50 lb/ac or 100 lb/ac). Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

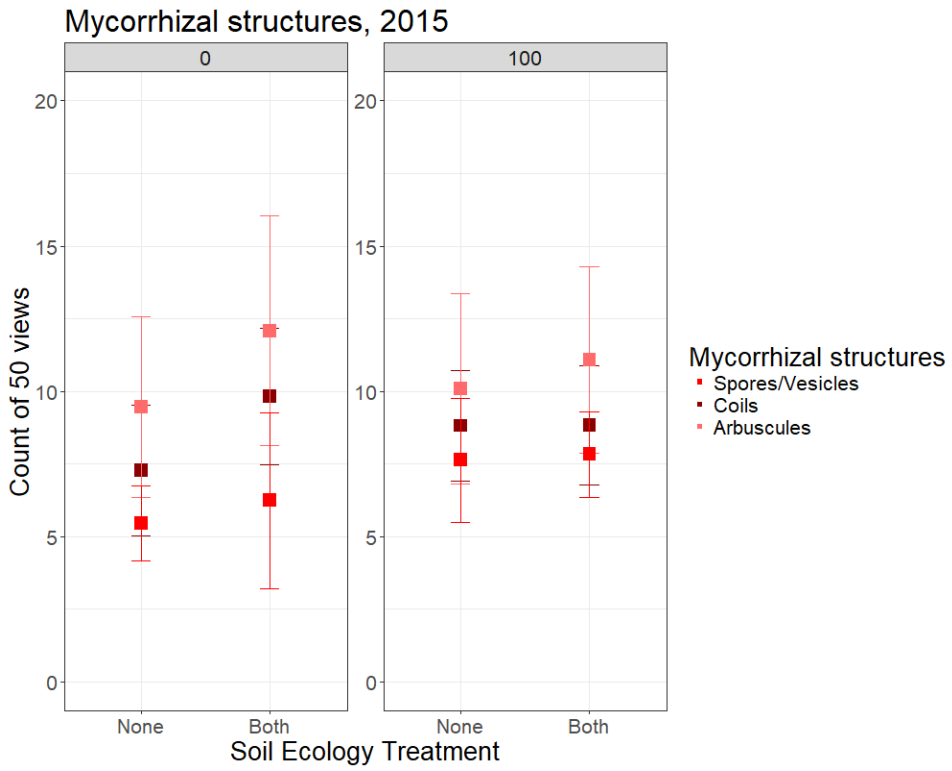


Figure 7

Mean mycorrhizal structures in the 2-year pots with standard error bars. There were no statistically significant differences due to experimental treatments. Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

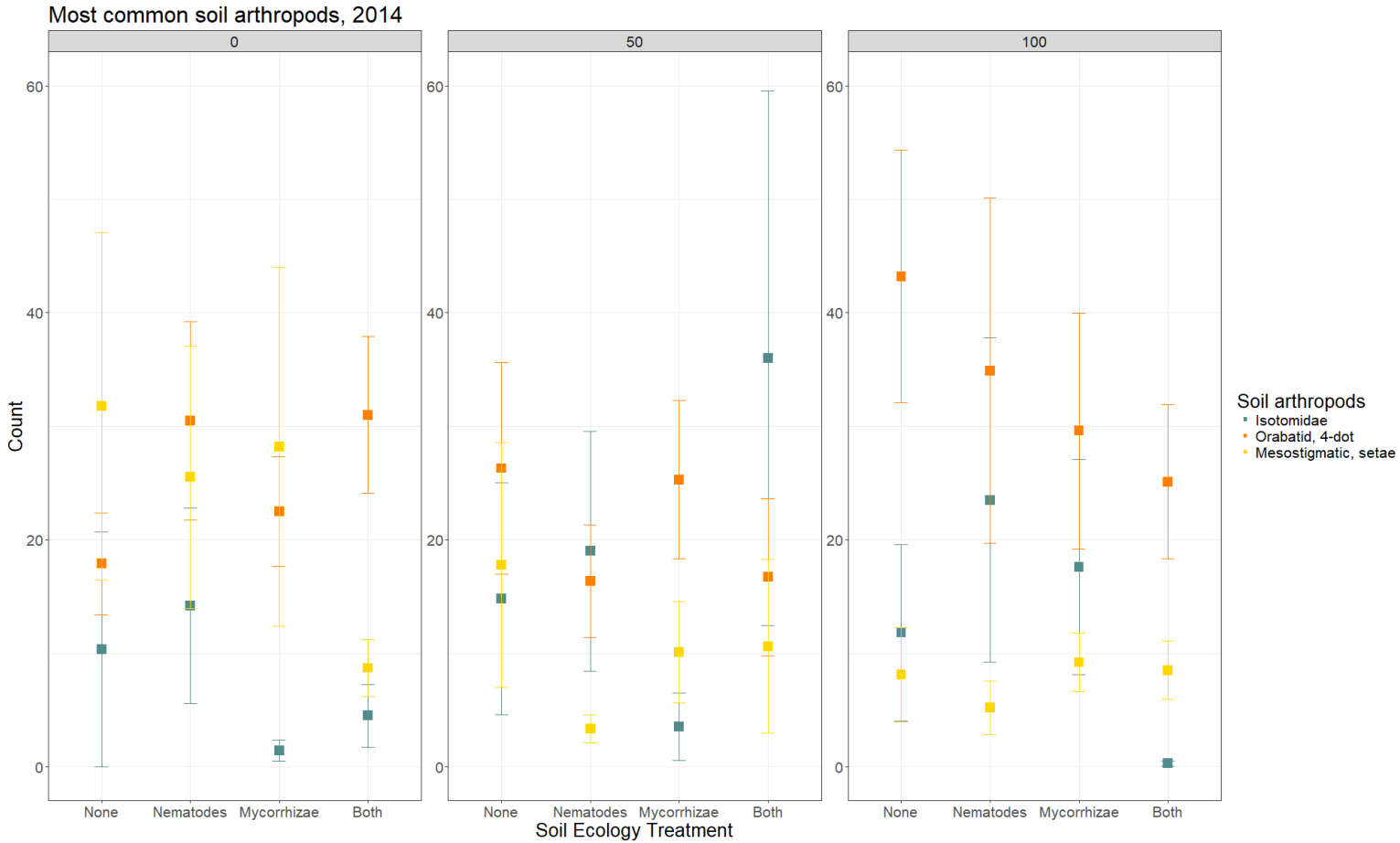

Most common soil arthropods, 2014
0
50
100
Count
60
40
20
0
None
Nematodes
Mycorrhizae
Both
Soil Ecology Treatment
Soil arthropods
Isotomidae
Orabatid, 4-dot
Mesostigmatic, setae

Figure 8

(Figure 8 continued)

Mean count of three common soil arthropod morphospecies in the 1-year pots with standard error bars. Soil arthropod morphospecies did differ due to fertilization level, and these three soil arthropods show a trend of different abundances across the fertilizer gradient. Soil ecology treatments are no additions, mycophagous nematodes, mycorrhizal inoculum, or both nematodes and mycorrhizal inoculum. The graph boxes are divided by amount of N fertilizer (0 lb/ac, 50 lb/ac or 100 lb/ac). Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

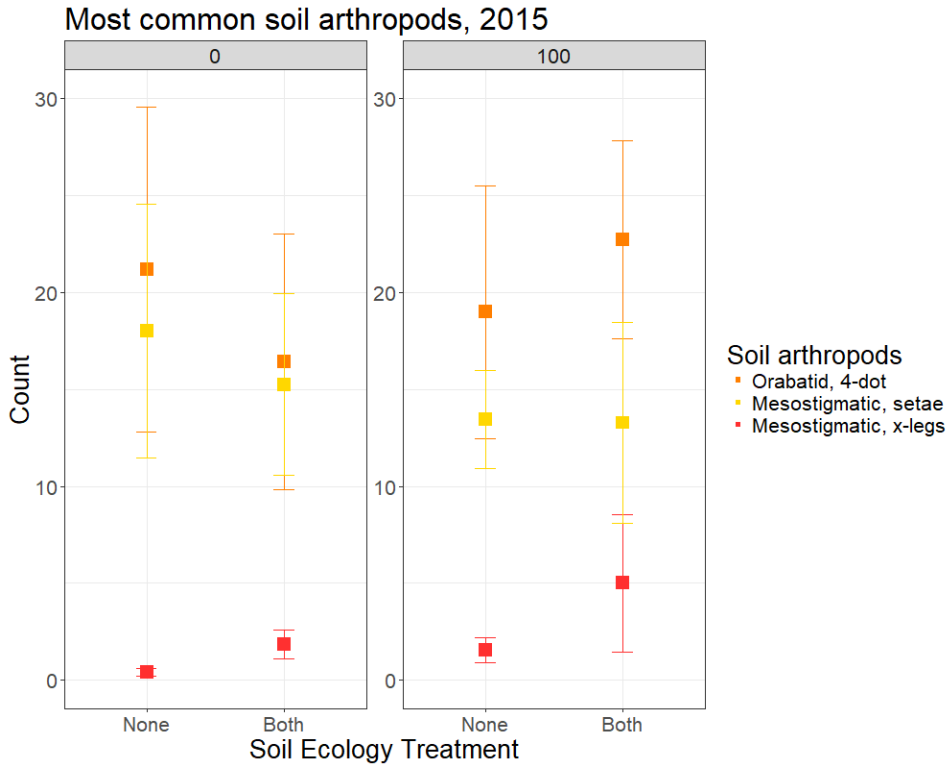


Figure 9

Mean count of three common soil arthropod morphospecies in the 2-year pots with standard error bars. There were no statistically significant differences due to experimental treatments. Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

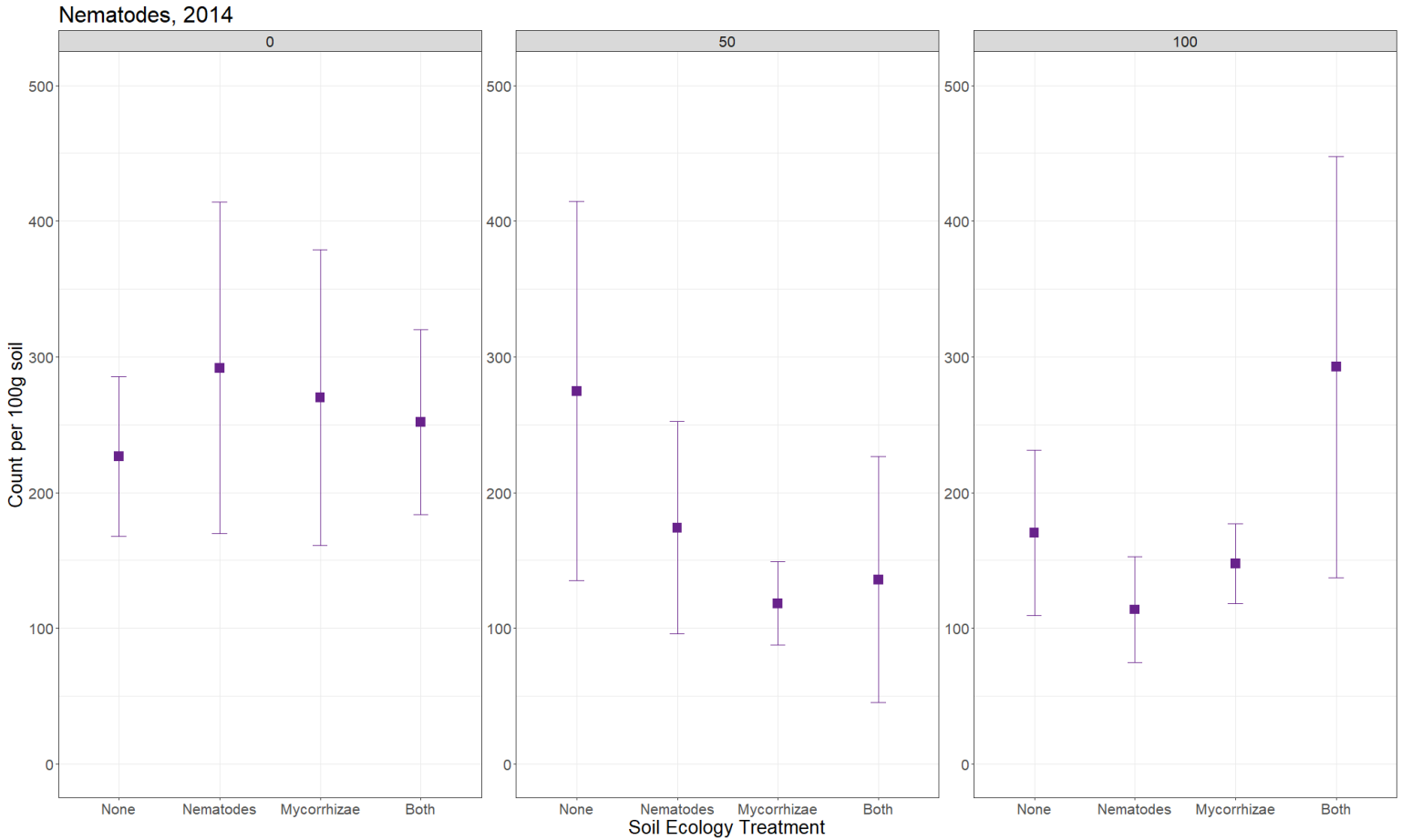

Nematodes, 2014
0
50
100
Count per 100g soil
500
400
300
200
100
0
None
Nematodes
Mycorrhizae
Both
Soil Ecology Treatment

Figure 10

(Figure 10 continued)

Mean nematode abundance in the 1-year pots with standard error bars. There were no statistically significant differences due to experimental treatments, however there were fewer nematodes in the fertilized treatments (50 lb/ac and 100 lb/ac) compared to the unfertilized treatment. Soil ecology treatments are no additions, mycophagous nematodes, mycorrhizal inoculum, or both nematodes and mycorrhizal inoculum. The graph boxes are divided by amount of N fertilizer (0 lb/ac, 50 lb/ac or 100 lb/ac). Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

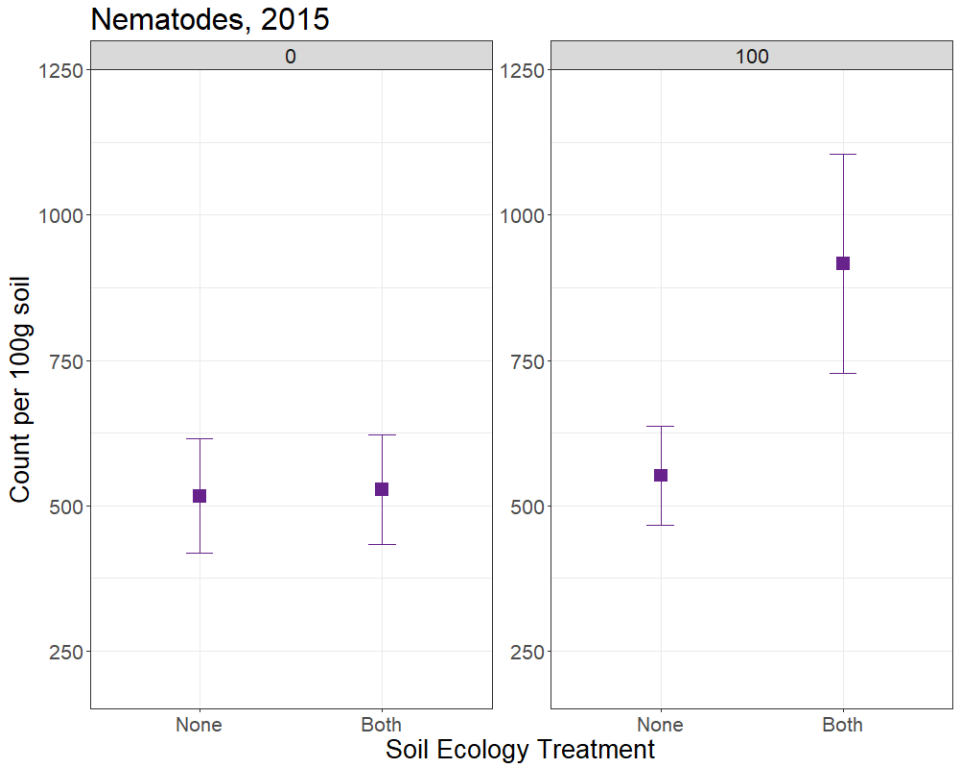


Figure 11

Mean nematode abundance in the 2-year pots with standard error bars. There were no statistically significant differences due to experimental treatments. However, the highest abundance was found in the fertilizer plus soil ecology treatments. Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). Results were analyzed using a linear model; p-values can be found in Table 3.

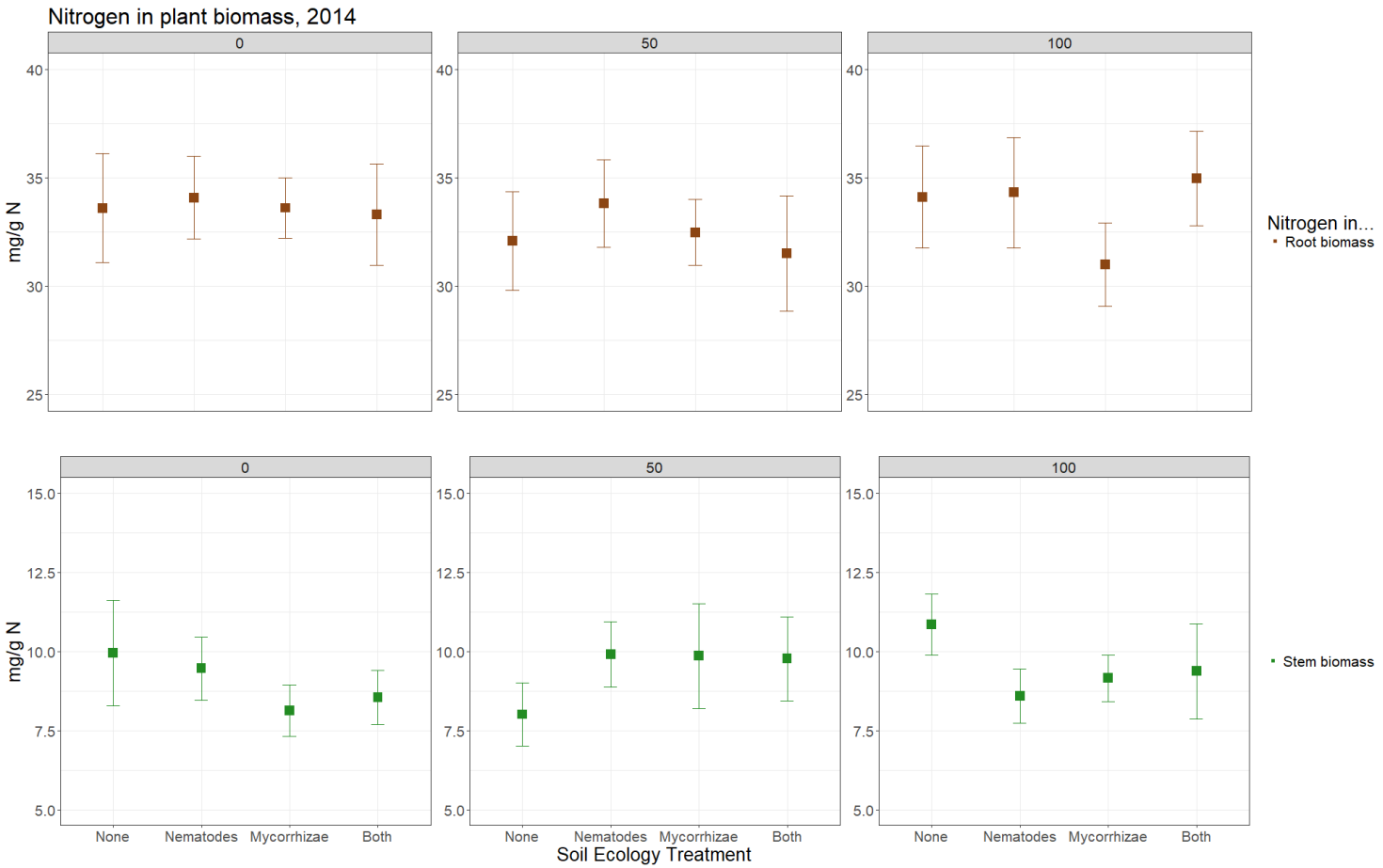

Nitrogen in plant biomass, 2014
0
50
100
mg/g N
40
35
30
25
15.0
12.5
10.0
7.5
5.0
None
Nematodes
Mycorrhizae
Both
Soil Ecology Treatment
Nitrogen in...
Root biomass
Stem biomass

Figure 12

(Figure 12 continued)

Mean N in switchgrass biomass for the 1-year pots with standard error bars, stem and root tissue separated. Soil ecology treatments are no additions, mycophagous nematodes, mycorrhizal inoculum, or both nematodes and mycorrhizal inoculum. The graph boxes are divided by amount of N fertilizer (0 lb/ac, 50 lb/ac or 100 lb/ac). Results were analyzed together using nonparametric MANOVA; p-values can be found in Table 3.

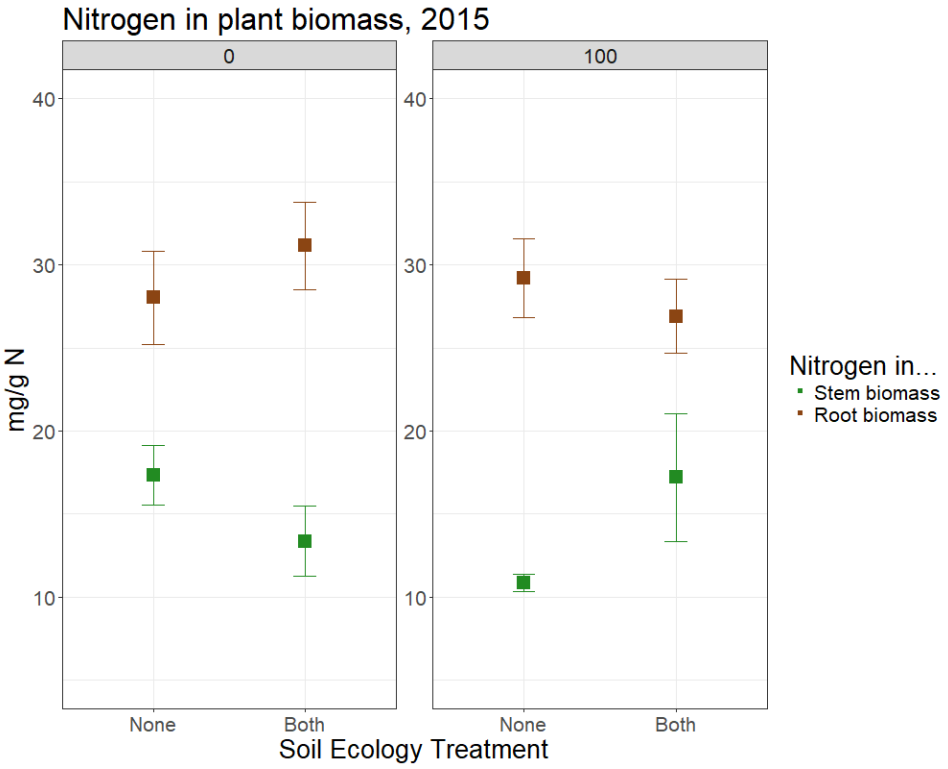


Figure 13

Mean N in switchgrass biomass for the 2-year plots with standard error bars; stem and root tissue separated. Seed biomass was not included in digests for plants that produced seeds. The interaction of fertilizer and soil ecology had a significant effect, which can be seen here in the graph with the reversal of the higher N content in "Both" for the unfertilized pots but in "None" for the fertilized pots. Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). Results were analyzed using nonparametric MANOVA; p-values can be found in Table 3.

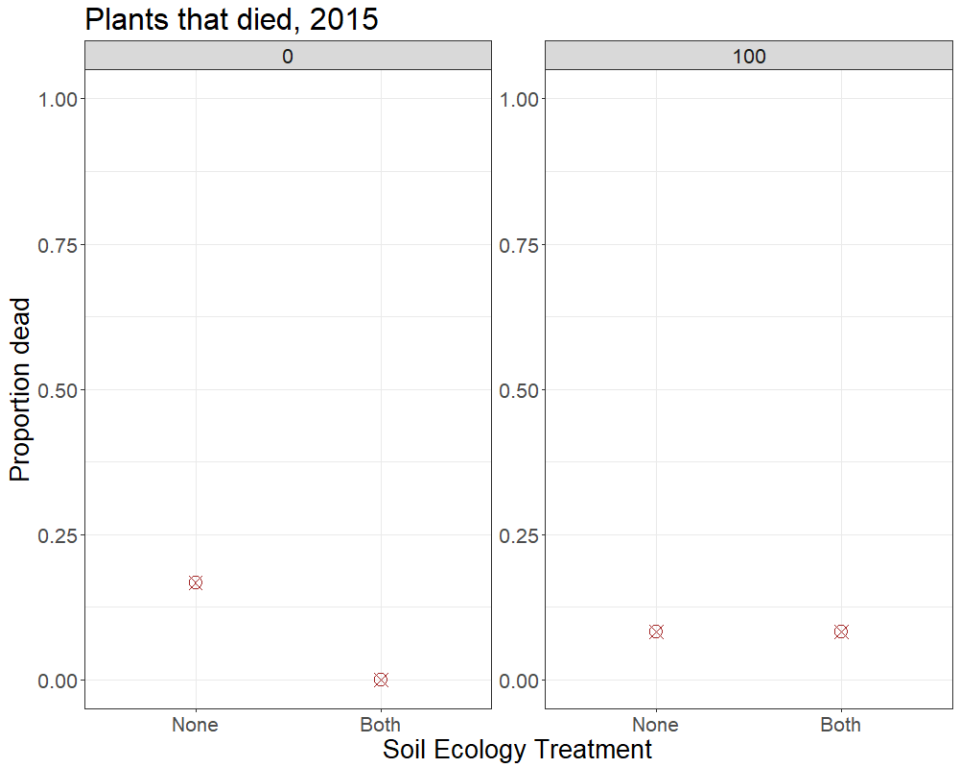


Figure 14

Proportion of plants that died grouped by treatment type (N = 4 of 48) shows no statistically significant correlation between treatment type and number of plants that died ($P > 0.05$). Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). Results were analyzed with generalized linear models and a binomial distribution.

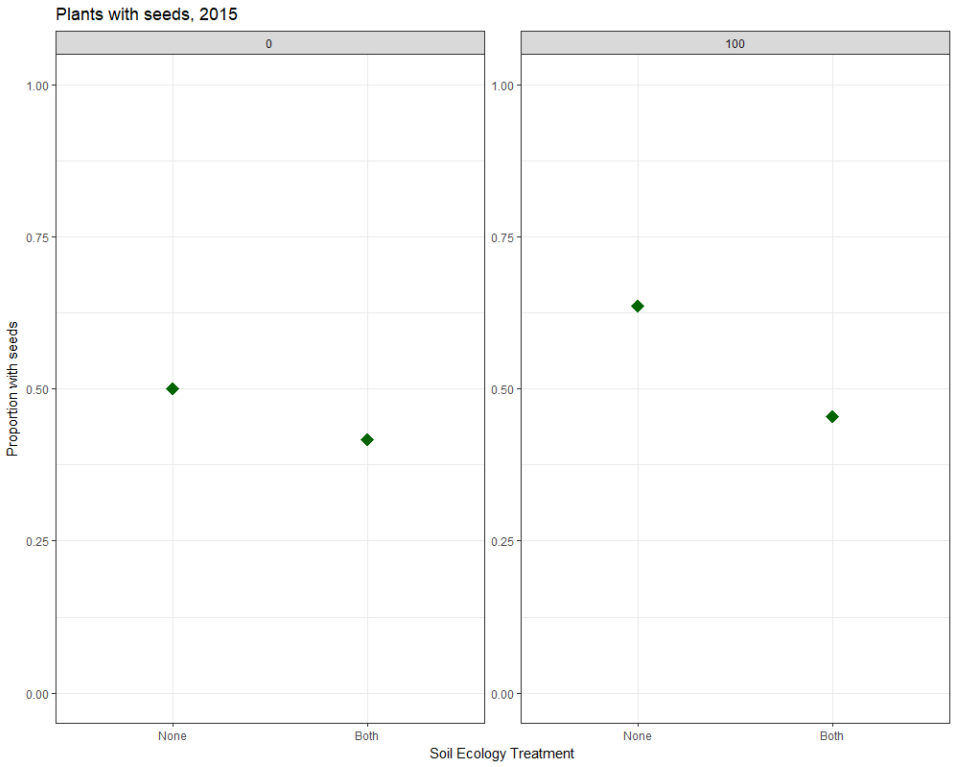


Figure 15

Proportion of plants that produced seeds grouped by treatment type (22 of 44 living plants). The number of plants that produced seeds was slightly higher in the fertilized plots, and was slightly reduced in both fertilizer treatments for pots with soil ecology additions. However, these trends were not statistically significant ($P > 0.05$). Soil ecology treatments were either no addition of nematodes and mycorrhizal inoculum, or both. The graph boxes are divided by amount of N fertilizer (0 lb/ac or 100 lb/ac). Results were analyzed with generalized linear models and a binomial distribution.

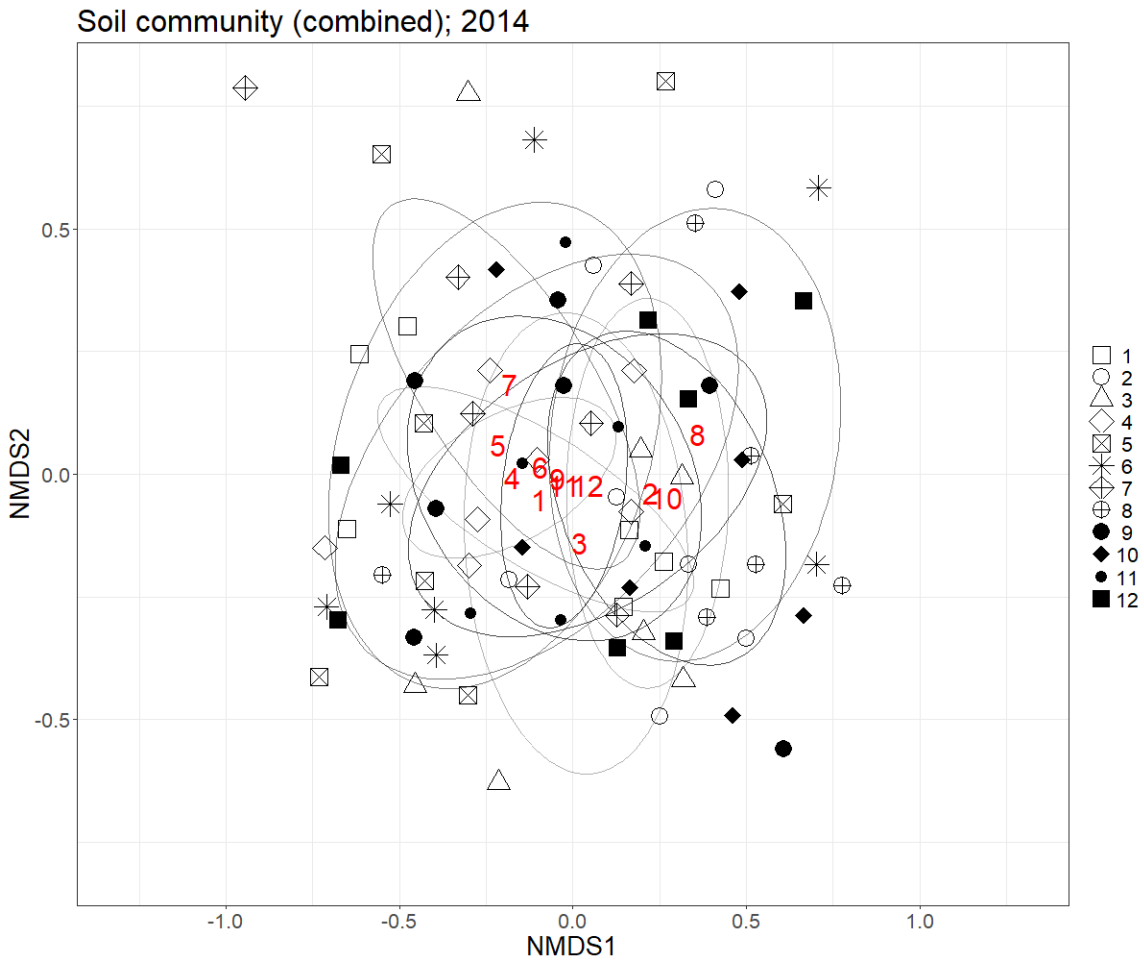


Figure 16

Non-metric multidimensional scaling (NMDS) for the soil community data for 2014. Each column was scaled to itself to reduce the chance of one variable skewing the results. There are no separations between treatments in any way.

Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Nematodes
3	0	Mycorrhizae
4	0	Both
5	50	None
6	50	Nematodes
7	50	Mycorrhizae
8	50	Both
9	100	None
10	100	Nematodes
11	100	Mycorrhizae
12	100	Both

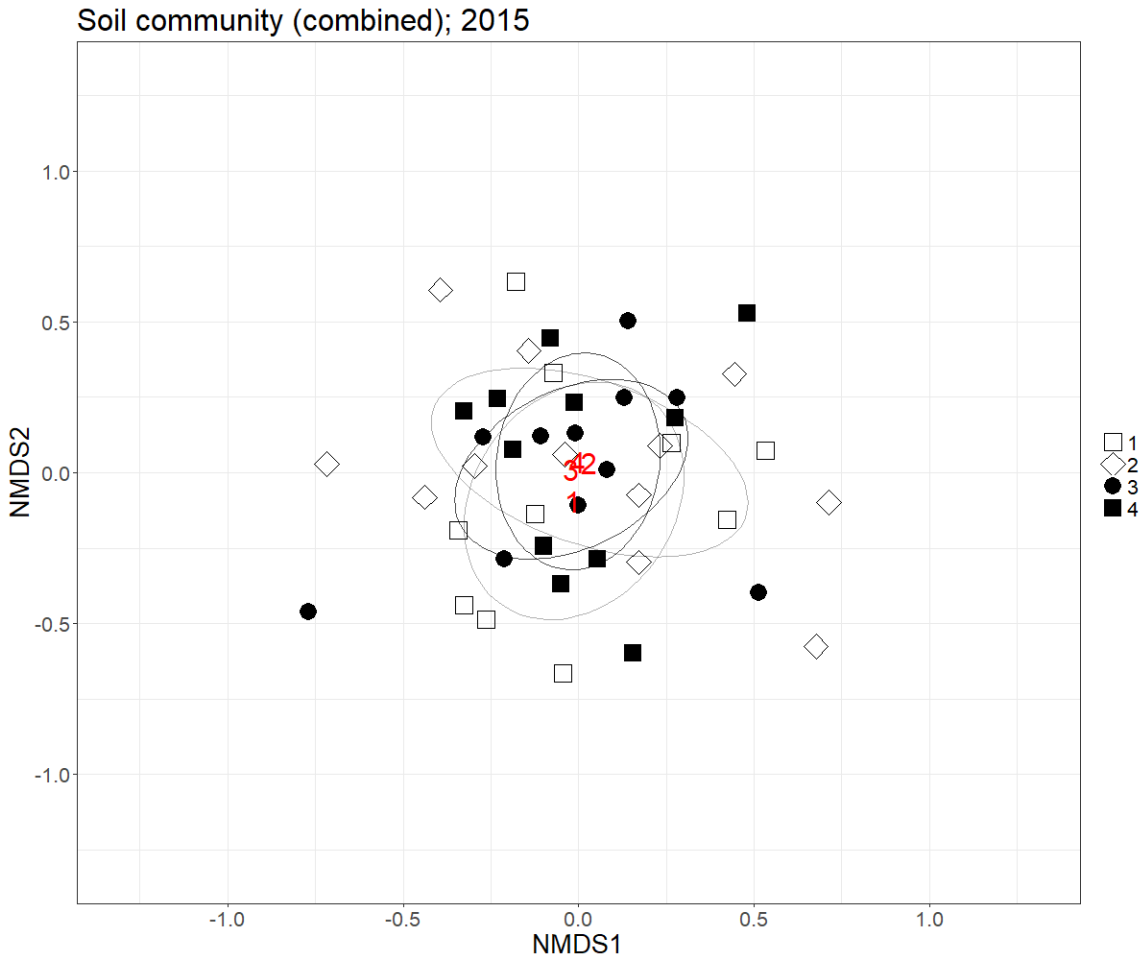


Figure 17

Non-metric multidimensional scaling (NMDS) for the soil community data for 2015. Each column was scaled to itself to reduce the chance of one variable skewing the results. There are no separations between treatments in any way.

Treatment	N (lbs/ac)	Soil Ecology
1	0	None
2	0	Both
3	100	None
4	100	Both

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Chapter 4: Edaphic manipulation of the soil community of biofuel switchgrass (*Panicum virgatum*) in three soils shows resilience of plant and soil community

Abstract

Panicum virgatum is a potential biofuel crop that is tolerant of many different soils. Previous experiments by the author have shown that the soil community and *P. virgatum* are resilient to nitrogen (N) fertilizer additions in prime agricultural soil. However, in less nutrient-rich soils, where biofuel crops should be grown to prevent competition with food crops, *P. virgatum* may show a greater response to changes in the soil conditions. This experiment investigates whether *P. virgatum* growth responds differently to soil manipulations (adding N fertilizer and commercial mycorrhizal inoculum) in three soils: "prime farmland", "farmland of local importance", and "not prime farmland" as rated by the Natural Resources Conservation Service farmland classification system. Additionally, the soil was used for two consecutive years with no additional manipulation to test for soil exhaustion. A 3x2x2 factorial design greenhouse experiment was conducted to test the hypothesis that switchgrass biomass yields and soil community would respond with different effect size or direction to edaphic manipulations in three soils.

In the first year, measurements of soil factors were different with statistical significance due to soil type. Higher extractable nitrate (NO_3) in fertilized treatments additionally was statistically significant. Statistically significant differences in measurements of plant factors (biomass and N content) were primarily due to soil type. While both stem and root N content were higher in fertilized treatments, the trend was not statistically significant. Soil community factors (mycorrhizal structures, soil arthropod morphospecies, carbon utilization of the microbial community, and nematode abundance) which were

primarily multivariate data, were only different with statistical significance due to soil type, not inoculation or fertilization.

In the second year, plants were grown in the same soil as in the first year to test for lag effects; no additional manipulation occurred. Soil extractable nutrients were lower than in the first year ($P < 0.05$). Within the second-year-only analyses of soil factors, statistically significant difference were primarily due to soil type. However, lower amounts of extractable phosphate (PO_4) were statistically significant in inoculated treatments. Plant factors were different with statistical significance only for soil type. Soil community factors showed stronger response to inoculation than in the first year, in addition to the statistically significant differences attributable to soil type.

These results showed the resilience of switchgrass and the soil community to perturbation across all soil types. While there were signs that the soil was approaching a state of exhaustion, this was not reflected in plant biomass yields. The lag response that was found suggests that treatment effects build up over time; since inoculation was not statistically significant in the first year but was in the second year for BIOLOG ecoplate results and nematode abundance. This building effect is worthy of further investigation. The response of multiple factors to soil type rather than fertilization or inoculation suggests that soil texture has a role as a driver of ecological communities, but this result does not align with other field-based studies in soil ecological research. Most importantly, these results support previous findings that show switchgrass to be suitable for a range of soil type, which is ideal for a potential biofuel crop.

Introduction

Switchgrass (*Panicum virgatum*), a potential biofuel crop, is said to tolerate many soil types. The ability to grow in soils suboptimal for crop production means that switchgrass should not compete directly with food crops for limited land resources. Switchgrass may even enhance marginal farm land through its deep root system. Previous research by the authors (Baumgarten, 2020b, c) on the soil community of switchgrass was conducted in prime farmland, rated by the Natural Resources Conservation Service (NRCS). However, since the ideal location for switchgrass production is marginal farmland, the conclusions may be of limited practical value. Thus, in the greenhouse experiment described here, the hypothesis is that the effects of fertilization and inoculation on the soil community will change when switchgrass is grown in soils less fertile than prime farmland.

The question of marginal lands versus good farm soil is critical for multiple reasons. First, in a growing world, the competition of biofuel crops with food crops is a significant concern (Fargione et al., 2010; Godfray et al., 2010). Research has shown that biofuel produced from food crops, such as sugarcane and corn, causes a rise in food price (Fargione et al., 2010; Searchinger et al., 2008). Biofuel production was thought to be a culprit in the 2007 - 2008 spike in food price (Mueller et al., 2011). Subsequent research suggested in general, the relationship between food crops and energy crops is non-linear, and to say that biofuels caused a direct price increase of food was an oversimplification of the issues that drive food price (Ajanovic, 2011; Aké, 2017; Tomei & Helliwell, 2016). Despite this conclusion, it is still suggested that using dedicated energy crops such as switchgrass ameliorates potential competition with food crops. However, even if the energy crops can be grown on less fertile soil, agricultural changes are filtered through the decisions of individuals (Burli et al., 2019; Jin et al., 2019). Farmers may still seek to grow the most

possible resource by growing biofuel crops on prime farm land. Thus, research into the most sustainable ideals of biofuel production can help farmers make decisions to align with sustainability and farm profitability.

The economic pressure on farmers can lead to direct land clearing (DLUC) for production of the biofuel crop, or indirect land clearing (IDLUC) to make space for food crop production that is offset by the production of the biofuel crop (Khanna et al., 2011; Searchinger et al., 2008). Any transformation of relatively undisturbed habitats into crop production will cause carbon (C) emissions (Searchinger et al., 2008). The ecological degradation and C loss from the soil associated with disturbance from DLUC and IDLUC can be great enough to offset any positive gains from using a renewable resource for energy (Khanna et al., 2011; Searchinger et al., 2008). Because the process of creating biofuels is complex (Demain, 2009; Dien et al., 2018; Galán et al., 2019; Rabbani et al., 2018), and the balance of net energy gain and neutral or negative C emissions is critical to the sustainability of the fuel, the potential C debt associated with DLUC and IDLUC cannot be dismissed. Although this topic is not addressed beyond this paragraph, it relates to switchgrass being a crop worthy of further investigation.

Switchgrass has the potential to enhance C storage in the degraded soils of marginal agricultural lands through its deep roots (Khanna et al., 2011). Targeting switchgrass for production on marginal land is key to ensuring net C absorption in the production of the biofuel (e.g., Baumgarten, 2020a). The term "marginal farmland" does not have the same definition same across all literature (Gopalakrishnan et al., 2011). Marginal farmland initially was defined purely economically, implying that the economic gain from growing crops does not offset the costs of production. Now the term usually includes some measure of soil health, such as being highly erodible, since the Conservation Reserve Program (CRP) was

enacted (Gopalakrishnan et al., 2011). Two studies find that switchgrass does in fact improve soil compared to previous use as annual cropland (Liebig et al., 2008) and compared to similar land used for corn biofuel production (Stewart et al., 2015). These findings support the assertion that switchgrass will also improve the soil in certain marginal soils.

The general definition of marginal land as areas where the economic gain from growing crops does not offset the costs of production (Gopalakrishnan et al., 2011) implies a nutrient deficit in comparison to prime farm land for common crops. Liebig's law of the minimum also related to the idea of nutrient deficits in the soil. Liebig's law was developed in the 1840's and was connected to the idea that soil resources could be exhausted by agriculture. Liebig thought the growth of individual plants was directly proportionate to the most limiting mineral nutrient in the soil (Usher, 1923). Although Liebig's initial description verbatim is not accurate, the overall message is still relevant to crop nutrient management and ecology (Ferreira et al., 2017; Hiddink, 2005; Treseder & Allen, 2002). Recent research shows that the microbial community responds differently to N fertilization in soils poor in N versus those rich in N, with some communities expanding in the N poor soil but remaining stable in the N rich soil (Wang et al., 2019). In contrast, Danger et al. (2008) suggest that communities do not follow Liebig 's law directly, rather the components of the community adjust to match the resources present. Thus, the addition of N fertilizer could differentially affect both the soil community and plant growth in soils with dissimilar mineral composition because inherent N-limitation will be different.

There is a long history of the concept of soil exhaustion. From concluding that early civilizations failed because of salinization of the soil to the hypothesis that medieval farmers literally plowed away the fertile topsoil, there are many examples of humans failing to preserve the soil. The fact that productive soils can undergo changes that lead to crop

failure, with a major component being the loss of N, has been known for a long time (e.g., Usher, 1923). Current concepts of soil exhaustion are multi-faceted and inherently include concepts of the soil order, which makes an overarching definition harder to determine. Soil exhaustion components include nutrient limitation, erosion, human activities causing damage such as deforestation and tillage, and ecological impacts such as over-fertilization with N and P (e.g., Armesto et al., 2009; Jordán et al., 2010; McDowell & Sharpley, 2003; Obalum & Obi, 2010). A large component of the current definition of soil exhaustion relates to Liebig's concept of nutrient limitation and considers the nutrient availability of the macro- and micro- nutrients necessary for plant growth. Best-management fertilization regimes are typically founded on this concept of supplementing the plant growth with the proper level of nutrients given the constraints of the local soil, with N being the most commonly applied macronutrient (e.g., Cao et al., 2018; de Ponti et al., 2012). In a recent meta-analysis, Trivedi et al. (2016) found that agricultural areas and adjacent natural areas have the same level of soil N, which they argue that is due to N fertilization additions. The idea of soil exhaustion leads to the question driving this research experiment: If switchgrass fields are to remain productive for 20 years, will soil exhaustion become more important as the fields age, and will N fertilizer become more crucial to sustain biomass yields?

This experiment uses three soils: prime farm soil, marginal farm soil, and poor farm soil. These descriptions of the three soils are based on the combination of farmland classification by the NRCS and the New Jersey (NJ) Soil Health Assessment (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). These soils have fundamentally different nutrient profiles due to their composition. Nutrient availability in soils relates to the particles that make up the soil: clay, silt and sand. Clay particles have much more reactivity than sand particles and can reduce leaching of

nutrients by binding them. Although the specific makeup of the soil was not the driving question of this research, it seems feasible that fertilizer may be less effective in soils with different inherent structure. Previous research showed that in prime farm soil switchgrass was minimally responsive to fertilization and the soil community was resilient to perturbation by fertilization (Baumgarten, 2020b, c). The hypothesis for this experiment is that in soil types with lower inherent nutrient availability compared to prime farm soil, the soil community will respond differently to fertilization, and that will in turn impact plant growth.

Some research has suggested that trophic cascades can mask responses to experimental treatment due to the response showing at a higher trophic level than expected (Coleman et al., 2004; Milton & Kaspari, 2007). Trivedi et al. (2016) propose that microbial communities can shift in advance of obvious physical and chemical changes as a marker of the degradation due to agriculture. Similarly, it is reasonable to anticipate that the greenhouse pots will attract communities of soil animals that can colonize new locations. It is reasonable to expect that field-collected soils that are only treated by homogenization will have remnants of the soil community from the original field site, particularly eggs, microbes, and other small remnants are capable of surviving the disruption of removal, transport, and homogenization. Research supports that members of the soil community are able to migrate into islands, such as revegetated soil or moss clumps on rocks (Åström & Bengtsson, 2011; Meloni & Varanda, 2015; Smith, 2013). Therefore, it is reasonable to expect experimental manipulations of edaphic conditions to affect the entire soil food web, for there to be a differential gradient within the soil community to measure, and to account for these various possibilities by measuring multiple trophic levels.

Further detail for the hypothesis of this experiment is that the addition of N fertilizer and a commercial inoculum will have different effect sizes in a marginal farm soil as well as in a poor farm soil when compared to the prime farm soil that was used in previous experiments (Baumgarten 2020b, c). The predicted results are that adding fertilizer and mycorrhizal inoculum will have linear and additive effects on plant growth and soil conditions. For example, mycorrhizal inoculation individually would lead to an increase in plant biomass yields, fertilizer addition alone would lead to a similar increase, and the combined effect would be that of the two individual effects added together. Soil community measures are predicted to respond to changes in the plant and soil factors by forming a different community (for soil microarthropods), having different abundance (for nematodes), having different structures (for mycorrhizal colonization), and utilizing different C resources (for enzymatic profile of microbial community). The smallest effect size is predicted in the prime farm soil, and a larger effect in the poor farm soil. However, the direction of the effects might be reversed between prime and poor farm soil because the additions will be modulated through the initial conditions of each soil.

To test this hypothesis, a greenhouse experiment was conducted with a 3x2x2 factorial design using three field collected soils, added N fertilizer, and/or added mycorrhizal inoculum. Factors directly from the soil itself were measured to model how the treatments were changing the edaphic conditions. Plant factors of biomass yield and tissue N content were measured to see how the treatments were affecting plant health. Multiple factors within the soil community were measured to capture the potential wide-ranging impact of the treatments across trophic levels in the soil.

Methods

Seeds were germinated in three different homogenized soils: prime farm soil, marginal farm soil, and poor farm soil (describe in detail in next paragraph). Treatments (Table 1) of fertilizer (0 lb/ac or 100 lb/ac—45.4 kg/ac) and a commercial mycorrhizal inoculum (sterilized or regular) were added after seedlings were well established (5 cm to 15 cm tall). Plants were grown for three months post treatment application, then harvested for total biomass (stem and root tissue). The soil was used for multiple measurements of the soil community: soil arthropods, nematodes, mycorrhizal colonization of plant roots, and microbial community function. The soil from each treatment (N = 4) was homogenized and stored in a plastic container over the winter. Seedling survival was less than expected (six seedlings died); only enough plants survived for three complete replicates of each treatment (N = 3). The second year, soil was redistributed. Because of the destructive measurements in the first year, pots were filled 1/4 with gravel then topped with the treatment soil (3/4 of the pot) and there was only enough soil for three replicates (N = 3). In 2016, the experiment was run from May through August. In 2017, the experiment was run from June through September.

The three soils used were collected in April, 2016 from locations where switchgrass was present (Figure 1). Prime farm soil (henceforth called "farm") was collected from nearby a switchgrass biodiversity study established and maintained by Dr. Stacy Bonos (Baumgarten, 2020b) at the Rutgers University Adelphia Extension Farm in Freehold, NJ (40.227053, -74.252517). Soil type was immediately adjacent to the Freehold sandy loam in Baumgarten (2020b): Holmdel sandy loam (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). It is rated the same as Freehold sandy loam; rated "good" for crop production by the NJ Soil Health Assessment

and "prime farmland" by the NRCS farmland classification system (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). The marginal farm soil was collected from an extension farm with an agreement with Rutgers University in Somerset, NJ (40.474351, -74.531560), where Dr. Bonos set up the same experiment as at Adelphia Farm; however, the experiment was not actively maintained. This marginal farm soil has shale parent material, thus will be called "shale" henceforth. Soil series is Klinesville channery loam, rated as "farmland of local importance" by the NRCS farmland classification system, and rated as "poor" by the NJ Soil Health Assessment (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). Finally, switchgrass commonly grows in disturbed areas throughout the Pine Barrens, so sand was collected from the forest edge of the Rutgers Pinelands Field Station in Pemberton, NJ (39.916069, -74.597176). Soil series is Evesboro sand; rated "not prime farmland" in the NRCS farmland classification system, and "poor" in the NJ Soil Health Assessment (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture, 2020). Henceforth, this soil will be called "sand". Soils were homogenized using a 1 cm screen.

Much work has gone into the breeding of switchgrass to match potential local conditions. A variety of switchgrass recommended for the northeastern United States, the Cave-in-Rock variety, was used in this experiment. Seeds in both years were germinated in the pots. Three seeds were planted per pot. After about two weeks, seedlings were thinned to one per pot. In 2016, additional plants were sprouted in extra pots, and two plants needed to be replaced before treatments were added. In 2017, additional plants were germinated at the same time in sterile sand in Hummert Growing trays. If a seedling unexpectedly failed, it

was replaced with a seedling from the sand, with the roots washed gently to remove any soil particles (N = 4).

Pots were 5 cm diameter PVC pipe cut to 18" (45.7 cm) with window screen at the bottom to hold soil in. These were placed into plastic drinking cups with 1.5" (3.8 cm) of gravel in the bottom to ensure that each pot was isolated and did not receive nutrients or water from neighboring pots.

Addition of inoculum and fertilizer in 2016 occurred three weeks after germination and were added at the same time. Plant seedlings were 5 cm to 15 cm tall. Mycorrhizal inoculum treatments were composed of a powdered inoculum that contained 7 *Glomus* species; brand Diehard Endo Drench. Mycorrhizal inoculum was added at a rate of 5 g per pot; the same weight of sterilized inoculum was added to the null treatments. Inoculum was sterilized in a 3 L batch for 120 sec in a high-power commercial microwave (Ferriss, 1984). Fertilizer was YaraLiva Tropicote 15.5-0-0, derived from Ammonium Calcium Nitrate Double Salt. Pots were fertilized at a rate of 0 lb/ac, or 100 lb/ac (45.4 kg/ac). N fertilizer was dissolved in 25 mL bottles of water. After addition of the powdered inoculum, fertilized water was added, and then additional water was added to bring the pots up to a consistent moisture level. Throughout the remainder of the growing season, pots were watered evenly.

Experimental breakdown occurred when the plant stem biomass was clipped after 12 weeks of growth. Pots were watered an equal amount 24 hours in advance of the experimental breakdown to assist with the partitioning of soil for experimental procedures. Plant biomass was dried for four days at 70°C and weighed. All weights were calculated based on oven-dried material. If the samples were in a paper bag, the dried bag was first weighed in total. The plant mass was then removed and the empty bag weighed in order to ensure that any plant biomass lost in the transition process did not result in an inaccurate

weight. The plant biomass was left stored in the paper bag at room temperature until it was acid digested for N content. This method was used in Baumgarten (2020c).

Total N was calculated by digesting 0.1 g of ground and homogenized—with coffee grinders—plant biomass (roots and shoots separately) in 5 mL of Kjeldahl solution. The samples were heated at approximately 370°C until the solution cleared entirely (average run time: 12 hours). These liquids were diluted with DI water to 20 mL, then stored in 25 mL plastic bottles in a freezer until the liquids could be analyzed. One switchgrass sample (collected from Adelphia Farm experiment [Baumgarten, 2020b]) was included in each digest run, in addition to a blank. Additionally, NIST apple leaf 1515 was digested in three of the digestion runs to get a clearer picture of digest precision. These standards were used to standardize all of the digest runs to each other. N analysis was conducted using a Shimadzu C:N analyzer. A standard curve was run on each day that a batch of samples was analyzed in order to check machine efficiency. This method was used in Baumgarten (2020c).

Soil nutrients were extracted for extractable N—both ammonium (NH_4) and nitrate (NO_3)—and phosphate (PO_4) within 24 hours of collection (for initial values) or experimental breakdown for both growing seasons. Ten grams of soil was extracted in 25 mL of Mehlich extractant for phosphorus and KCl for N. Soil was collected from the middle 10" (25 cm) of the pot in 2016 and the bottom 6" (15.25 cm) in 2017. Soil was homogenized before being portioned out for extraction. Soil and extract solutions were shaken at 200 RPM for 60 minutes, and then vacuum filtered through #2 filter paper to separate soil from extract liquids. Extracts were frozen until they could be analyzed. Colorimetric assays for PO_4-P, NH_4-N and NO_3-N were conducted in 96 multiwell plates using an AccuScan plate reader (Thermo Scientific). PO_4-P was determined with the Malachite green method of D'Angelo et al. (2001) and Jeannotte et al. (2004), NH_4-N with sodium nitroprusside and

dichloroisocyanuric acid and NO_3-N with vanadium chloride and Griess reagents using the methods described by Hood-Nowotney et al. (2010).

Soil arthropods were collected from the top 3 cm of the pots; the goal in 2016 being to conserve soil to be used in 2017. The 3 cm cores were inverted in Berlese funnels. The cores were dried for five days, with the heat/lights being gradually turned up to full strength. In this set up, the soil arthropods crawled downwards, away from the light, and towards the attractive 70% methanol mixture with 10% glycerol, which both killed and preserved them. Arthropod morphospecies were assessed with a dissection microscope. To increase reliability of soil arthropod morphospecies identification, only one person did the identification, photos and notes from earlier results were reviewed and the data reclassified as needed, and peer reviewed works were used to guide identification. Collembola were fairly straightforward to identify to family (using Christiansen & Bellinger, 1998). However, mite morphospecies may have been more heterogeneous. Mites were essentially identified to sub-order (using Dindal, 1990), and then further divisions to morphospecies were only loosely based on published keys. This method was used in Baumgarten (2020b, c). Soil in pots was drier at the initiation of the extraction process in 2017 based on observation.

Nematodes were extracted from homogenized soil from the middle 10" (25 cm) of the pots in 2016 and the bottom 6" (15.25 cm) of the pots in 2017. Nematodes were extracted with two different methods. In 2016, nematodes were extracted from 5 g of soil placed on top of filter paper and a screen in glass funnels. In 2017, 50 g of soil were placed on coffee filters and plastic chicken wire baskets in shallow dishes of water. Both methods are a modification of the Oostenbrink filter method, which utilizes the nematodes' natural movement to separate them; the nematodes move through the soil and filter, enter the water portion of the setup and descend to the bottom. In both set-ups, the soil was in contact with

water for the entire extraction period of 24 hours. In 2016, the entire amount of liquid containing extracted nematodes was assessed under a dissecting microscope. In 2017, the liquid collected was standardized to 200 mL, homogenized, then 20 mL was assessed for nematodes present. This method was used in Baumgarten (2020b, c).

Roots for mycorrhizal assessment were collected from the bottom 6" (15.25 cm) of the pots. Roots were gently removed from most of the soil and stored in plastic storage bags in the fridge for no more than 24 hours before preparation. The roots were washed to remove all soil; then placed in 10% KOH to clear the cells. After five days at room temperature, the roots were removed and washed three times in tap water. Then the roots were placed in 1% HCl for 1 min in preparation for the staining. The roots were stained in 0.05% trypan blue in lactoglycerol for two days at room temperature. Trypan blue selectively stains fungal tissue and not plant tissue (Brundrett et al., 1984). Finally, roots were stored in lactoglycerol until mycorrhizal colonization could be assessed with a modification of the magnified intersections method on a compound microscope (Giovannetti & Mosse, 1980; McGonigle et al., 1990). This method was used in Baumgarten (2020b, c).

Enzymatic profile of soil microbial community was assessed using BIOLOG ecoplates after both growing seasons. Measuring microbial community function through measurements of enzyme activity is a time-efficient way to address some ecological questions (Burns et al., 2013; Wang et al., 2015). This colorimetric method evaluates the enzyme production for 31 known substrates. Soil was collected from the roots used for mycorrhizal colonization assessment; it was soil that was clinging to the roots after they were gently removed from the bulk of the soil. A suspension of 1 g soil in 99 mL water was shaken for 20 min and allowed to sediment for 30 min in a fridge. A 150 μl aliquot of suspension was dispensed into each well of the BIOLOG ecoplate and incubated at room temperature.

Plates were immediately read at 590 nm for background, and again after three and five days of incubation. Background absorption was subtracted from the final absorbance value for each cell and then corrected for the control. Pattern of absorbance values for all substrates was used in a multivariate analysis, Principle Component Analysis (PCA), to compare C source utilization between treatments. This method was used in Baumgarten (2020b).

Organic matter (OM) content was calculated using loss-on-ignition (LOI) techniques. Three grams of oven dried soil was placed in pre-weighed oven dry crucibles and then in a muffler furnace, heated at 550°C for four hours, and then cooled and weighed to calculate the loss. pH was measured for oven dried soil, placed in a 50-50 ratio with water, using an Oakton Ion 700 pH meter with an Orion probe. Both OM and pH were measured just before being potted and after each growing season.

Statistical analyses were performed in R version 3.5.2 (R Core Team, 2019) and RStudio version 1.1.463 (RStudio Team, 2020). Packages used include vegan (Oksanen et al., 2019), MASS (Venables & Ripley, 2002), tidyverse (Wickham et al., 2019), ggfortify (Horikoshi & Tang, 2018; Tang et al., 2016), Rmarkdown (Allaire et al., 2020; Xie et al., 2018), knitr (Xie, 2020; 2015; 2014), and lme4 (Bates et al., 2015). Linear models were run for all single measurements of data and a model was compared where the interaction of soil, fertilization, and inoculation was included versus the interaction not included. Akaike information criterion (AIC) was used to select the model with the better fit for the data. A lower AIC value is taken to mean the model is of better quality, and simplicity of the model is a factor, although if AIC are within 3 units of each other, the models are theoretically even in their ability to explain the data (e.g., Gutierrez & Heming, 2018). Multivariate analysis of variance (MANOVA) and principle component analysis (PCA) were performed on combined data to better analyze and visualize the complex data.

Results

Soil measures

Soil OM content was highest across all soil types for the initial reading (Figure 2a, Table 2), and lowest after the 2017 growing season. T-test showed that sand did not differ significantly by year, but farm differed significantly between all three dates and shale differed significantly between initial values and the two post-growing season measurements. Shale had the highest OM content, and sand the lowest (Table 3). In 2016, all three soils differed significantly; shale has the highest OM and sand the lowest. The best-fit linear model predicted that mycorrhizal inoculum causes a slight non-significant increase in organic matter and N fertilization causes a slight non-significant decrease (Figure 2b). In 2017, all three soils were significantly different, with shale having the highest OM and sand the lowest. The best-fit linear model predicted non-significant slight decrease due to inoculation and a non-significant slight increase due to fertilization, both being the opposite trend of what occurred in 2016 (Figure 2c, Table 3).

Across all three dates, pH measurements for farm were between 4.24 and 6.38; pH measurements for sand were between 3.95 and 5.69; and pH measurements for shale were between 4.13 and 5.68. Initial values of pH were highest for farm and lowest for sand (Figure 3a, Table 2). NRCS Web Soil Survey data showed farm has pH of 4.6, sand has pH of 4.3, and shale has a pH of 5.3, which were in the range of thr measurements. Initial values were higher than after both growing seasons, but pH was higher in 2017 than in 2016. Additionally incongruent was that the magnitude of difference in farm soils between 2016 and 2017 is larger than for sand and shale. pH in 2016 was highest in farm and lowest in sand (Table 3). pH in 2017 similarly was highest in farm and lowest in sand (Table 3). The best-fit linear model predicted a non-significant increase in pH due to inoculation, but no

change due to fertilization (Figure 3b). In 2017, mycorrhizal inoculation was associated with a non-significant decrease and fertilization was associated with a moderately significant ($P < 0.1$) increase in pH (Figure 3c).

All three soil extractable nutrients were highest after the growing season in 2016 and much lower after the 2017 growing season. Initial values of NH_4 were higher in sand and shale, and lower in farm (Figure 4a, Table 2). Initial values of NO_3 were the opposite—highest in farm and lowest in sand and shale (Figure 5a, Table 2). Initial values of PO_4 were low for all three soils (Figure 6a, Table 2). At the end of the growing season in 2016, NH_4 was highest in the sand, and was slightly lower in farm than shale (Figure 4b). Best-fit linear model predicted non-significant increases due to both inoculation and fertilization (Table 3). NO_3 was highest in farm and lowest in shale, and there was a significant increase of 2 mg/g due to fertilization, and a non-significant increase due to inoculation (Figure 5b, Table 3). PO_4 was lowest in the farm, and highest in shale and sand, which were essentially even (Figure 5b). There was a significant increase due to inoculation, and a slight non-significant increase due to fertilization (Table 3). At end of the 2017 season, NH_4 was highest in sand and lowest in farm (Figure 4c). Best-fit linear model predicted a non-significant increase due to fertilization and no difference due to inoculation (Table 3). NO_3 was highest in the shale and lowest in sand (Figure 5c). Best-fit linear model predicted a moderately significant ($P < 0.1$) decrease due to inoculation, and basically no difference due to fertilization (Table 3). PO_4 was highest in sand and lowest in shale (Figure 6c). Mycorrhizal inoculation led to a significant decrease in the best-fit linear model, and fertilization caused a slight non-significant increase (Table 3).

Plant measures

The three soils have different textures. Post growing season, the different textures were obvious in the plant pots; the plants produced visibly different root structures in response (Figure 7). In both 2016 and 2017, plants produced the lowest stem and root biomass in the shale (Figure 8). In 2017, two plants did not grow well, and thus were subject to wetter soil than the rest of the plants, because pots were watered evenly. However, on average, plants produced slightly more biomass in 2017. In 2017, root structure also showed significant differences between soil types in the formation of root-rings at the base of the pot. Seventeen pots developed a root ring: 1 in farm, 10 in sand, and 6 in shale. The presence/absence of root-rings was analyzed with a binomial general linear model, and showed that soil type was significant, but not the treatment types (Figure 9).

Stem biomass in 2016 was significantly higher in farm compared to sand and shale, and biomass was lowest in shale (Table 4). The best-fit linear model for the 2016 stem data predicted a non-significant decrease in production due to both mycorrhizal inoculation and fertilization, although slightly more negative for inoculation (Figure 10a). Root biomass in 2016 was significantly lower in shale compared to sand and farm, and was highest in sand but not significantly higher than farm (Table 4). The best-fit linear model for the root data predicted a non-significant decreased in root production due to fertilization, and a non-significant increase due to inoculation (Figure 10a, Table 4). In 2017, stem biomass in farm and sand was essentially the same, while shale produced significantly less (Figure 10b). The best-fit linear model predicted a non-significant increase due to both inoculation and fertilization (Figure 10b, Table 4). Root biomass in 2017 was highest in sand, and lowest in shale (Figure 10b, Table 4). Best-fit linear model predicted a small (non-significant) increase due to fertilization and a larger (but still non-significant) increase due to inoculation (Table

4). Since the biomass of each plant is very likely co-varied, MANOVA was also performed for the biomass data. In 2016, soil was a significant factor ($P < 0.05$), but inoculation and fertilization were not. In 2017, soil was a significant factor, as was the interaction of soil with fertilization ($P < 0.05$), but inoculation and fertilization alone were not significant.

Both stem and root biomass N content in 2016 (Figure 11a) were significantly higher in fertilized treatments. There was significantly higher N content in sand, and a lower amount in both farm and shale (Table 4). Best-fit linear model predicted a non-significant increase in N content of both stem and roots due to inoculation. Root N content in 2016 was the highest in farm and the lowest (by a small amount) in sand, but the soils were not significantly different from each other (Figure 11a, Table 4). In 2017, stem N content was significantly lower in sand compared to shale and farm, and was non-significantly highest in shale (Figure 11b). Best-fit model for stem N content predicted a non-significant decrease due to inoculation and a non-significant increase due to fertilization (Figure 11b, Table 4). Root N content was not significant due to any treatments, and was highest in shale and lowest in sand (Figure 11b). Best-fit model for root N content predicted a significant effect due to interactions, which indicated that inoculation did not have a consistent effect nor did fertilization (Table 4). Since stem and root N content likely co-vary, MANOVA was run on the two datasets concurrently. In 2016, no factor was significant. In 2017, soil was significant ($P < 0.05$), and the interaction of fertilization and inoculation was mildly significant ($P < 0.1$), but inoculation and fertilization alone were not significant.

The previous two measurements for roots and stems were multiplied together to get the total N (mg) in plant tissue. In 2016, total stem N was highest in farm and lowest in shale, and fertilization was associated with a non-significant increase in total N (Figure 12a, Table 4). Total root N was highest in farm, slightly lower in sand, and lowest in shale, and

inoculation was associated with a non-significant increase in total N (Figure 12a, Table 4). In 2017, stem total N was highest in farm and lowest in sand; fertilization was associated with an increase in total N ($P < 0.1$) and inoculation was associated with a non-significant decrease (Figure 12b, Table 4). Root total N was highest in sand and lowest in shale, and fertilization was associated with a non-significant increase and inoculation associated with a non-significant decrease in total N (Figure 12b, Table 4). Thus, for both years, total plant N was highest in farm and lowest in shale, although the trend was more pronounced in 2016.

Soil community measures

Total abundance of soil arthropods was higher in 2016 compared to 2017, although the number of morphospecies identified was the same (22 morphospecies in 2016 and 24 in 2017). In 2016, the most morphospecies were found in the shale (Figure 13a). 8 of 22 morphospecies had higher abundance in the unfertilized treatments, 2 of 22 had higher abundance in fertilized treatments, 7 of 22 were about even or undeterminable between fertilized and unfertilized treatments, and 5 of 22 were found in low abundance in only one treatment type. However, MANOVA results showed that soil type was the only significant factor in the soil arthropod morphospecies community composition, inoculum and fertilizer were not (Figure 13b, Table 5). These results seem to be driven by a number of species that were unique to the shale and sand (Figure 13c). In 2017, shale had a slightly higher number of soil arthropod morphospecies compared to farm and sand (Figure 14a). 5 of 24 morphospecies were higher in unfertilized treatments, 2 of 24 were higher in fertilized treatments, 6 of 24 were about even or undetermined, four were found in low abundance in two treatments, and seven were found in low abundance in only one treatment. MANOVA results in 2017 showed that soil type was close to significant, but fertilization and inoculation

were not (Figure 14b). PCA showed that the ellipses across soil types overlap much more than in 2016 (Figure 14c, Table 5).

Mycorrhizal structures occurred in all treatment types. In 2016, arbuscules, coils and vesicles were highest in sand and lowest in farm, in contrast to spores which were less abundant but had the highest counts in farm (Figure 15). Inoculation treatment correlated with both an increase and a decrease in mycorrhizal structures across all soil types. 2017 mycorrhizal structures were more variable than 2016. Spores were highest in farm and lowest in sand, but coils and arbuscules were approximately even across all soil types. Vesicles were inconsistent; they showed high amounts in one farm treatment (+ inoculation and + N) and one sand treatment (- inoculation and - N) (Figure 16). MANOVA results in 2016 showed that soil was mildly significant at $P < 0.1$ level, but not other treatments (Figure 17a, Table 5). PCA showed that the soil ellipses overlap a fair amount, but that farm was most associated with spores compared to the other soils (Figure 17b). MANOVA results in 2017 showed that no treatment was significant (Figure 18a, Table 5). Additionally, PCA showed much overlap between the ellipses, although sand was slightly more associated with arbuscules compared to the other two soils (Figure 18b).

BIOLOG ecoplates showed differences in microbial functional community due to soil type and inoculum at $P < 0.1$ but not N in 2016 (Figure 19, Table 5). In 2016, PCA ellipses overlapped a fair amount. However, farm seemed to be associated with positive values of A, and X; shale was more associated with L, M, U, and H; sand was associated with G, F and L. In 2017 differences were significant due to soil and inoculation (Figure 20, Table 5). In 2017, PCA showed sand separating from the other two soils. Farm was more associated with A, X, G and Y; shale was more associated with Q, H, N, D, C; and sand was

more associated with U, Z, V, B, and L. See Table 6 for C source associated with the alphabetical short letter ID used here and in the figures.

Nematode abundance was highest in sand in 2016 (Figure 21a). Sand was significantly different from both farm and shale, but they were not different from each other (Table 5). Linear model predicted a non-significant increase in abundance due to inoculation and a non-significant decrease due to fertilization. Nematode abundance was highest in farm in 2017, and lowest in both sand and shale (Figure 21b). Linear model showed soil type and mycorrhizal inoculation to be significant factors (Table 5). Inoculation led to an increase in abundance, and fertilization led to a non-significant decrease.

Discussion

An overview of the factors analyzed with linear models showed that in general, inoculation and fertilization led to small increases in the measured factors in 2016, although they were primarily non-significant changes (Table 7). However, in 2017 inoculation was more associated with small decreases in measured factors. Soil type caused more significant differences than the addition of fertilizer or inoculation. Soil type was associated with a similar amount of significant differences in measured factors in 2017 as it was in 2016. Almost all the initial measurements were significantly different due to soil type (Table 2). These results show that this experimental treatments and methods were altering the soil in measurable ways (P-values in Table 3). However, there were not cascading effects in the plant growth or the soil community that indicate a response to the shift in the soil conditions

In general, there were not important differences in LOI and pH (Figure 2 and 3). Although OM was not associated with significant differences due to N fertilizer or inoculation, it was decreasing over the three time periods. These data correlate with the idea

of exhaustion of the soil. Since continued fertilization is associated in much research with decreasing soil pH, as far back as the 1920's (Usher, 1923) and in a current meta-analysis in agricultural settings (Geisseler & Scow, 2014), it seems incongruent that pH is not following that pattern in this experiment (Figures 5 - 7). However, given that pH and LOI were showing limited response to all treatments, perhaps the experimental manipulations, such as homogenizing field soil, had a dominant influence on these two factors that masked any other trends.

Sand had an unexpectedly higher extractable NH_4 content compared with the other soils (Figure 4). Although all levels dropped in farm and shale after the first plant crop, levels in sand remained higher compared to the other two soils. All levels again decreased after the second crop. The decrease in NH_4 over time is likely related to plant uptake and exhaustion of the soil. Fertilizer application seemed to have the greatest effect on sand, which could fit with the hypothesis of the most nutrient-limited soil needing fertilization. One possible factor explaining these results is that something in the shale soil is immobilizing NH_4.

The initial level of NO_3 in sand and shale seems exceptionally low (Figure 5). After the first crop, levels in sand and shale were higher than the original soil. It is possible that either (a) original NO_3 values were incorrectly low in these two soils or (b) there was a priming effect on nitrification due to soil disturbance during homogenization and potting that occurs more strongly in sand and shale versus in the N-rich farm soil. After the first crop, farm soil was generally higher than other soils and all soils showed a significant response to fertilizer addition. For both farm and sand soil there was an increase in NO_3 correlated with mycorrhizal inoculation in unfertilized soils but a slight decrease in fertilized soils. Shale showed a similar pattern, but to a very limited magnitude. This pattern essentially disappeared in the farm and sand soils after the second crop, probably due to increased crop

uptake of N enhanced by mycorrhizae in the more depleted soil. Extractable NO_3 was significantly lower after the second crop in all soils. Another factor of note is that after the second crop, sand NO_3 was extremely low again, possibly showing that this nutrient-limited sand is the most sensitive to soil exhaustion. If soil that is exhausted changes the quickest in response to fertilization, or has the highest absolute value of fluctuation in extractable nutrients compared to nutrient rich soil, then the fluctuations in NH_4 and NO_3 in the sand are more reasonable.

The higher extractable N content in sand did not translate into plant N content (Figure 12). It is surprising that sand, which theoretically has less available nutrients than farm and shale soils, does not show effects from the treatment as was hypothesized. Another surprising finding is that sand had the highest NH_4 at the end of the growing season 2016. Perhaps nitrification could have been inhibited in the sand thus slowing the breakdown of NH_4; Haynes and Naidu (1998) describe poorly aggregated soils as susceptible to periods of slow nitrification post-fertilization. However, given that all the pots were subject to the same environmental conditions, it seems odd for that to be the explanation. Perhaps the plants differentially used NH_4 over NO_3 or vice versa in the three soils (e.g., Bassirirad, 2000; Christodoulou et al., 2019; Zhang et al., 2019). In simplified conditions, Woods et al. (1982) find that amoebae grazing bacteria increase N mineralization but nematode grazing does not linearly increase N mineralization; this suggests that it is possible that an unmeasured aspect of the soil community was influencing the incongruent measurements in extractable N.

Initial values of extractable PO_4 were very low in all soils (Figure 6). Values for all soils after the first crop were higher than initial values, though sand and shale soil were highest. These values could have been a result of a priming effect due to soil disturbance during homogenization and potting. Extractable PO_4 was essentially the same between the

first and the second crop in farm soil, but declined in sand and shale such that PO_4 values were similar between soils after the second crop. The effect of mycorrhizal inoculation was to have significantly lower P levels in mycorrhizal treatments after the second crop; probably due to the efficiency of arbuscular mycorrhizae for P uptake. This effect was shown in both fertilized and unfertilized soils, except for fertilized sand. Given that extractable PO_4 dramatically changed over the course of the experiment, future experiments should include the explicit hypothesis that P is relevant even if it is not manipulated directly. For instance, Houlton et al. (2008) propose that P-limitation as well as temperature limitation relates to the high prevalence of N-fixing plants in the tropics, which highlights the conclusion that the interconnectedness of these nutrient cycles is important.

While the biomass yield results were not statistically significant, the trends may be relevant. Inoculation correlated with a decrease in stem biomass and an increase in root biomass in 2016 (Figure 10a). These data make sense because mycorrhizal associations are expected to have a cost to plants in nutrient rich conditions. Similarly, in 2017, there was a non-significant trend that both fertilization and inoculation increase stem and root biomass (Figure 10b). This trend could align with the fact that the experimental additions are helping the plants survive in the more nutrient-limited soil. If these trends are due to mycorrhizal associations, it would be expected to see a trend of higher AMF structures in at least the unfertilized and inoculated treatments. For sand, there does seem to be a correlation in 2016 with higher arbuscules, coils and vesicles in inoculated treatments, and a decrease in yield for the same treatments (Figure 15). However, in 2017 there does not seem to be any trends that align between mycorrhizal structure and biomass yield (Figure 16).

Though in general the soil community measurements did not exhibit clear trends, there are a few interesting things to note. Nematode abundance is the only factor that

showed the same pattern of an increase in response to inoculation and a decrease in response to fertilization for both years (Figure 21). Two soil community factors suggest that time is an important factor. Both the BIOLOG ecoplate results and the nematode abundance were significantly different due to inoculation in 2017, but not in 2016, which could indicate a magnifying effect of inoculation over time (Figure 19, 20, and 21). Conversely, both soil arthropods and BIOLOG ecoplate results were most strongly responsive to soil type. It is not surprising that the BIOLOG ecoplate results generally showed a strong soil effect over treatment effects as the microbial community has developed to those soil conditions, so it would be expected that the microbial community would be relatively resilient to homogenization. Soil arthropods showed a strong separation in community in 2016 but not in 2017 (Figure 13 and 14), which perhaps is a response to the decline in soil nutrients.

Signs of soil exhaustion were predicted for this experiment, and some trends supported this hypothesis while others contradicted it. Plant total N content was essentially the same between years (Figure 12), although per gram it was slightly lower in 2017 (Figure 11); this suggests that the soil was not exhausted to the point of affecting plant growth. Similarly incongruent with the theory of soil exhaustion is the fact that biomass production was higher in 2017 versus 2016 (Figure 10). The most likely explanation is that the plants fared better because of better light conditions; the experiment was conducted a month later (from July to September instead of June to August). However, given that the extractable nutrients decreased over the course of the experiment, it would be reasonable to expect to find lower biomass production in 2017, which was not the case. The formation of root rings in sand and shale soils in 2017 (Figure 9) suggests the plants were seeking richer soil, which does support the idea that those soils were headed towards nutrient exhaustion. The

BIOLOG results show in a PCA the sand community separated more from the shale and farm in 2017 (Figure 20) than in 2016 (Figure 19). If sand was more exhausted, the greater separation in the microbial function analysis could be a result of nutrient limitation. The soil arthropod community showed less separation between the communities in 2017, which similarly could be a response to lower soil nutrients. Additionally, the overall abundance of soil arthropods also declines between the two years; although, as previously mentioned, at the time of extraction the soil pots were visually less moist, which could affect the extraction process.

Clearly, soil type is the strongest factor. These results do not match a study in Canada prairie soils that concluded that agricultural practices and land use significantly impacted AMF community composition and diversity but soil type did not (Bainard et al., 2014). However, AMF community was not addressed in this study, only the degree of root colonization and the mycorrhizal structures. Additionally, the horticultural inoculum that was applied may not perform the same way a natural mixed inoculum would perform. Zinati et al. (2011) found that commercial inoculum did not perform as well for ericaceous shrubs as adding inoculum of soil from an ericaceous community. Similarly, a large study in France also found that soil community members were more associated with land type (i.e. forest vs. meadow vs. crop) and agricultural intensity rather than soil type across a variety of soil types and conditions (Cluzeau et al., 2012).

At the most fundamental level, soil texture relates to soil type. The three soils have very different textures. The NRCS web soil survey lists the median of soil particle size for the three soils as: Farm—69% sand, 24% silt, and 8% clay; Shale—43% sand, 40% silt and 18% clay; Sand—92% sand, 5% silt and 4% clay. Most notable is the difference in clay content. Clay is a great ion exchange medium which may be binding P and could have an

influence on N which could either decrease fertility (absorbing) or increase (de-absorbing) nutrients. This exchange capacity would be higher in shale than the other soils. Similarly, the three soils had different pH. The results of the BIOLOG ecoplates (Figure 19 and 20) correlate with other research that shows pH to be a strong limiting factor for bacteria (Castle et al., 2016; Rousk et al., 2010; Turner et al., 2019). But though pH can affect both bacterial and fungal communities, and the soil texture is undeniably different, were differences between soils attributable to these factors, or is it a case of correlation and not causation?

The focus of this research experiment on ecology makes sense (Baumgarten, 2020a, b), but if soil pedology is as strong of a factor as it was in this experiment, then soil and plant ecology may need to shift to include pedology in the fundamental measurements included in experimental design and analysis. If fundamentally soil texture is a strong driver of soil community and plant community, then any study that does not measure this and accidentally measures communities with different soil structure could be falsely attributing conclusions to one factor instead of soil structure. However, the same caveat applies to this experiment; an unexpected effect from the homogenization and potting process could be correlated with soil type and thus, results could be falsely attributed to the soil type.

Two things stand out to change if this experiment were repeated. First, given the complicated results that were found, it is unlucky that the initial design to have 5 replicates did not succeed due to seedlings' dying. Thus, higher replication would be a priority. Second, because there were signs of soil exhaustion, but it did not affect plant biomass production, a longer duration—even one more season—would be valuable. Had the experiment continued, at what point would the lack of extractable nutrients and soil exhaustion prove devastating for plant growth?

Because this experiment was fully factorial, and there were statistically significant differences among treatments, any differences within soil due to inoculation and fertilization should have been measurable. The strong signal from soil type should not have hidden signals from experimental treatments. The original hypothesis was that contrasting soil types would respond differently to treatments depending on fertility and structure. There are no clear flaws in the experimental design for the questions that were being asked or the in types of analyses that were done. Therefore, the conclusions hold.

However, is it possible that we as ecologists are over-simplifying the questions of what-drives-what and who-grows-where? If the ecological world is driven by cycles, like the N, P, C and water cycle, are we measuring experimental treatments and results with these cycles in mind? For example, do the extractable nutrients at the time of measurement capture the cyclical pattern of a particular locality? Are we treating the interconnectedness of these cycles with enough brevity? Even in this experiment in a more controlled greenhouse setting, the complexity of the interplay of these cycles seems to be affecting the results.

Conclusion

The hypothesis of this experiment was that the addition of N fertilizer and a commercial inoculum would have different effect sizes and potentially reverse effects due to interactions in three soils: a sandy-loam classified as prime farm land, a shale-parent-material soil classified as marginal farmland, and sand classified as poor farmland. However, the best-fit statistical model generally did not include interaction terms, and thus the treatments were having a similar effect in all three soils. It was predicted that adding fertilizer and inoculum would have linear and additive effects, which was found for some soil and plant factors.

However, the responses to experimental manipulations were often not strong enough to be statistically significant.

Soil community measures were expected to respond to changes in the plant and soil factors by forming a different soil microarthropod community, nematode abundance, mycorrhizal structures, and enzymatic profile of microbial community. Often, the soil community measurements were responsive primarily to soil type and not experimental manipulations. Mycorrhizal inoculation led to a significant change in 2017 in the microbial community enzyme profile, so it is possible that there was a lag effect. Additionally, there were some indicators of a changing response of soil community and plant root growth that could be explained by declining soil nutrients that might lead to soil exhaustion if the experiment had been continued.

These results show the resilience of switchgrass and the soil community to perturbation across all soil types, because extractable nutrients were significantly affected by experimental treatments in 2016 and the plant biomass yields were slightly higher in 2017. The responses to soil type versus inoculation and fertilization across all measurement types suggests that soil texture may play a significant role in the factors that were measured. This makes sense given the wealth of data on how soil particles interact differently (i.e. reactivity of clay particles versus sand particles), but does not match some comprehensive studies of soil ecology in field settings across a range of soil types and land uses. Follow-up research on priming effects from transferring soil from field to greenhouse conditions could be very interesting. Another important follow-up is further exploring the close relationships of nutrient cycling (i.e. N, P, C) in regulating plant and soil communities rather than looking at just one nutrient. The lag response that was found in the microbial community enzymatic profile and nematode abundance suggests that treatment effects can build up over time, but

in light of previous research where there was not a clear building effect through time (Baumgarten, 2020b), this effect needs further investigation. However, one undeniable message is that these results support previous findings that show switchgrass to be suitable for a range of soil type, which is ideal for a potential biofuel crop.

Tables

Table 1

This showed the soil type and additions for the 12 treatment types. Each treatment (i.e. f1) was collected, homogenized, and held in a plastic shoe box over the winter between 2016 and 2017. The treatments were redistributed to the same pots/location as the first year.

Type	**Soil**	**Nitrogen fertilizer**	**Inoculum**
f1	farm	0 lb/ac	-
f2	farm	0 lb/ac	+
f3	farm	100 lb/ac	-
f4	farm	100 lb/ac	+
sa1	sand	0 lb/ac	-
sa2	sand	0 lb/ac	+
sa3	sand	100 lb/ac	-
sa4	sand	100 lb/ac	+
sh1	shale	0 lb/ac	-
sh2	shale	0 lb/ac	+
sh3	shale	100 lb/ac	-
sh4	shale	100 lb/ac	+

Table 2

P-values from linear models for the five soil factors with soil type with time included as a fixed variable. The three times are: initial measurement, post growing-season 2016, post-growing season 2017. "0.000" values indicate a p-value of less than 0.001.

	OM	pH	NH_4	NO_3	PO_4
Sand	0.000	0.000	0.000	0.000	0.390
Shale	0.000	0.001	0.000	0.000	0.408
2016	0.009	0.000	0.680	0.649	0.000
2017	0.000	0.060	0.007	0.000	0.000
Sand:2016	0.191	0.001	0.022	0.000	0.019
Shale:2016	0.191	0.057	0.004	0.000	0.009
Sand:2017	0.189	0.915	0.007	0.000	0.643
Shale:2017	0.235	0.951	0.000	0.000	0.080

Table 3

Soil factors

a) all factors for 2016 b) all factors for 2017.

These tables show the p-values calculated from best-fit linear models for the five factors related to the soil itself. Organic matter content (OM), pH, Ammonia (NH_4), Nitrates (NO_3) and Phosphate (PO_4). No interaction terms between the experimental treatments were statistically significant. "0.000" values indicate a p-value of less than 0.001.

a)

	OM '16	pH '16	NH_4 '16	NO_3 '16	PO_4 '16
Sand	0.000	0.052	0.000	0.032	0.000
Shale	0.000	0.215	0.057	0.001	0.000
Fertilized	0.291	0.971	0.142	0.000	0.577
Mycorrhizal Inoculum +	0.619	0.641	0.470	0.335	0.015

b)

	OM '17	pH '17	NH_4 '17	NO_3 '17	PO_4 '17
Sand	0.000	0.000	0.044	0.003	0.054
Shale	0.000	0.000	0.345	0.003	0.081
Fertilized	0.377	0.087	0.763	0.715	0.809
Mycorrhizal Inoculum +	0.366	0.215	0.990	0.057	0.012

Table 4

Plant factors

a) all factors from 2016 b) factors from 2017 c) Root N from 2017.

Best-fit linear models were used to calculate p-value results for 6 factors relating to plant growth: plant stem biomass (Stem), stem N content (Stem N), root biomass (Root), and root N content (Root N), Total stem N and Total root N. Some interactions between treatments for Root N were statistically significant in 2017, but all other analyses did not have statistically significant interaction terms. "0.000" values indicate a p-value of less than 0.001.

a)

	Stem '16	Root '16	Stem N '16	Root N '16	Total stem N	Total root N
Sand	0.000	0.718	0.116	0.285	0.031	0.805
Shale	0.000	0.007	0.806	0.472	0.000	0.002
Fertilized	0.661	0.152	0.041	0.020	0.305	0.845
Mycorrhizal Inoculum +	0.491	0.898	0.540	0.660	0.932	0.655

b)

	Stem '17	Root '17	Stem N '17	Total stem N	Total root N
Sand	0.981	0.041	0.000	0.000	0.650
Shale	0.005	0.243	0.153	0.124	0.915
Fertilized	0.275	0.648	0.520	0.073	0.365
Mycorrhizal Inoculum +	0.654	0.352	0.181	0.344	0.827

(Table 4 continued)

c)

	Root N '17
Sand	0.303
Shale	0.002
Fertilized	0.068
Mycorrhizal Inoculum +	0.589
Interaction Sand:N	0.892
Interaction Shale:N	0.002
Inteaction Sand:Inoc	0.207
Interaction Shale:Inoc	0.019
Interaction N:Inoc	0.091
Interaction Sand:N:Inoc	0.255
Interaction Shale:N:Inoc	0.002

Table 5

Soil community factors

a) 2016 factors b) 2017 factors c) 2017 mycorrhizae d) nematodes for both 2016 and 2017 Adjusted p-values for the multivariate data was calculated using nonparametric MANOVA for three soil community factors: microbial community with BIOLOG ecoplates (Ecolog), soil arthropod community (Soil Arthropods), and mycorrhizal structures. Nematodes p-values were derived from the best-fit linear model. "0.000" values indicate a p-value of less than 0.001.

a)

	Ecolog '16	Soil Arthropods '16	Mycorrhizal Structures '16
Soil	0.002	0.001	0.029
Nitrogen	0.355	0.078	0.976
Inoculum	0.691	0.324	0.761

b)

	Ecolog '17	Soil Arthropods '17
Soil	0.001	0.071
Nitrogen	0.805	0.142
Inoculum	0.061	0.880

(Table 5 continued)

c)

	Mycorrhizal Structures '17
Soil	0.102
Nitrogen	0.908
Inoculum	0.844
Interaction (Soil:N)	0.020
Interaction (Soil:Inoculum)	0.409
Interaction (N:Inoculum)	0.021
Interaction (Soil:N:Inoculum)	0.307

d)

	Nematodes '16	Nematodes '17
Sand	0.000	0.000
Shale	0.101	0.003
Fertilized	0.743	0.165
Mycorrhizal Inoculum +	0.491	0.050

Table 6

C sources of the BIOLOG ecoplates and short hand version used in the document.

carbon source	short
Water	Water
B-Methyl-D-Glucoside	A
D-Galactonic Acid T-Lactone	B
L-Arginine	C
Pyruvic Acid Methyl Ester	D
D-Xylose	E
D-Galacturonic Acid	F
L-Asparagine	G
Tween 40	H
i-Erythritol	I
2-Hydroxy Benzoic Acid	J
L-Phenylalanine	K
Tween 80	L
D-Mannitol	M
4-HydroyBenzoic Acid	N
L-Serine	O
a-Cyclodextrin	P
N-Acetyl-D-Glucosamine	Q
y-Amino Butyric Acid	R
L-Threonine	S
Glycogen	T
D-Glucosaminic Acid	U
Itaconic Acid	V
Glycyl-L-Glutamic Acid	W
D-Cellobiose	X
Glucose-1-Phosphate	Y
a-Keto Butyric Acid	Z
Phenylethyl-amine	AA
a-D-Lactose	BB
D_L-a-Glycerol Phosphate	CC
D-Malic Acid	DD
Putrescine	EE

Table 7

Summary of the results, with red/bold signs indicating significance of P<0.1. See tables 2-5 for P-values. Fertilization (+N) and inoculation (+I) do lead to increases in measured factors, but these changes are primarily non-significant.

Initial	Farm	Sand	Shale		+N	+ I
LOI	**0**	**-**	**+**		na	na
pH	**+**	**-**	**0**		na	na
NH4	**-**	0	**+**		na	na
NO3	**+**	**-**	0		na	na
PO4	-	-	-		na	na
2016						
LOI	**0**	**-**	**+**		-	+
pH	**+**	**-**	0			+
NH4	**-**	**+**	0		+	+
NO3	**+**	0	**-**		**+**	+
PO4	**-**	0	**+**		+	**+**
Stem (g)	**+**	0	**-**			-
Root (g)	0	**+**	**-**		-	+
Stem N	0	+	-		**+**	+
Root N	+	-	0		**+**	+
Stem Tot N	**+**	0	**-**		+	
Root Tot N	**+**	0	**-**			+
Nematodes	0	**+**	**-**		-	+
2017						
LOI	**0**	**-**	**+**		+	-
pH	**+**	**-**	**0**		**+**	-
NH4	-	**+**	0		+	
NO3	**0**	**-**	**+**			**-**
PO4	0	**+**	**-**		+	**-**
Stem (g)			**-**		+	+
Root (g)	0	**+**	-		+	+
Stem N	0	**-**	+		+	-
Root N	0	-	+			
Stem Tot N	**+**	**-**	0		**+**	-
Root Tot N	0	+	-		+	-
Nematodes	**+**	-	0		-	**+**

Figures

Figure 1

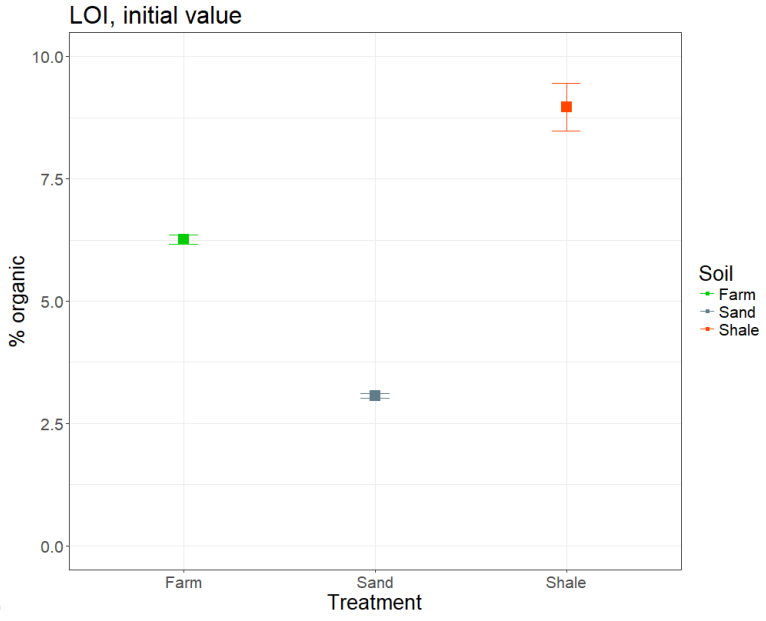
Somerset, NJ: Klinesville channery loam

Freehold, NJ: Holmdel sandy loam

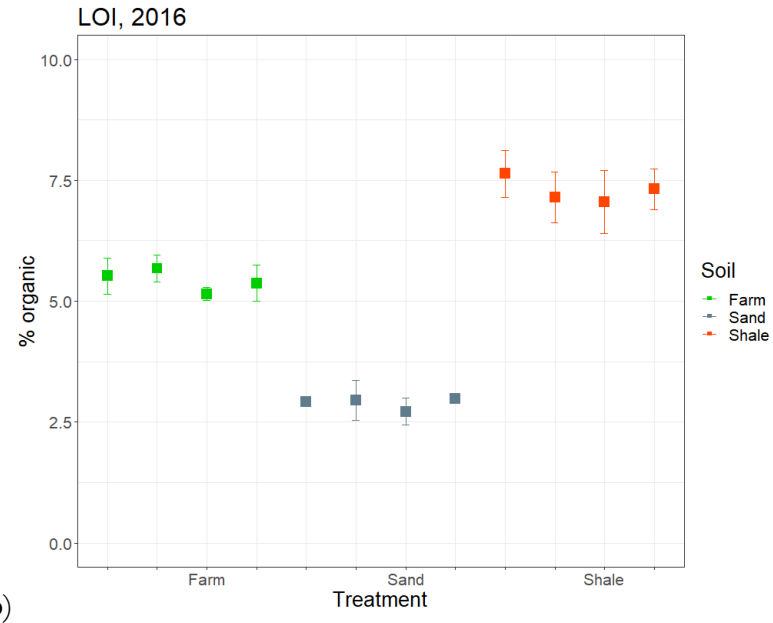
Pemberton, NJ: Evesboro sand

The three locations where soil was collected for experimental procedures. Prime farm soil was collected from the Rutgers University Adelphia Extension Farm in Freehold, NJ (40.227053, -74.252517). Soil series was Holmdel sandy loam. The marginal farm soil was collected from an extension farm with an agreement with Rutgers University in Somerset, NJ (40.474351, -74.531560). Soil series was Klinesville channery loam. Poor farm soil was collected at the Rutgers Pinelands Field Station in Pemberton, NJ (39.916069, -74.597176). Soil series was Evesboro sand.

Figure 2

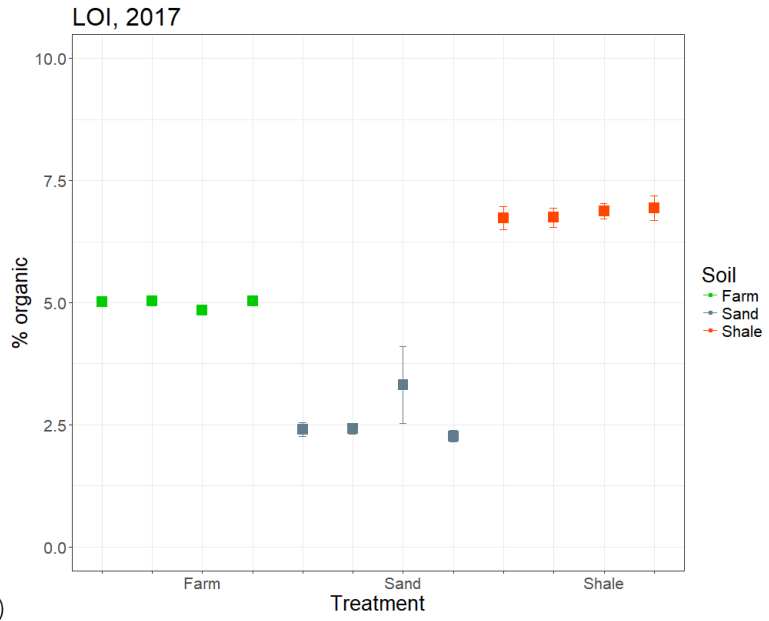

LOI, initial value
10.0
7.5
5.0
2.5
0.0
% organic
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

a)


LOI, 2016
10.0
7.5
5.0
2.5
0.0
% organic
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 2 continued)



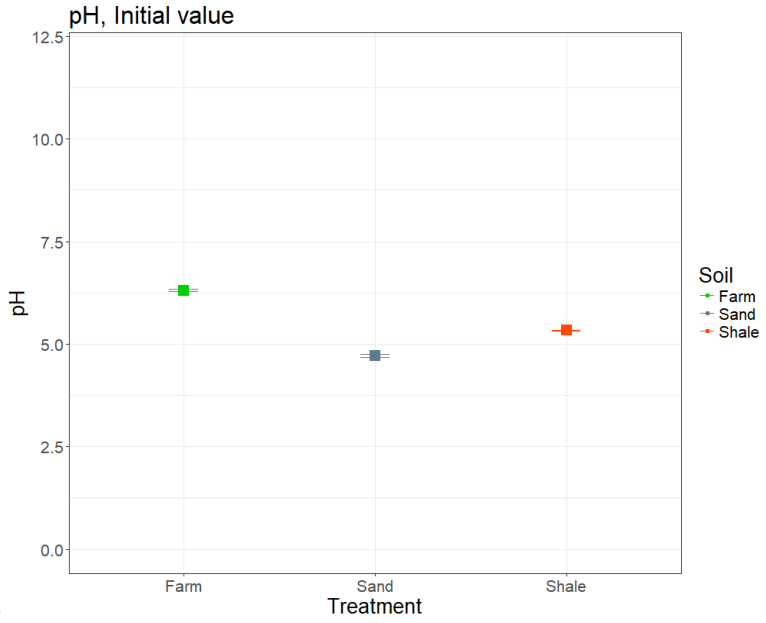
c)

a) Initial organic matter content values were significantly different between the three soils and were significantly different from the values in 2016 ($P < 0.05$), and 2017 (at $P < 0.1$).

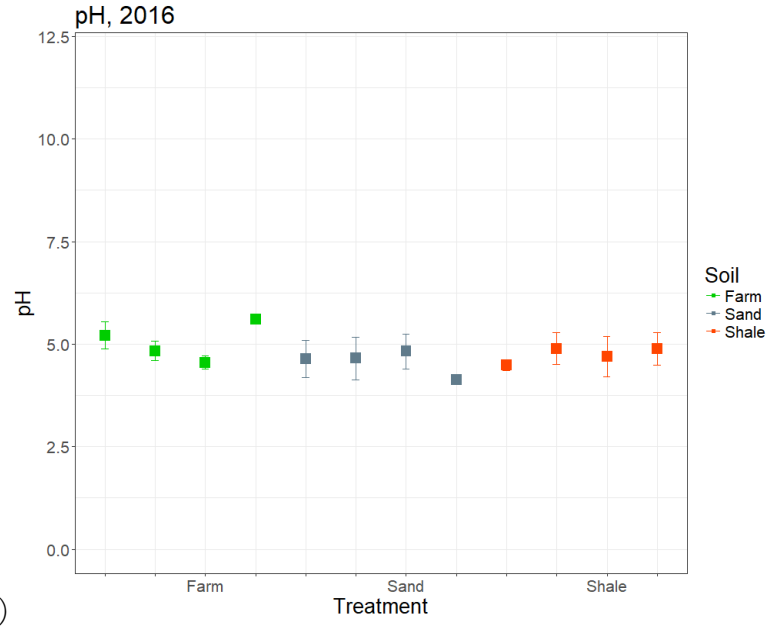
b) Post-growing season 2016; organic matter content was significantly different between all three soils, with shale the highest and sand the lowest. There was a non-significant increase due to inoculation and a non-significant decrease due to fertilization.

c) For organic matter post-season 2017, linear model supported that all three soils were significantly different from each other, but that there were no significant differences associated with fertilization or inoculation. T-test also supported that the three soils differed with statistical significance. Shale again had the highest OM content and sand the lowest.

Figure 3

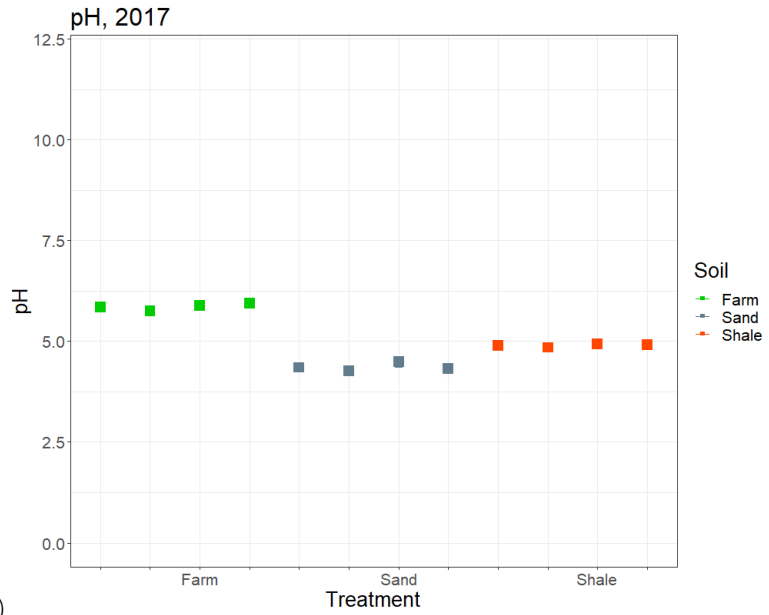

pH, Initial value
12.5
10.0
7.5
5.0
2.5
0.0
pH
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

a)


pH, 2016
12.5
10.0
7.5
5.0
2.5
0.0
pH
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 3 continued)



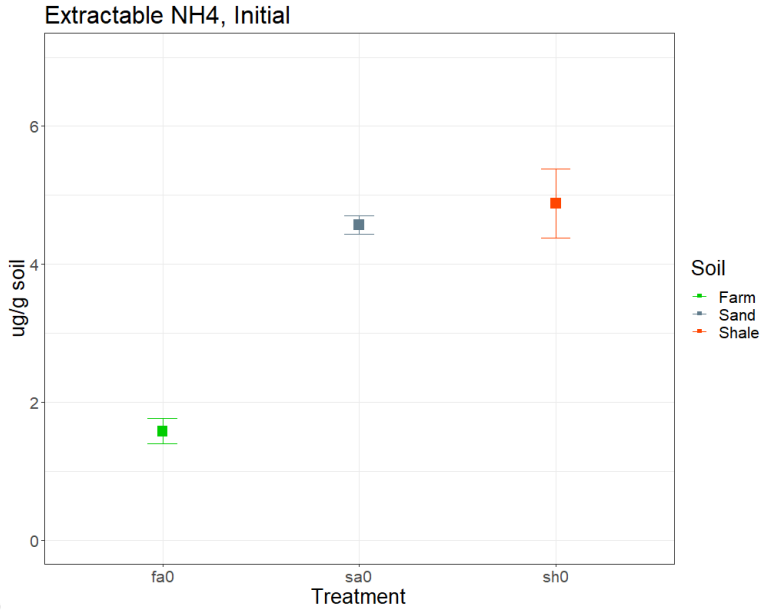
c)

a) Initial values of pH were highest in farm and lowest in sand. Linear model supported that the soil types had a statistically significant difference from each other and that the initial readings were statistically significant as higher than the readings post-growing season 2016, but were non-significantly higher than the post-growing season 2017 measures (averaged over mycorrhizal and fertilizer treatment).

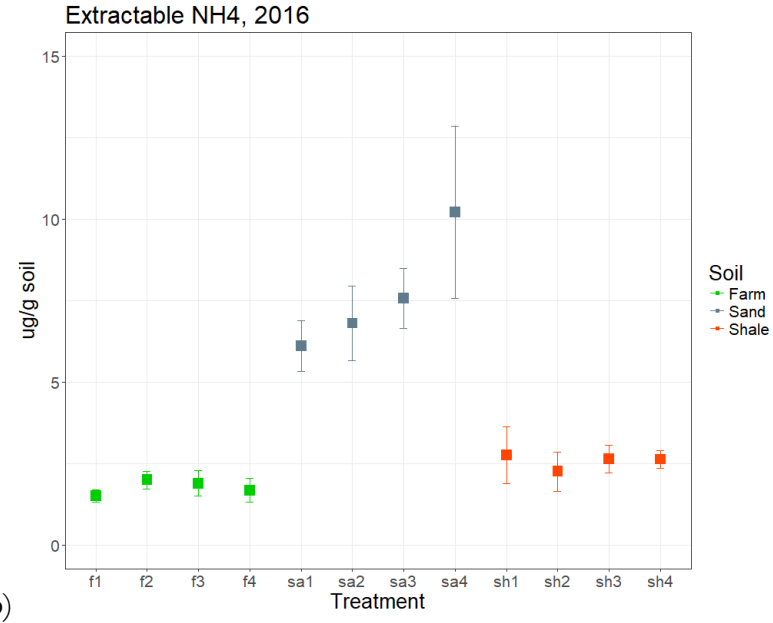
b) Post-growing season 2016. Linear model showed farm soil had a higher pH than sand soil ($P = 0.052$), and shale was in between the two. Mycorrhizal inoculation was associated with a non-significant increase in pH, but fertilizer showed no trend.

c) Post-growing season 2017. Linear model showed that soil type was significant between all three soils, fertilizer was associated with a higher pH at the $p < 0.1$ level, and mycorrhizal inoculation was associated with a non-significant decrease in pH.

Figure 4

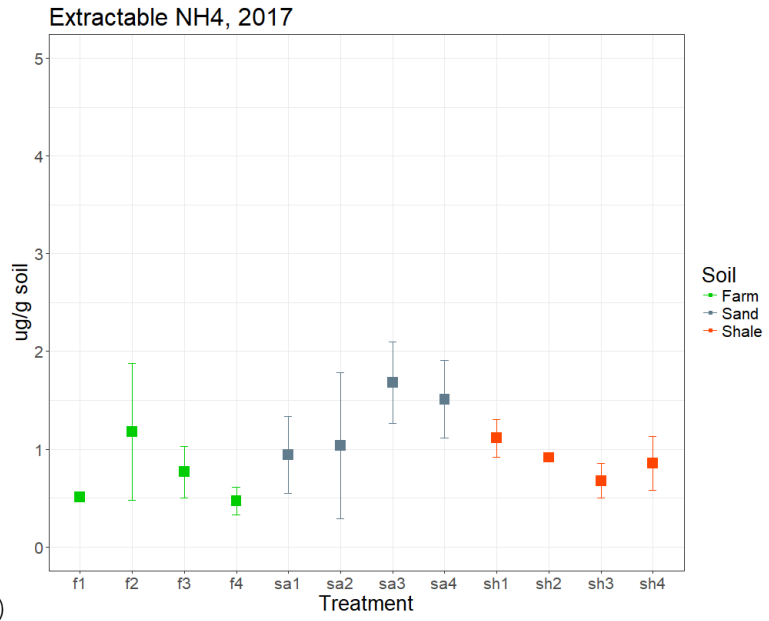

Extractable NH4, Initial
ug/g soil
0
2
4
6
fa0
sa0
sh0
Treatment
Soil
Farm
Sand
Shale

a)


Extractable NH4, 2016
ug/g soil
0
5
10
15
f1
f2
f3
f4
sa1
sa2
sa3
sa4
sh1
sh2
sh3
sh4
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 4 continued)



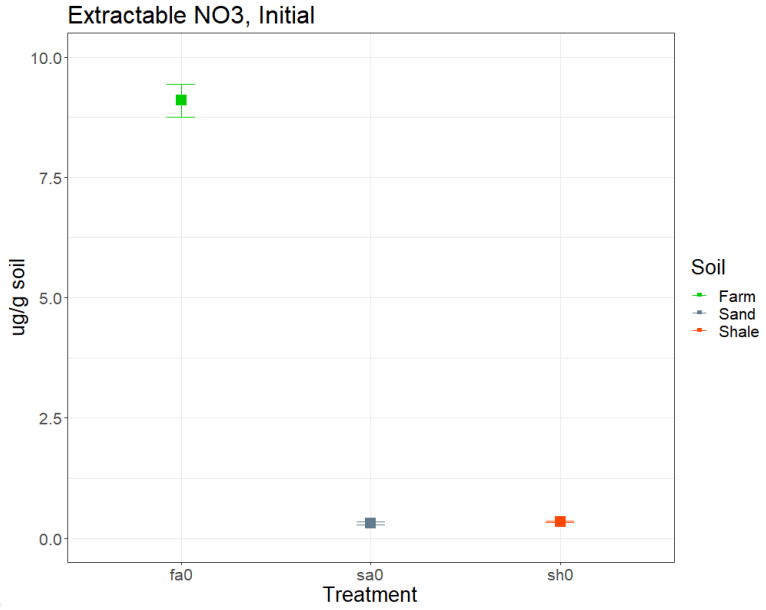
c)

a) Initial NH_4 showed highest values in sand and shale and lowest values in farm soil.

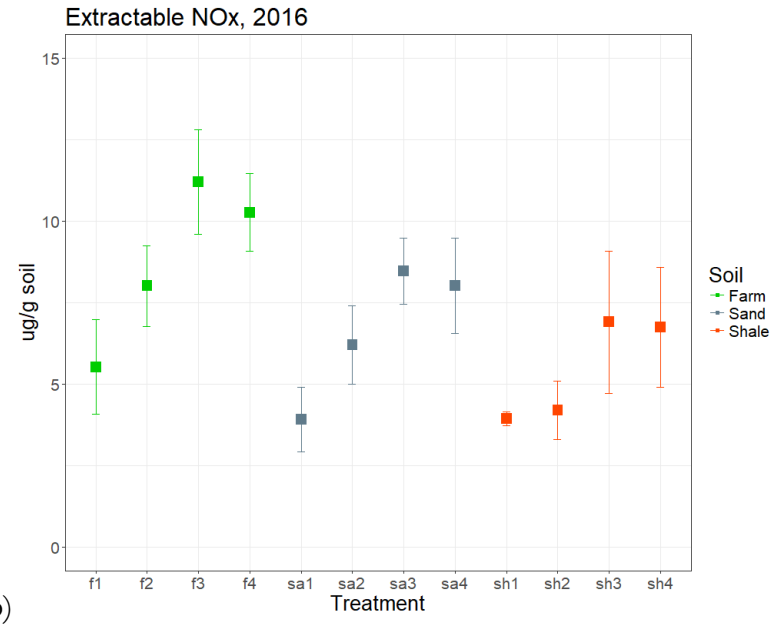
b) Post-growing season in 2016, extractable NH_4 was significantly higher in sand than in farm and shale (farm being the lowest). Linear model did not support any statistically significant differences due to fertilization or inoculation treatments.

c) Post-growing season NH_4 was much lower than post-season 2016. Linear model showed that sand has significantly higher NH_4 than farm soil, and shale was in between (non-significant). Fertilization and inoculation had statistically non-significant effects.

Figure 5

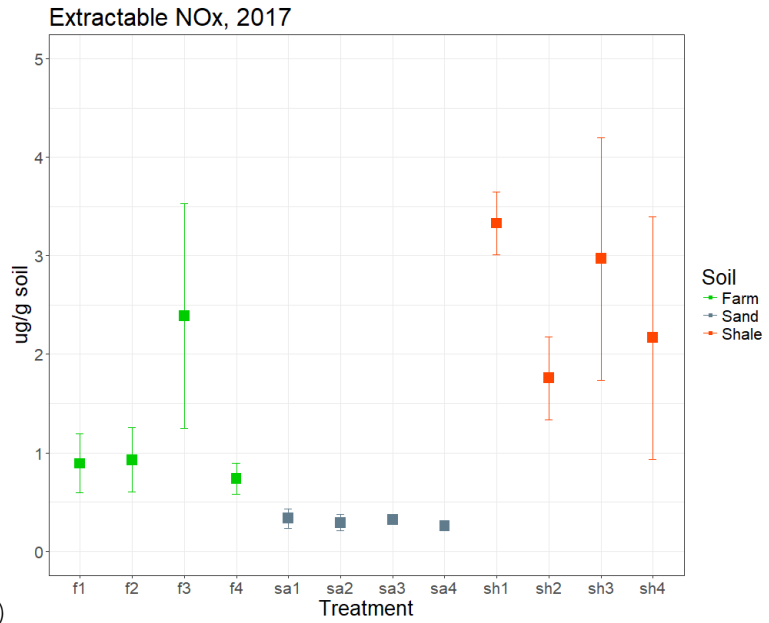

Extractable NO3, Initial
10.0
7.5
5.0
2.5
0.0
ug/g soil
fa0
sa0
sh0
Treatment
Soil
Farm
Sand
Shale

a)


Extractable NOx, 2016
15
10
5
0
ug/g soil
f1
f2
f3
f4
sa1
sa2
sa3
sa4
sh1
sh2
sh3
sh4
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 5 continued)



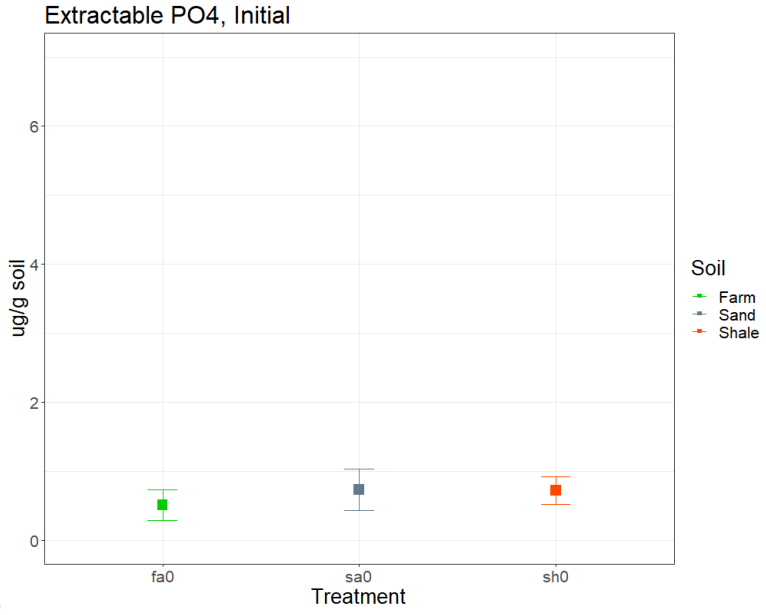
c)

a) Initial NO_3 showed lowest values in sand and shale and highest value in farm soil.

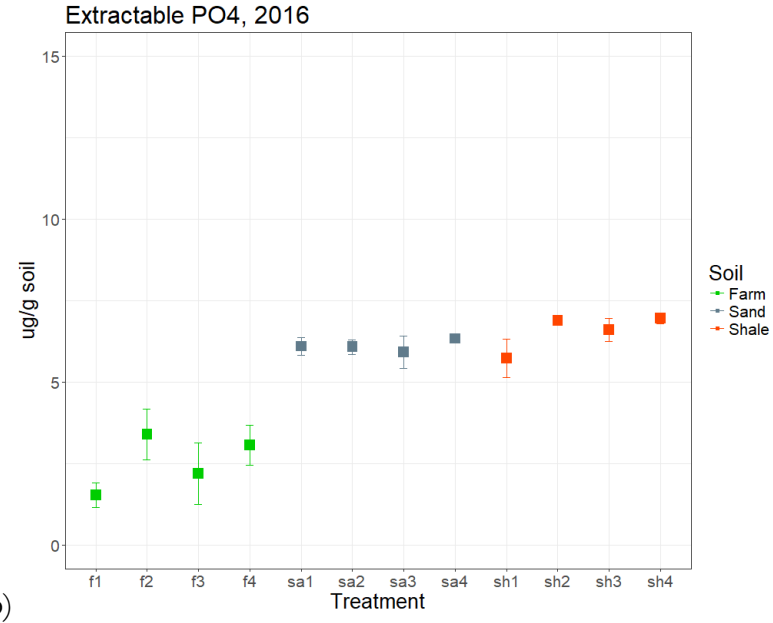
b) Post-growing season in 2016, extractable NO_3 was significantly higher in fertilized treatments, and was significantly higher in farm soil compared to shale soil.

c) Post-season 2017 extractable NO_3 was lower than post-season 2016. Linear model supported that all three soils were significantly different, with shale having the highest and sand the lowest extractable NO_3. There was a mildly significant trend of lower NO_3 due to inoculation ($P < 0.1$) and no statistically significant difference due to fertilization.

Figure 6


Extractable PO4, Initial
ug/g soil
0
2
4
6
fa0
sa0
sh0
Treatment
Soil
Farm
Sand
Shale

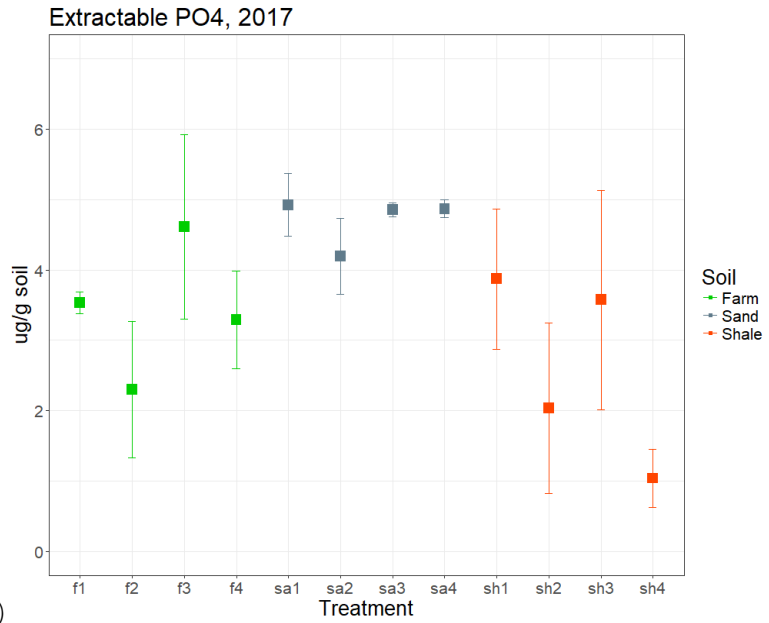
a)


Extractable PO4, 2016
ug/g soil
0
5
10
15
f1
f2
f3
f4
sa1
sa2
sa3
sa4
sh1
sh2
sh3
sh4
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 6 continued)

c)

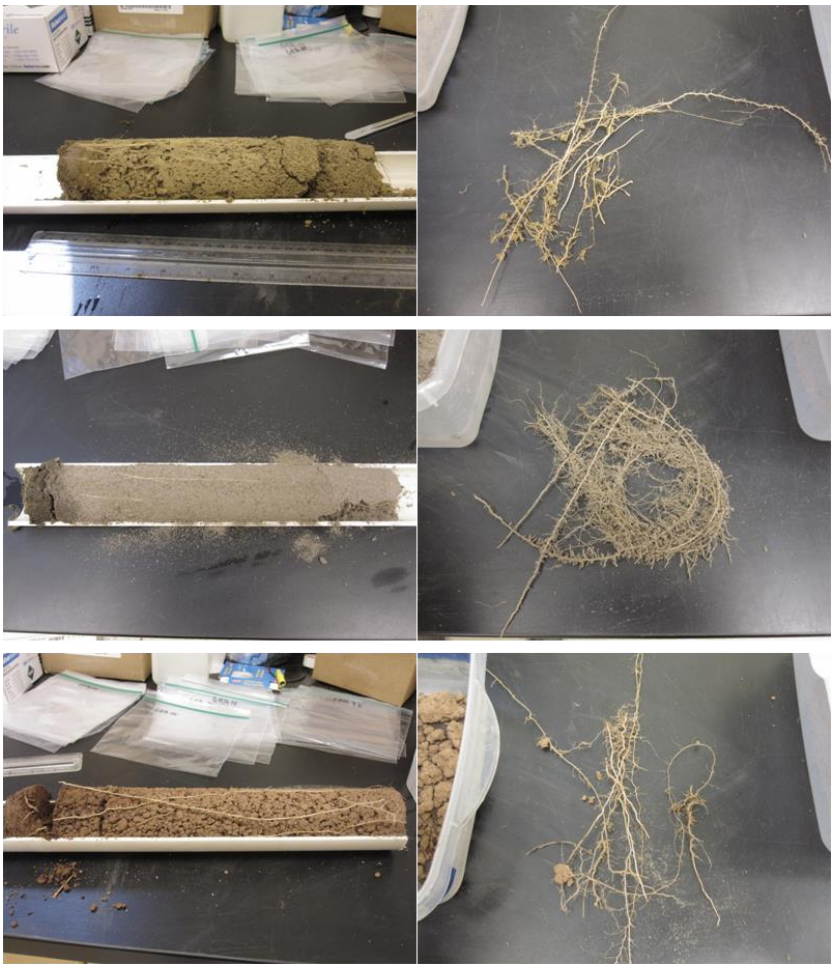


a) Initial PO_4 measured showed not much present in any of the soil types.

b) Post growing season 2016, PO_4 was significantly lower in farm soils compared to sand and shale (which were essentially even). Mycorrhizal inoculation was associated with a significantly higher amount of extractable PO_4.

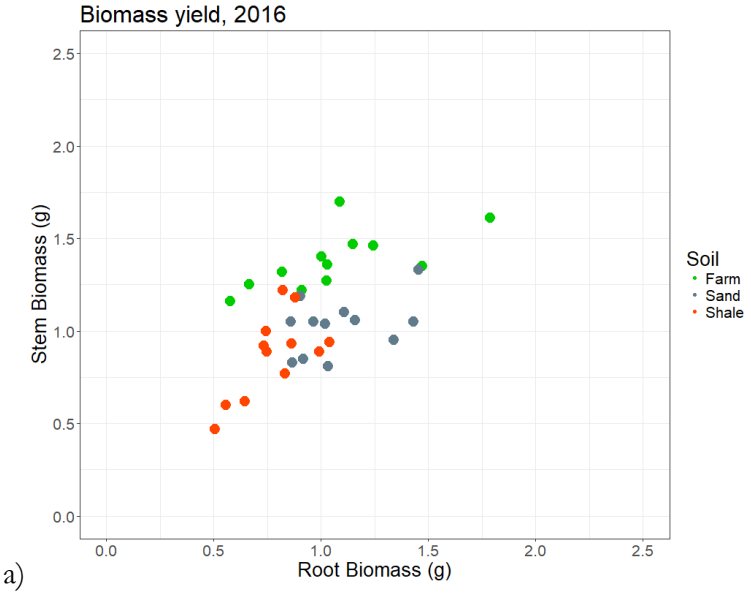
c) Post-season 2017 extractable PO_4 was lower than post-season 2016. Linear model showed mild significance ($P<0.1$) for sand having the most extractable PO_4 and shale the least. Fertilization had no significant effect. Mycorrhizal inoculation had a significant effect of decreasing PO_4.

Figure 7

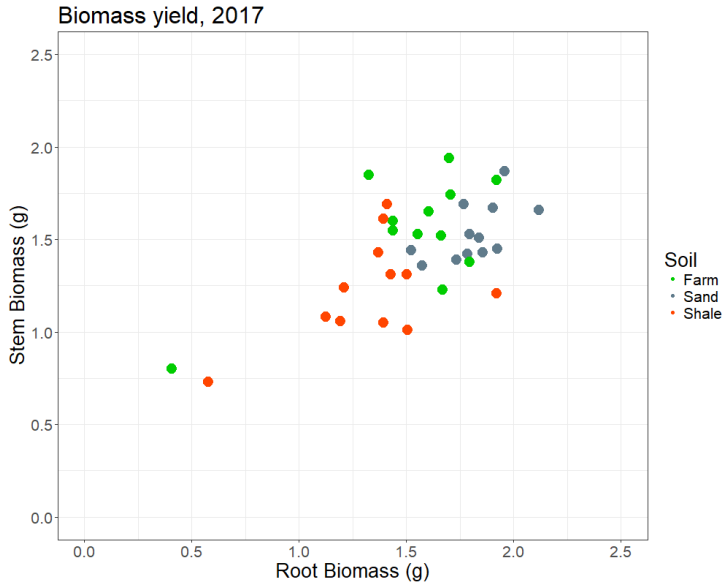


The three soils in 2016, during the breakdown of the experiment. There is a visible texture difference between the soils. Different root morphologies between the soil types can be seen as well in the photographs. Most notable is that fine roots were much more obvious and likely took up a higher percentage of total root area in the sand soil. From top to bottom: Farm soil, roots grown in farm soil, sand, roots grown in sand, shale soil, roots grown in shale.

Figure 8



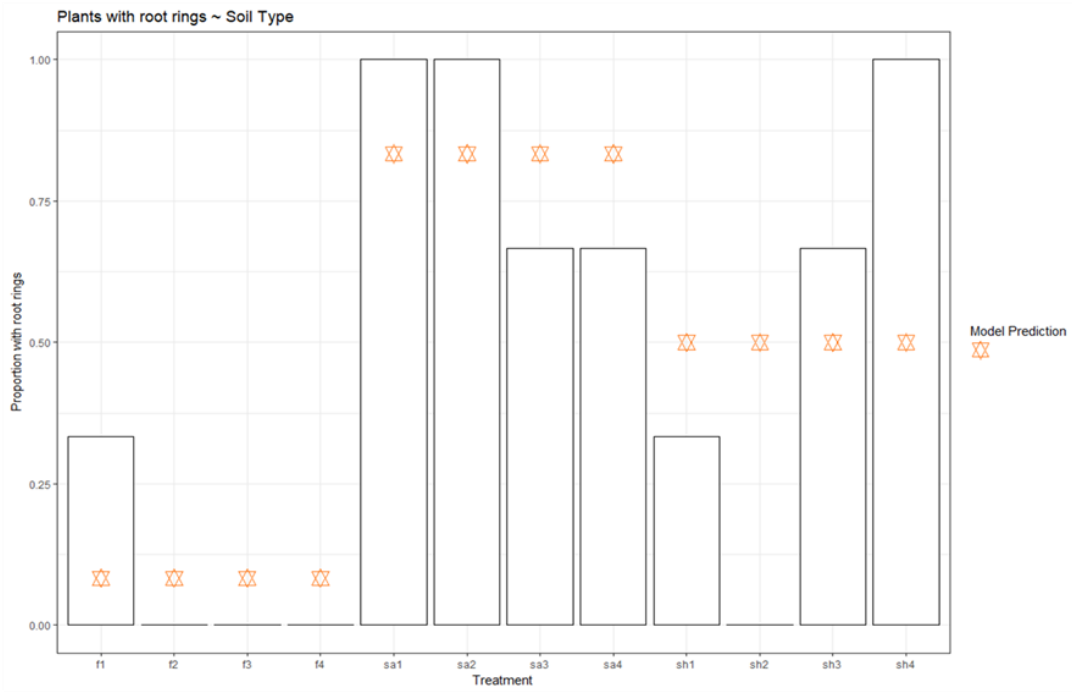
a)



b)

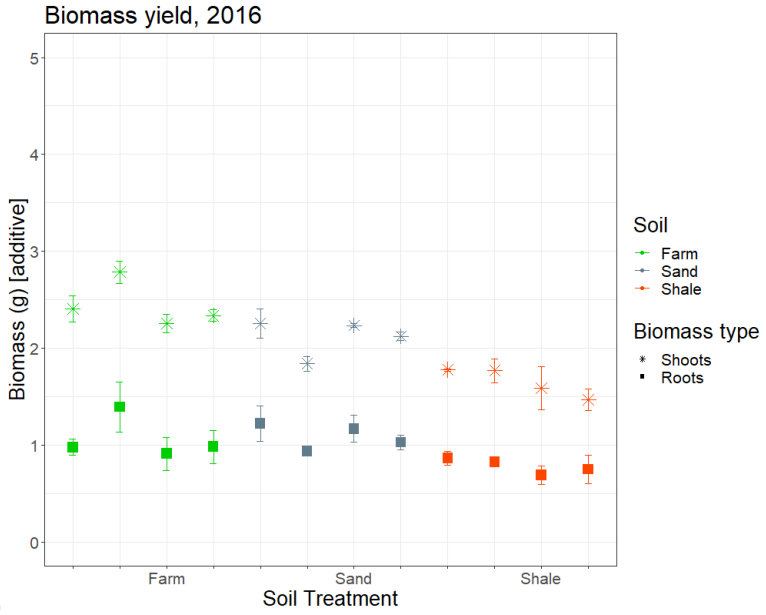
a) Root vs shoot biomass for 2016. Plants produced the lowest biomass in the shale soil. b) Root vs shoot biomass in 2017. The two outlier points are the plants that struggled. Similar to 2016, the shale soil consistently yielded plants with lower biomass. Neither year had statistically significant results for the differences in biomass yield.

Figure 9

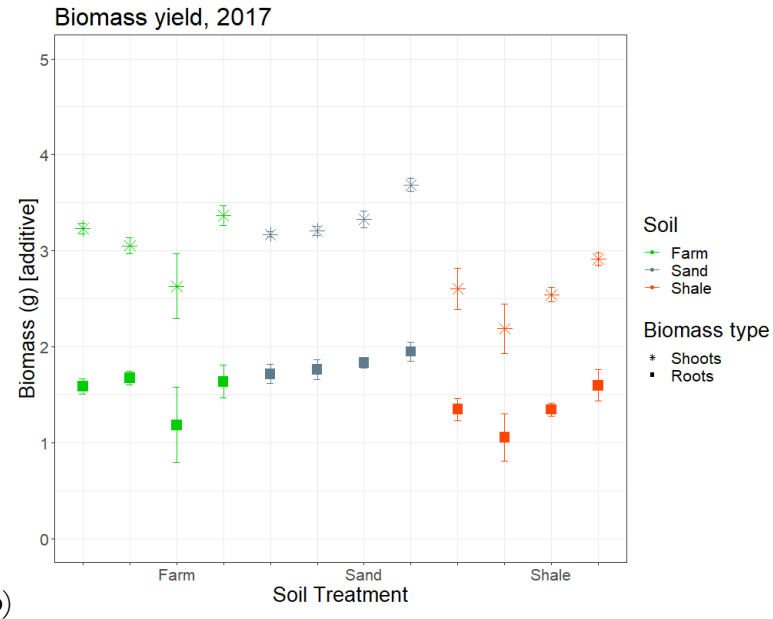


Proportion of treatments (N = 3 per treatment type) in 2017 with root ring present compared to the model prediction of the best-fit model. Soil type but not treatment was significant. The presence of a root ring suggests that the plant roots were in a foraging strategy, suggesting that the nutrients in the soil were approaching exhaustion. Root ring presence was highest in sand treatments (the center 4 bard of the graph), second highest in shale, and very low in the farm soil.

Figure 10



a)



b)

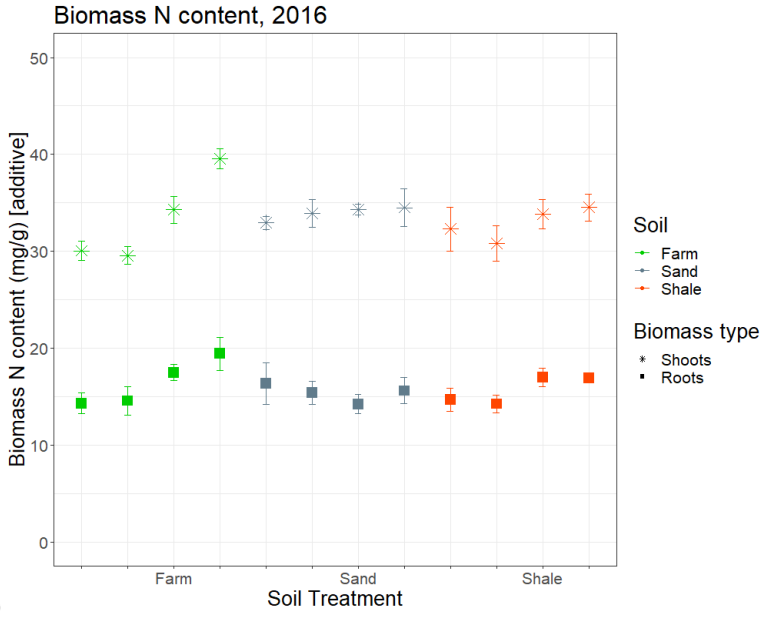
a) Plant biomass production for 2016. Biomass yields for stems and roots were analyzed separately. Linear model predictions for the biomass as an individual factor showed that farm and sand have significantly higher stem biomass than shale, and farm and sand had significantly higher biomass than shale for root biomass. Inoculation had a non-significant

(Figure 10 continued)

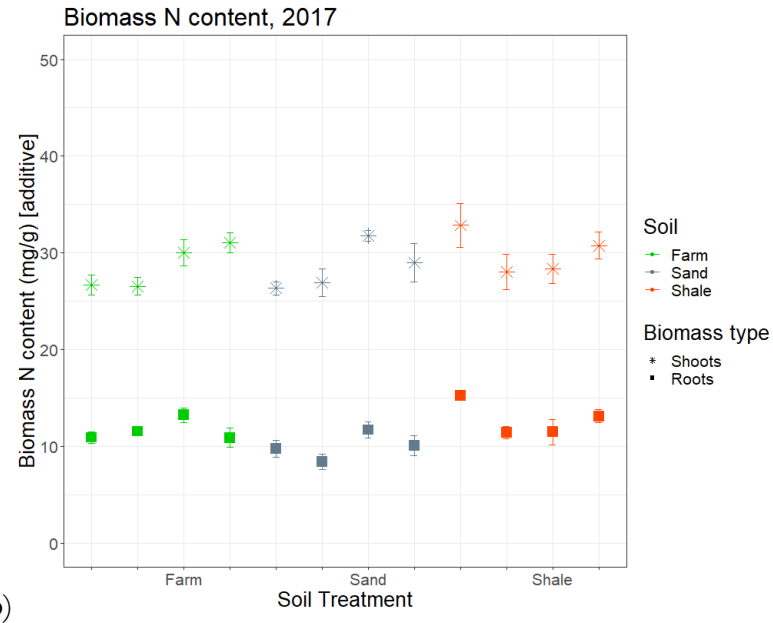
negative effect on stem biomass and a small non-significant positive effect on root biomass. Fertilization had barely an effect on stem tissue and a non-significant negative effect on root biomass.

b) Plant biomass production for 2017. Biomass yields for stems and roots were analyzed separately. Linear model predictions for the biomass as an individual factor showed that farm and sand have significantly higher stem biomass than shale, and sand had significantly higher biomass than shale for root biomass. Both fertilizer and inoculation had non-significant positive impacts on stem and root tissue, with the effect of inoculation being larger in root biomass.

Figure 11



a)



b)

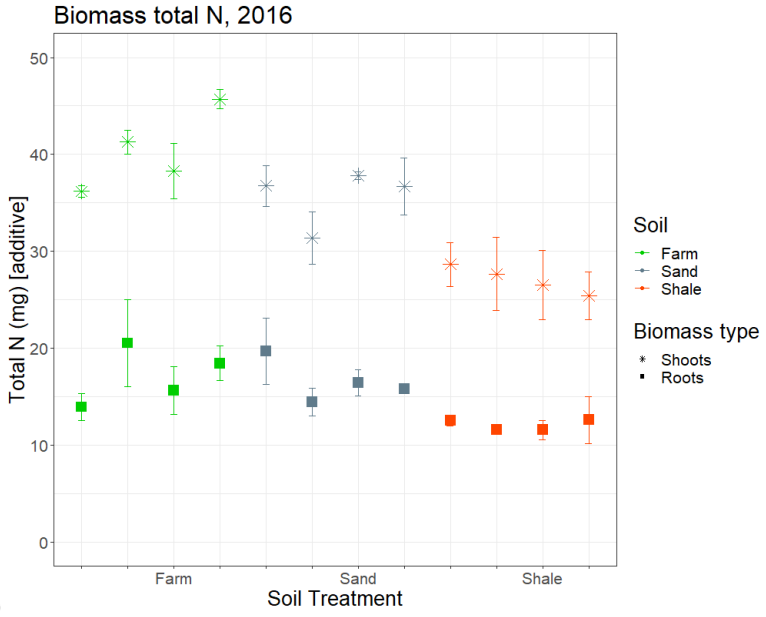
a) N content of plant biomass production for 2016. N content for stems and roots was analyzed separately. Linear model predictions for the N content of stems and roots as individual factors showed that soil does not have an effect on N content. Fertilization had a

(Figure 11 continued)

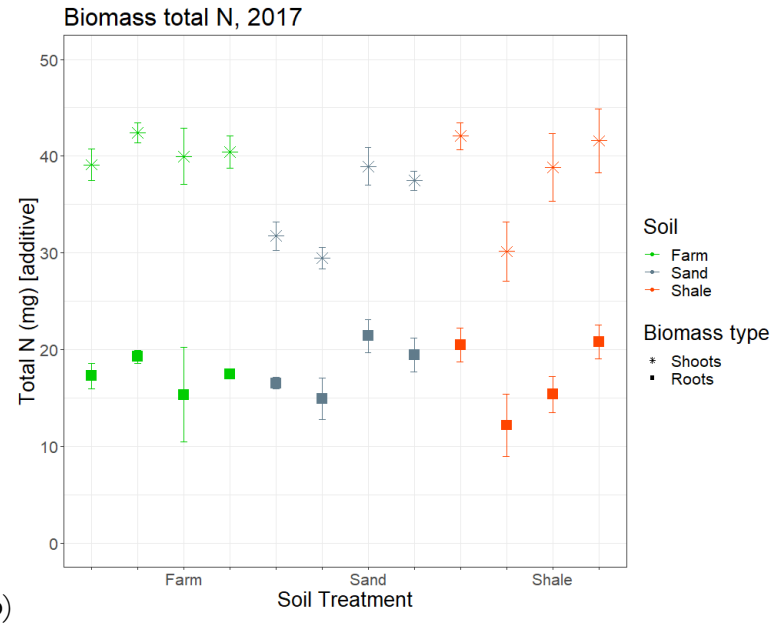
significant positive impact on stem and root N content. Inoculation had a non-significant positive effect on stem and root N content.

b) N content of plant biomass production for 2017. N content for stems and roots was analyzed separately. Linear model predictions for the N content of stems and roots as individual factors showed that N content of stem tissue was significantly lower in sand compared to farm and shale soil. However, there was no statistically significant difference due to soil type on root N content, although roots in the sand had the lowest N content. Fertilization had a non-significant positive impact on stem N content and a variable effect on root N content. Inoculation had a non-significant negative effect on stem N content and a variable effect on root N content.

Figure 12



a)



b)

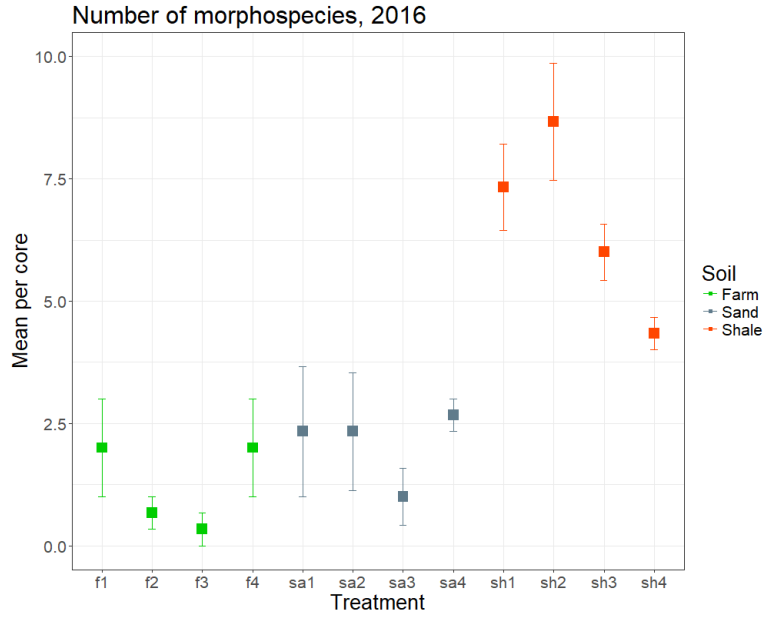
a) Post-season 2016 total biomass N content. Best-fit linear model predicted that stem tissue total N is higher due to fertilization, but not with statistical significance, and predicted no trend due to inoculation. Farm soil had the highest and shale the lowest total N content; all soil types were associated with differences that had statistical significance ($P < 0.05$). For

(Figure 12 continued)

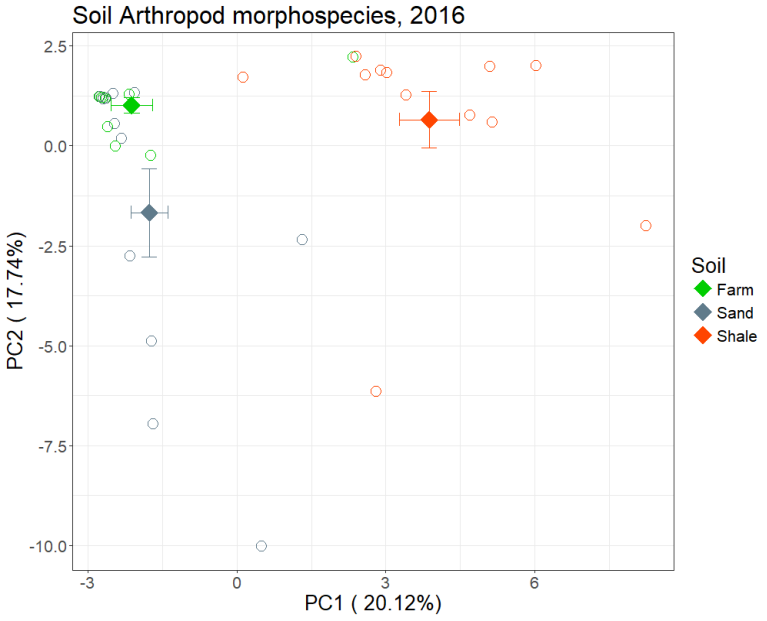
root tissue, best-fit linear model predicted a non-significant increase due to inoculation and no trend due to fertilization. Farm and sand had similar total N and shale had the lowest ($P < 0.05$). When root and stem N were added together, farm had the highest N content and shale the lowest.

b) Post-season 2017 total biomass N content. Best-fit linear model predicted that fertilizer increased total N ($P < 0.1$) in stem tissue and mycorrhizal inoculum was associated with a non-statistically-significant decrease. Sand stem tissue had the lowest total N and farm tissue had the highest. For root tissue, best-fit linear model predicted that fertilization caused a non-significant increase and inoculation caused a non-significant decrease. Sand had the highest total N and shale the lowest. When root and shoot values were added together, farm had the most N and sand had the least, although it was similar to shale.

Figure 13

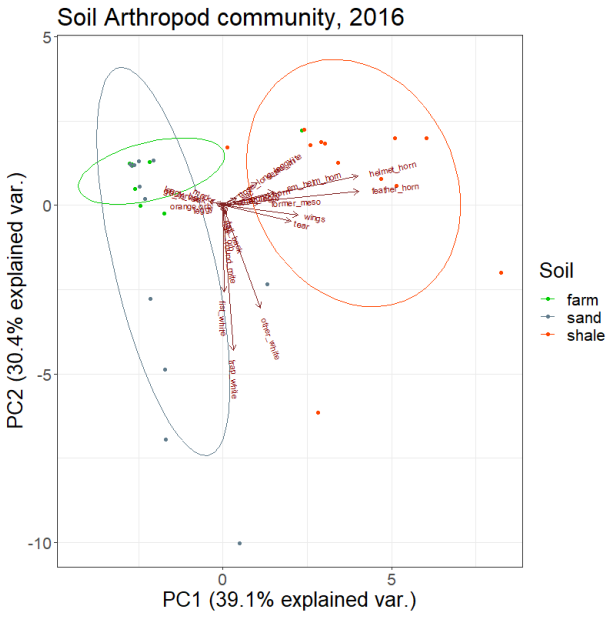

Number of morphospecies, 2016
Mean per core
10.0
7.5
5.0
2.5
0.0
f1
f2
f3
f4
sa1
sa2
sa3
sa4
sh1
sh2
sh3
sh4
Treatment
Soil
Farm
Sand
Shale

a)


Soil Arthropod morphospecies, 2016
PC2 (17.74%)
2.5
0.0
-2.5
-5.0
-7.5
-10.0
-3
0
3
6
PC1 (20.12%)
Soil
Farm
Sand
Shale

b)

(Figure 13 continued)

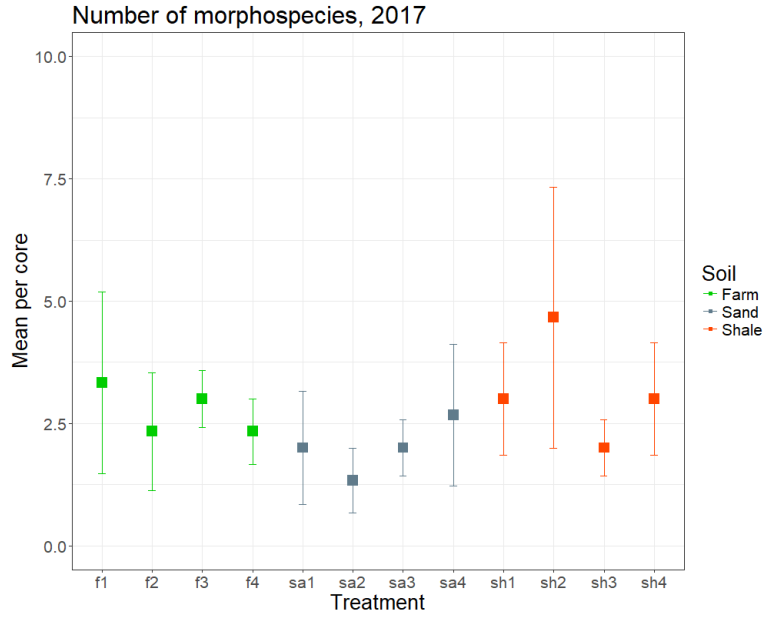


c)

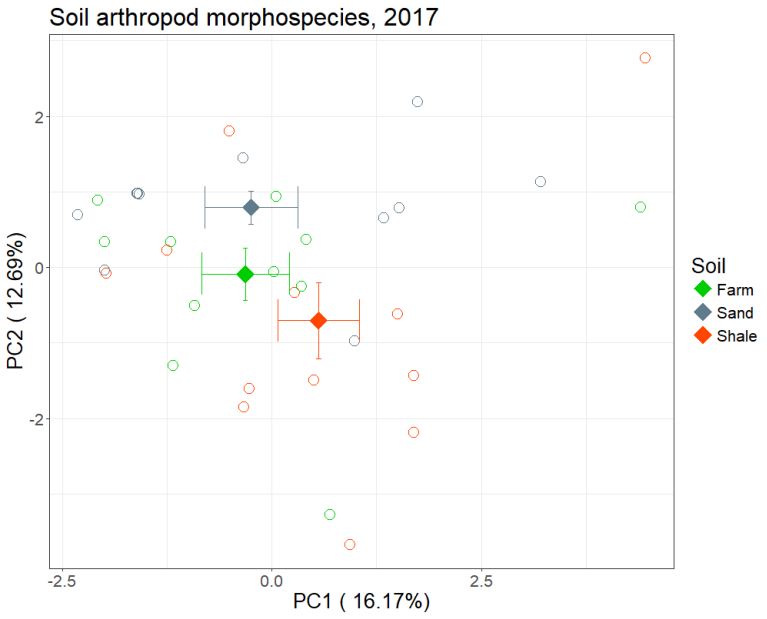
a) The mean number of soil arthropod morphospecies was highest in shale soil.

b) PCA of soil arthropod morphospecies abundance showing the centerpoint of each soil type. Community data was scaled using log transformation. Nonparametric MANOVA of data showed that soil was a significant factor in the difference between soil communities. N was significant at $P < 0.1$, and inoculation was not statistically significant. c) PCA of soil arthropod morphospecies with species directionality labeled and the larger space of each soil type highlighted by ellipses. A number of morphospecies were associated only with shale soils.

Figure 14

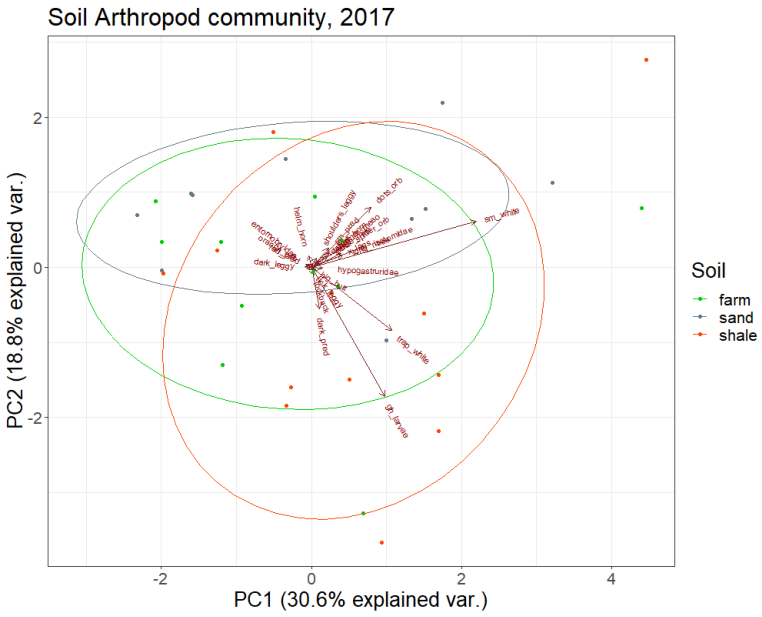

Number of morphospecies, 2017
Mean per core
10.0
7.5
5.0
2.5
0.0
f1
f2
f3
f4
sa1
sa2
sa3
sa4
sh1
sh2
sh3
sh4
Treatment
Soil
Farm
Sand
Shale

a)


Soil arthropod morphospecies, 2017
PC2 (12.69%)
2
0
-2
-2.5
0.0
2.5
PC1 (16.17%)
Soil
Farm
Sand
Shale

b)

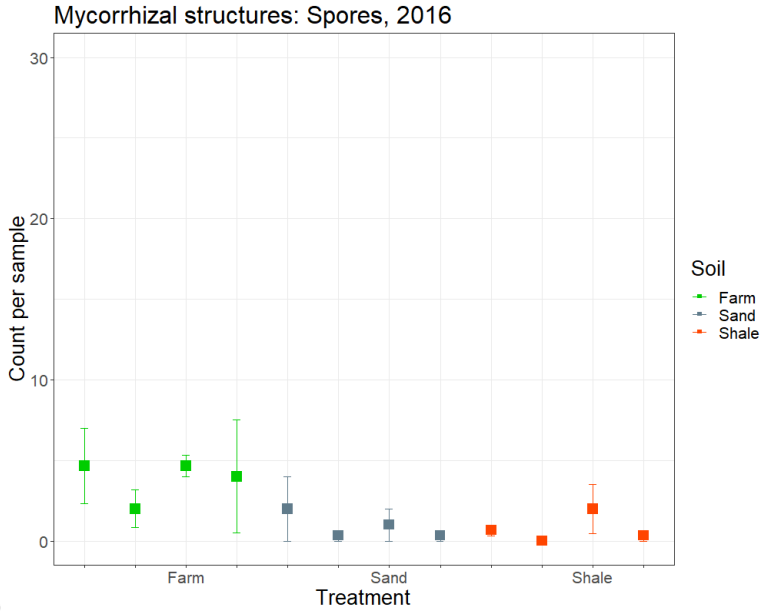
(Figure 14 continued)



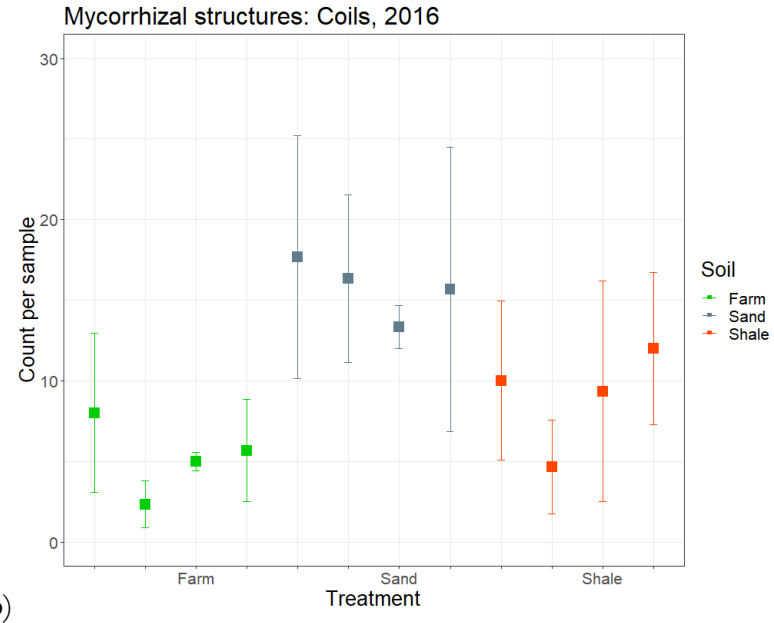
c)

a) The mean number of soil arthropod morphospecies was similar across soil types for 2017. b) PCA of soil arthropod morphospecies abundance for 2017 showing the centerpoint of each soil type. Community data was scaled using log transformation. Nonparametric MANOVA of data showed that soil was a significant factor at $P < 0.1$ in the difference between soil communities. N and inoculation were not statistically significant. c) PCA of soil arthropod morphospecies with species directionality labeled and the larger space of each soil type highlighted by ellipses. The ellipses overlaped much more than those for 2016.

Figure 15

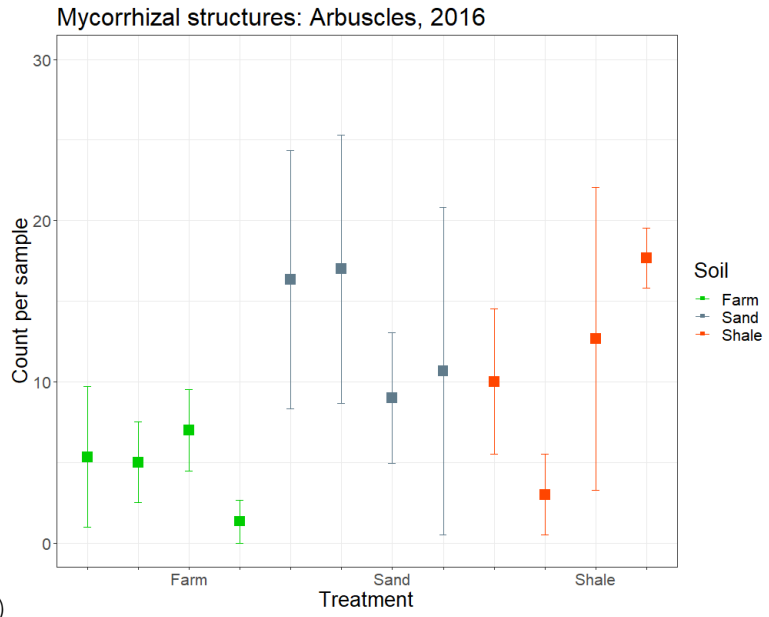

Mycorrhizal structures: Spores, 2016
Count per sample
30
20
10
0
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

a)

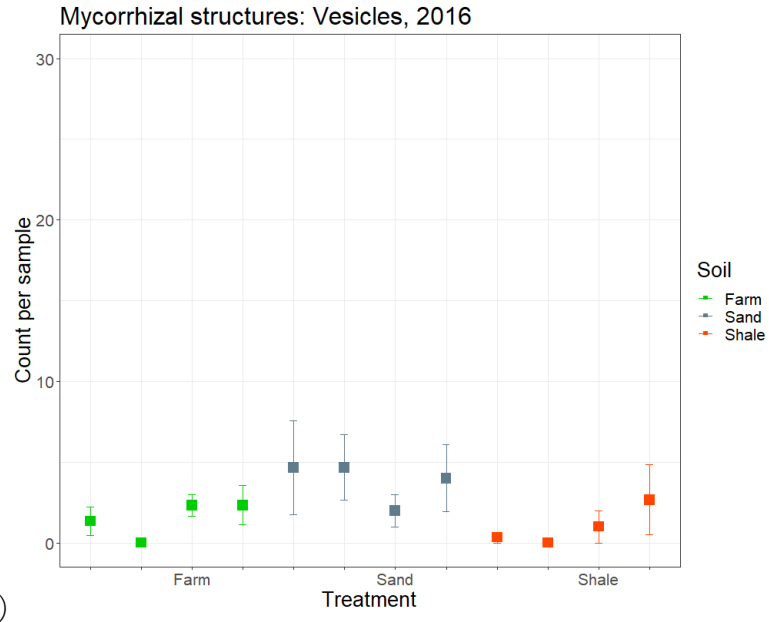

Mycorrhizal structures: Coils, 2016
Count per sample
30
20
10
0
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 15 continued)



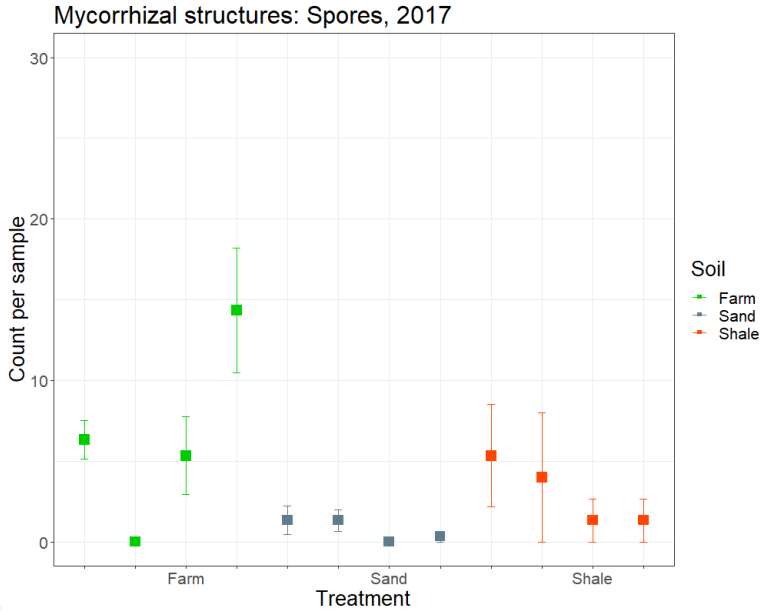
c)



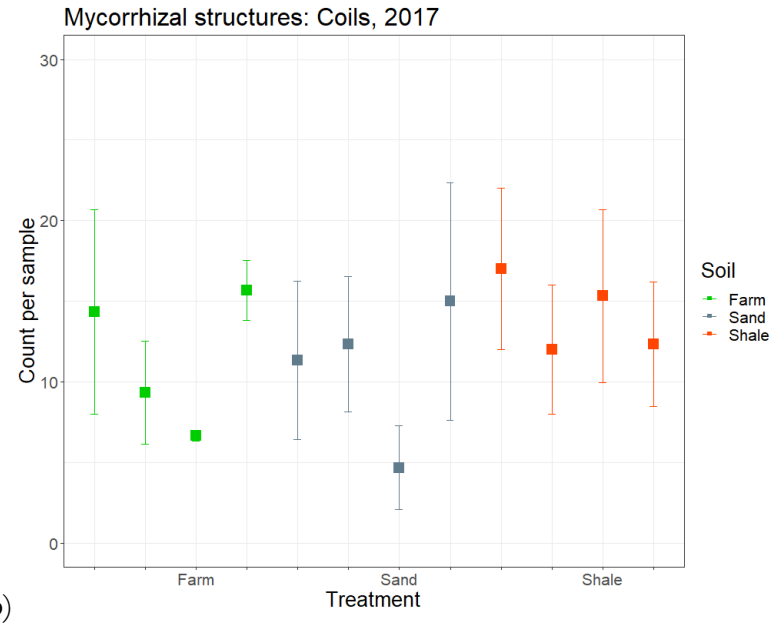
d)

Mycorrhizal structure count of 50 views for 2016. There was no clear trend due to inoculation nor fertilization. a) Spores were generally at low abundance. b) Coils were most present in sand soil. c) Arbuscules were higher in sand and shale soil compared to farm soil. d) Vesicles were in low abundance.

Figure 16

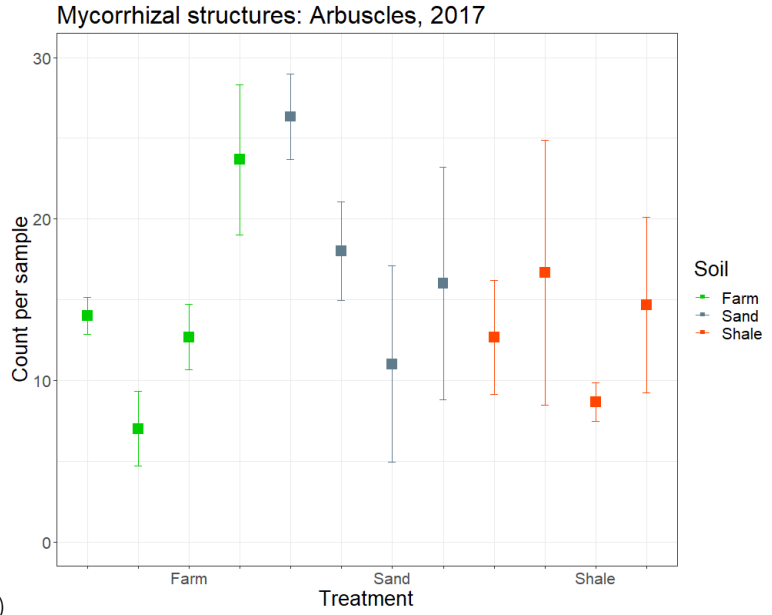

Mycorrhizal structures: Spores, 2017
Count per sample
30
20
10
0
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

a)

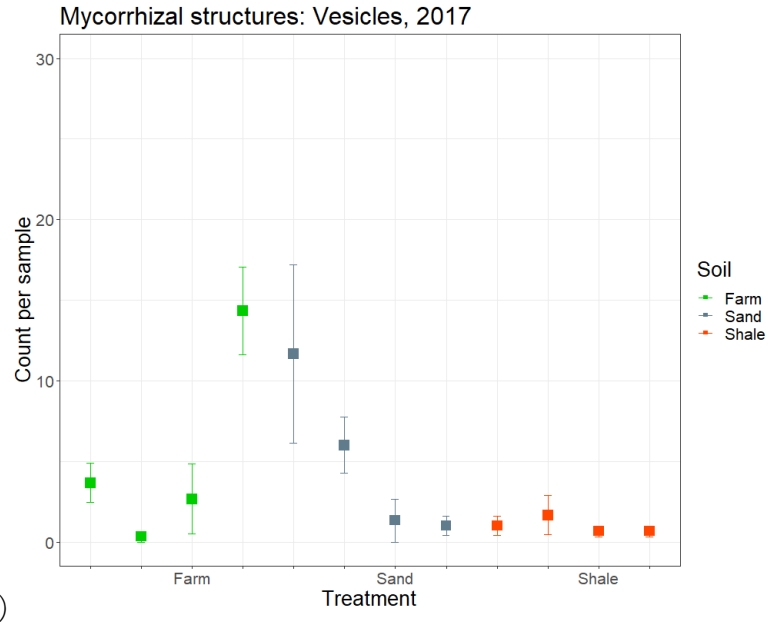

Mycorrhizal structures: Coils, 2017
Count per sample
30
20
10
0
Farm
Sand
Shale
Treatment
Soil
Farm
Sand
Shale

b)

(Figure 16 continued)



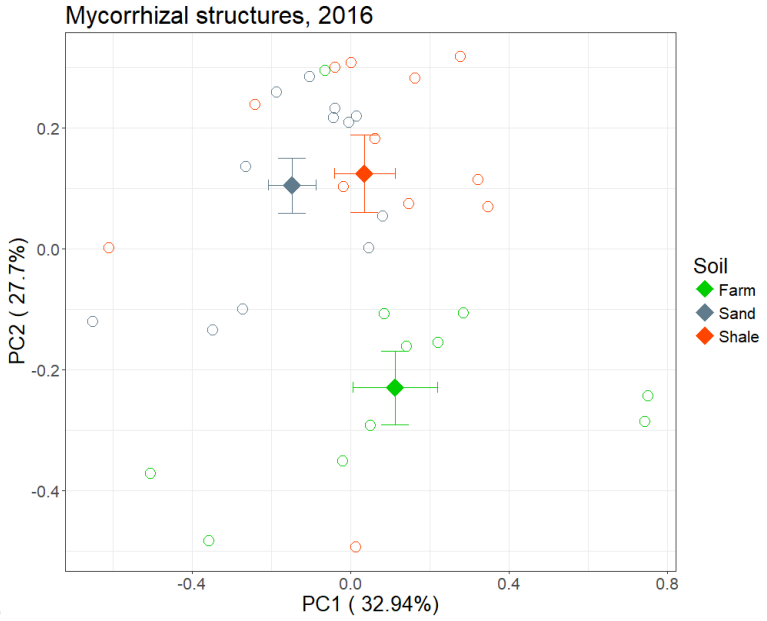
c)



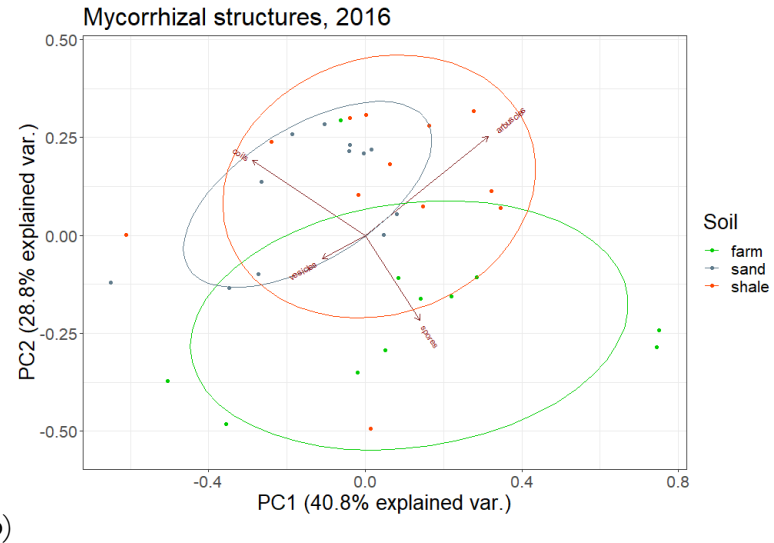
d)

Mycorrhizal structure count of 50 views for 2017. There was no clear trend due to inoculation nor fertilization, nor was there statistical significance. a) Spores were more present in farm and shale soil than in sand. b) Coils were at similar abundance in all soils. c) Arbuscules were at similar abundance for all soils. d) Vesicles were higher in farm and sand soil compared to shale soil.

Figure 17



a)



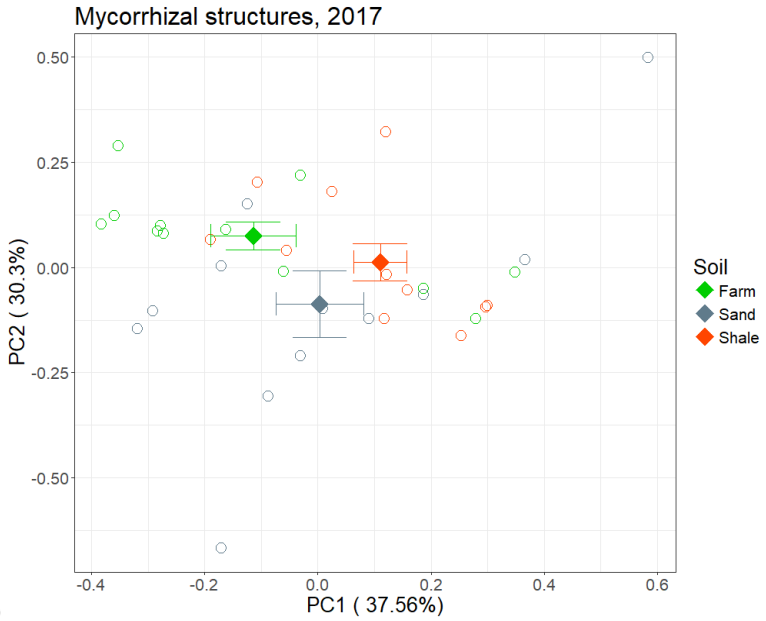
b)

Nonparametric MANOVA of mycorrhizal structures showed that soil type led to statistically significant differences between the mycorrhizal structures but inoculation and fertilization did not. a) PCA showing the centerpoints of the multivariate data suggests that farm soil was most different compared to sand and shale.

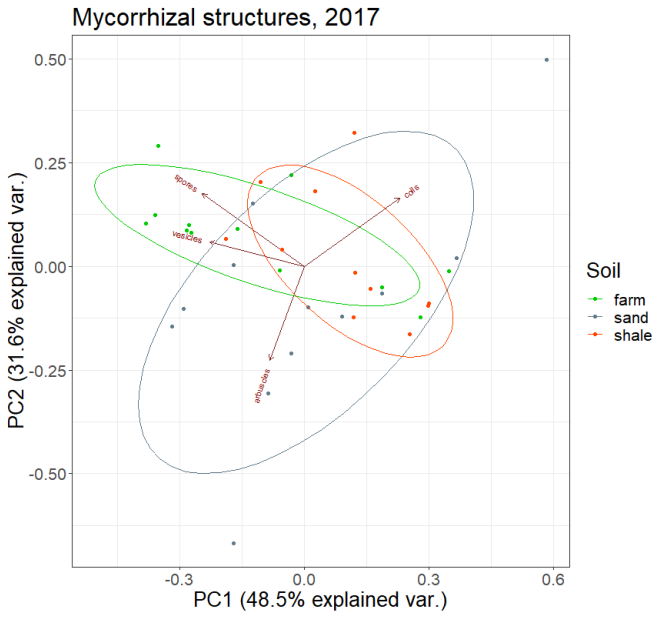
(Figure 17 continued)

b) PCA showing the directionality of the structure and the ellipses for the soil type showed the same thing, that farm was most distinct from sand and shale, and tended to be more associated with spores compared to sand and shale.

Figure 18



a)



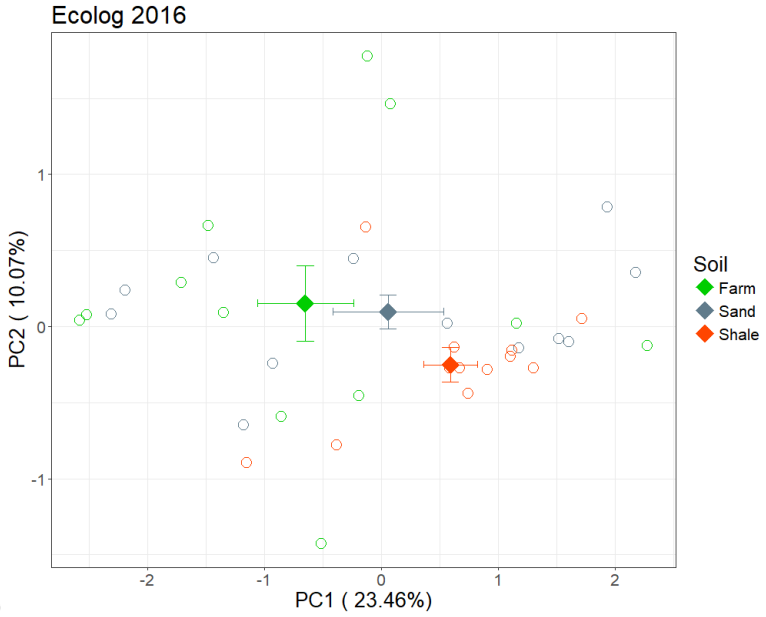
b)

MANOVA of mycorrhizal structures showed that the interaction of soil with fertilization and the interaction of fertilization with inoculation led to a different composition of mycorrhizal structures, but soil type, inoculation, and fertilization alone did not correspond with any statistically significant pattern in the mycorrhizal structures.

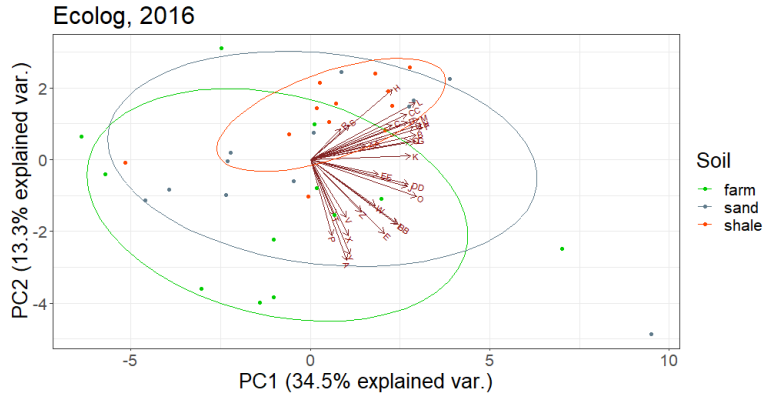
(Figure 18 continued)

a) PCA showing the centerpoint of the soil types showed no clear separation. b) PCA showing directionality of the mycorrhizal structures and the ellipses associated with the soils; there was much overlap between soil types.

Figure 19

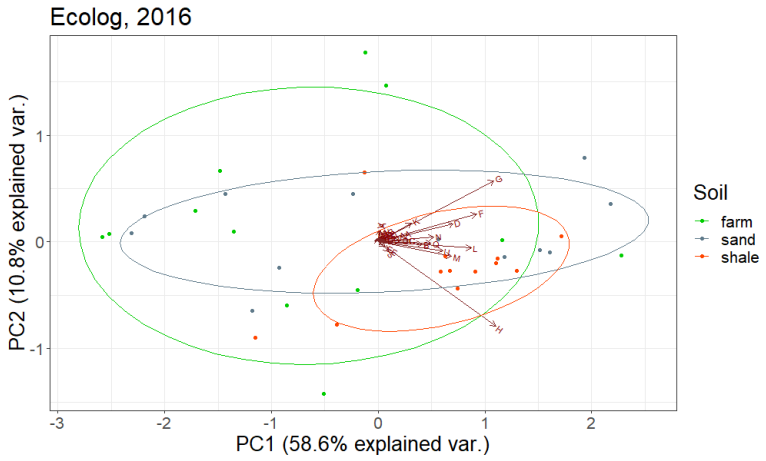

Ecolog 2016
PC2 (10.07%)
PC1 (23.46%)
Soil
Farm
Sand
Shale

a)


Ecolog, 2016
PC2 (13.3% explained var.)
PC1 (34.5% explained var.)
Soil
farm
sand
shale

b)

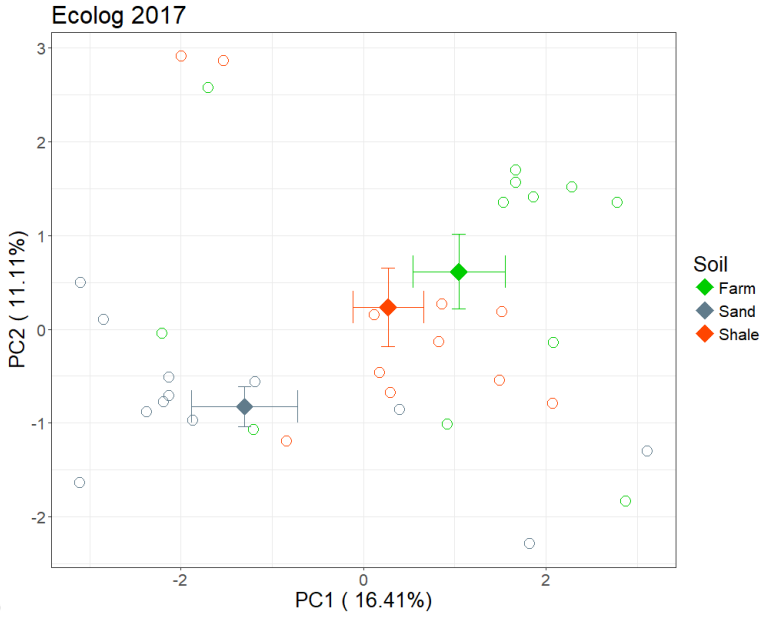
(Figure 19 continued)



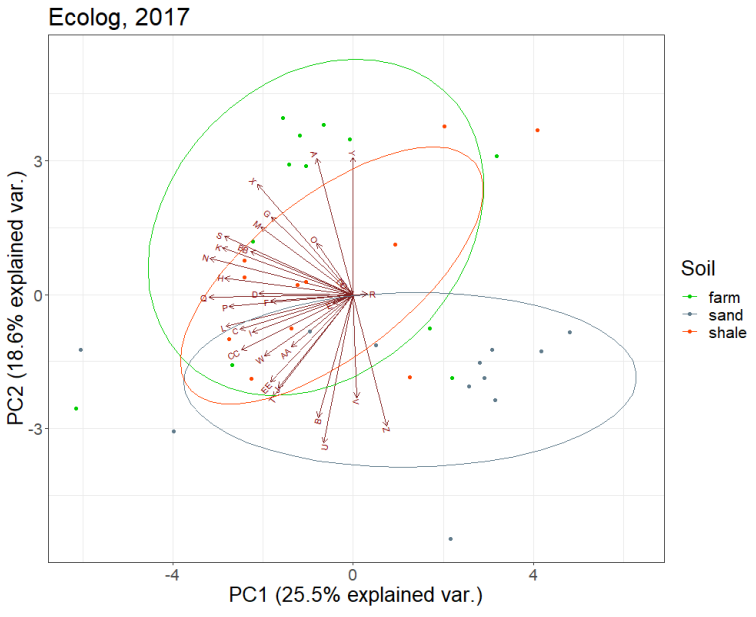
c)

a) MANOVA of BIOLOG ecoplates showed significance due to soil type but not inoculation nor fertilization treatment in 2016. This PCA showed the centerpoint of soil type and standard error. Data were adjusted using decostand function and normalize command. b) PCA of data showing directionality of the C sources and the ellipses associated with the soil type. Data was scaled using automatic option in prcomp. c) PCA showing the same thing as b, but the data was not scaled.

Figure 20

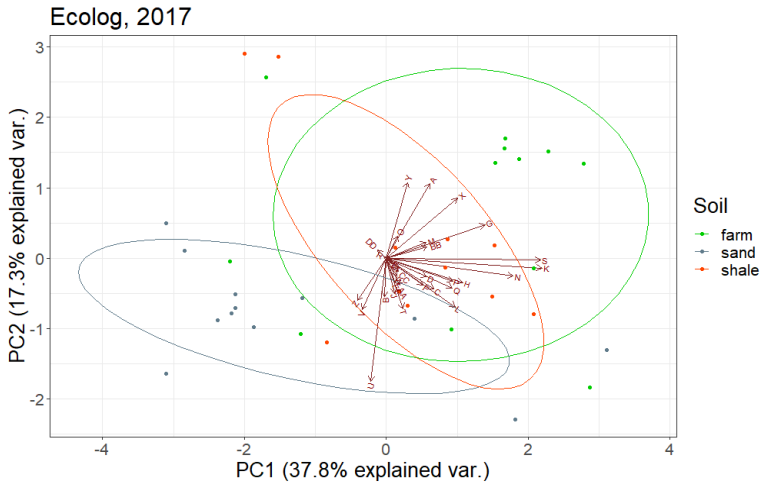

Ecolog 2017
PC2 (11.11%)
PC1 (16.41%)
Soil
Farm
Sand
Shale

a)


Ecolog, 2017
PC2 (18.6% explained var.)
PC1 (25.5% explained var.)
Soil
farm
sand
shale

b)

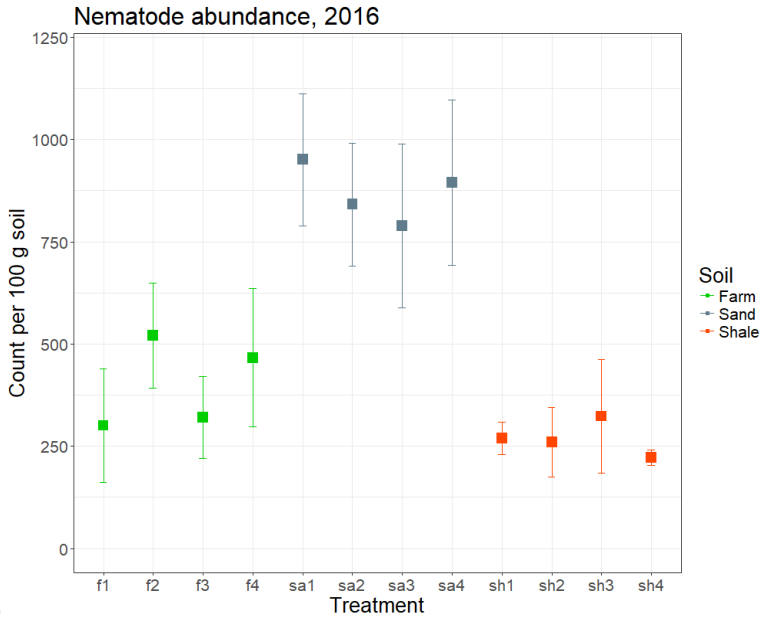
(Figure 20 continued)



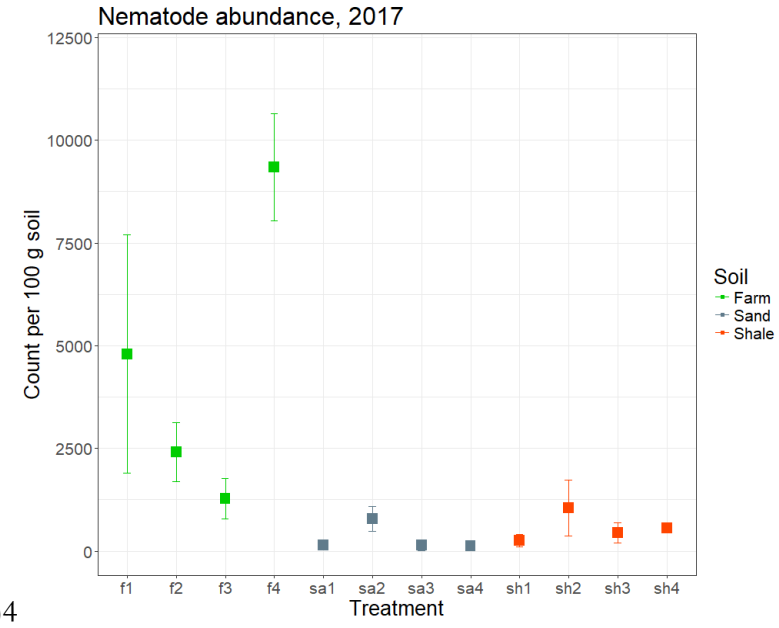
c)

a) MANOVA of BIOLOG ecoplates showed significance due to soil type and inoculation treatment in 2017. This PCA showed the centerpoint of soil type and standard error. Sand soil was more separate from the other two soils than it was in 2016. Data were adjusted using decostand function and normalize command. b) PCA of data showing directionality of the C sources and the ellipses associated with the soil type. Data scaled using automatic option in prcomp. c) PCA showing the same thing as b, but the data was not scaled.

Figure 21



a)



b)4

a) Nematode abundance in 2016. Sand had much higher nematode abundance than farm and shale soils, and shale had (not-significantly) the lowest abundance. Linear model showed non-significant increase due to inoculation and non-significant decrease due to fertilization.

(Figure 21 continued)

b) Nematode abundance in 2017. Abundance was estimated to be much higher than in 2016 partially because the method of collection was different. Farm soil had much higher nematode abundance than sand and shale soils, with sand having (not-significantly) the lowest abundance. Linear model showed significant increase in nematode abundance due to inoculation, and non-significant decrease due to fertilization.

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Chapter 5: Conclusion

Summary

The goal of this dissertation was to look at the micro-scale occurrences in the soil community of biofuel switchgrass to better understand the sustainability of the crop. Based on the literature, I concluded that soil ecology can inform best-management practices for sustainable switchgrass production. Because switchgrass forms associations with arbuscular mycorrhizal fungi (AMF), and AMF are a part of the complex soil community, using soil ecology in an agricultural context is interesting and relevant to both to current agricultural and ecological research (Baumgarten, 2020a).

In chapter 2, the hypothesis that yearly application of nitrogen (N) fertilizer would fundamentally shift the soil community of switchgrass was mostly disproven in a research field established 5 years prior to sampling (Baumgarten, 2020b). There were differences between the soil community of the reference area (unplanted, mowed farmland) and that planted with switchgrass. However, there was limited evidence that yearly applications of N led to a change in the soil community. There were instances where fertilization led to statistically significant differences in the data, but there were no consistent patterns, not even in plant biomass yields. There was evidence that soil microarthropod communities and mycorrhizal structures were changing though time, but the conclusions that can be drawn from these trends are confounded by the difficulty in statistically analyzing repeated-measurements. The conclusion was that fertilization does not lead to a clear, measurable, building change in the soil community.

In chapter 3, the hypothesis that there were plant-soil feedbacks in the switchgrass system due to the AMF-switchgrass relationship was disproven (Baumgarten, 2020c). In a

greenhouse mesocosm experiment, the edaphic community was manipulated for plants grown for one and two seasons. Any plant-soil feedbacks should have been measurable in the plant biomass yield measurements. There were limited responses of the soil community to experimental treatments, but the plants biomass yields did not differ with statistical significance between treatment types. When the soil community measurements were combined for analysis in non-metric multidimensional scaling (NMDS), the trends were not strong enough to create a pattern differentiating the experimental treatments with statistical significance. These results suggest that neither plants nor the soil community have a linear response to the experimental manipulations. Most striking is that the fertilizer addition (with a 0 lb/ac, 50 lb/ac, and 100 lb/ac treatment) did not affect plant biomass production.

In chapter 4, the hypothesis that edaphic manipulation would result in differing effect sizes in three distinct soils was not supported (Baumgarten, 2020d). The soils were conserved and used to grow plants for a second year to see if signs of soil exhaustion were revealed when the soils were more nutrient drained. While extractable nutrients declined over the two growing seasons, the response in the plant growth and soil community was less than expected, especially in the nutrient-poor sand. The plants were resilient to the experimental treatments, and produced slightly more biomass in the second year of growth. The soil community showed limited response to experimental treatments except to the differences in soil type.

Based on these results, best-management practice of annual N fertilization should be reconsidered to reduce the energetic cost of fertilizer because the plant biomass yields were resilient to edaphic manipulations. Reducing fertilizer applications would help ensure net energy gain in the production of the final biofuel product. However, the results also indicate the resilience of the soil community. This means that the current best-management practice

of applying low levels of N fertilizer is not outweighing the inherent capacity of the soil to support life. Most likely, there is a great benefit to the soil and soil community from the extensive root system of switchgrass.

Switchgrass: resilience and sustainability

In general, the results of each experiment support that switchgrass is rigorously resilient to the applied edaphic manipulations. The most striking observation is that switchgrass did not regularly produce more biomass under fertilized conditions. Although other researchers have found switchgrass to be unpredictable and to not consistently respond to fertilization (e.g., Duran et al., 2016; Jung & Lal, 2011; Miesel et al., 2017), it is still a best-management practice to apply fertilizer (e.g. Emery et al., 2017; McLaughlin & Kszos, 2005; Miesel et al., 2017). This resilience in the ability to grow without fertilization is key to switchgrass being a good biofuel crop, since fertilizers require energy in their production and application. A reduced use of fertilizers should result in a net energy gain for the end biofuel product (Baumgarten, 2020a). Additionally, the three-soil greenhouse experiment corroborates the plan for switchgrass to be produced on marginal farmland (e.g., Khanna et al., 2011; Liebig et al., 2008; Stewart et al., 2015), because biomass yields were equally good in sand and shale soils compared to farm soil (Baumgarten, 2020d).

Recent research, asking similar questions to those posed in this dissertation, found that switchgrass benefitted most from AMF when in low N conditions (Jach-Smith & Jackson, 2018). Additionally, under excess N-fertilizer application, switchgrass produced the same amount of biomass but had "luxury-consumption of N" (Jach-Smith & Jackson, 2018). Most notably, the authors concluded that: "These findings suggest that the use of N fertilizer should be limited in switchgrass cropping systems to promote AMF abundance and

function, which may supplant N fertilizer in providing adequate plant N." While this research does not show a benefit of AMF or N fertilization in nutrient-poor sand (Baumgarten, 2020c), the overall results support the conclusion of Jach-Smith and Jackson (2018).

There are three more issues that relate to the question of switchgrass as a biofuel crop, which are somewhat outside the fields of ecology and agriculture but are worth discussion to highlight the importance of cross-disciplinary research. Farmer choice, energy infrastructure, and engineering of a different form of N-fertilizer.

Farmer choice to produce switchgrass is part of the big picture that could not be addressed in this research. Burli et al. (2019) find that in Missouri, seeing demonstration plots of switchgrass positively influenced willingness to grow, but "having land under Conservation Reserve Program, lands that experienced flooding of water stress in recent years, or lands that confront erosion issues did not have a significant influence on farmer willingness". Additionally, in farmer adoption of the crop, the s-shaped curve of technology diffusion should be taken into account (Jin et al., 2019). One model in Indiana suggested that after about 10 years of increasing adoption of growing switchgrass, the negative experiences led to a lack of any farmers willing to plant the switchgrass, but a subsidy was able to stabilize the scenario (Jin et al., 2019). Ecological research that may benefit farmers through advising best-management practices is relevant to this complex issue of whether farmers will choose to plant switchgrass as a biofuel crop.

There is entropy behind the current energy infrastructure such that future energy systems might be most successfully built on current systems, rather than replacing the systems entirely. One underlying assumption in this dissertation is that switchgrass and other cellulosic materials are promising as a partial solution to the global reliance on gasoline.

However, given the prevalence of cellulosic material in our world, there may be other ways to efficiently use these materials rather than by being turned into ethanol. For instance, Yee et al. (2013) find promising use of switchgrass into ethyl *tert*-butyl ether as an additive to help gasoline combust more fully, which is a "shorter" production than trying to convert switchgrass into ethanol.

Finally, a major underlying assumption of this research is that the production of N fertilizer via the Haber-Bosch process is a critical weakness in the production of sustainable biofuel. However, if ammonium production could be decoupled from fossil fuels the fundamental question of this dissertation would be less crucial. Pfromm (2017) suggests mechanisms to make emissions-free ammonium by retrofitting current Haber-Bosch systems to use renewable electricity.

Agricultural impacts on soil

Two findings in this dissertation corroborate the idea that farming can be designed for less impact on ecological communities (e.g., Bender et al., 2016; Brussaard et al., 2007). First, the soil community abundance was higher in areas planted with switchgrass compared to mowed land adjacent to the planted areas (Baumgarten, 2020b). Second, fertilization was not necessary for switchgrass biomass yields, and a reduction in N fertilizer will reduce the indirect impacts on communities through leaching.

The results in this dissertation, that switchgrass is associated with a soil community that is resilient to edaphic manipulations, relate to carbon (C) storage. Most critically because the soil community is a crucial part of decomposition. However, the results presented in this dissertation may be limited in application because biomass was not removed yearly as it would be in commercial production; it was most often mown annually in the fall and the

residue left in place. Given that C storage is so integral to sustainability, future research should include the complete removal of biomass.

As previously discussed in Baumgarten (2020d), the idea of soil exhaustion is currently best understood through the idea that plants need a variety of nutrients to grow, and if one or more is limited, that limiting nutrient then limits the plant growth. Historically, in the 1800s, farming methods would turn virgin land into production and then to leave it fallow to rebuild the soil (Sutter, 2016). This method then was replaced by a holistic model of soil protection; farmers learned to rebuild the soil through animal husbandry/wastes (Sutter, 2016). However, the holistic model was relatively quickly replaced with the idea that various nutrients were key to "soil exhaustion" and thus could be treated more like a math problem with the specific nutrients— mainly N—being applied to soils (e.g., Sutter, 2016). This shift in thinking was related to Liebig's law of the minimum and the discovery that "soil exhaustion" was actually attributable to loss of N rather than a fundamental change in the mineral components of the soil (Usher, 1923). Clearly, this understanding was fundamental to the increase in agricultural production in the 20th century, yet, there are major problems that have resulted. Now, nutrient cycling is better understood and how human interactions have fundamentally shifted nutrient cycles can be calculated. The next step is to better understand how the major nutrient cycles are connected and to work towards rebalancing the nutrient cycles in agricultural productions. Nutrient cycles and interconnectedness will be explored more in the "pedology-ecology" section.

The cascading negative ecological effects that result from excess fertilization are hard to ignore as an ecologist. However, as fertilization is undeniably necessary, the positive aspects of N fertilization are worthy of mention. One review noted that even studies that observe how fertilization affects soil structure conclude that the benefits of fertilization are

much greater than any negative effects (Bronick & Lal, 2005). Although generally N fertilization or deposition is associated with acidification of the soil (Geisseler & Scow, 2014), Trivedi et al. (2016) found in a meta-analysis that agriculture actually tends to be more alkaline than natural areas across the globe, possibly due to liming practices. Similarly, they find that N levels are not dramatically lower in agricultural areas compared to natural areas, which they postulate is due to N fertilization (Trivedi et al., 2016). These two points show that agricultural amendments do solve the local problem that they are intended to solve. Although it is crucial to mitigate the cascading effects and negative changes that occur, the fact that the amendments work in the first place should not be ignored.

In conclusion, 14 years ago, McLauchlan (2006) said, "a complete understanding of ecosystem change during and after agriculture is currently lacking," which is still a relevant statement. Although many questions remain, the future is hopeful.

Soil community implications

The results of this dissertation speak to the resilience of the soil community to various manipulations. This is further supported by previous research. For example, two studies showed that soil arthropods can shift their diet but maintain abundance when fertilizers (N or N-P-K) are added (Gan et al., 2014; Lemanski & Scheu, 2014). Typically, the soil has more diversity than corresponding terrestrial communities (Bardgett et al., 2005; Wardle, 2006), which is interpreted to relate to ecological function. Setälä et al. (2005) describe "the high degree of generalism—even omnivory—in resource use among decomposer organisms" as a reason there seems to be little interspecific competition, and that relates to resilience to perturbation.

This dissertation could not completely address diversity due to the difficulty in identifying soil organisms to species (e.g., Sylvain & Wall, 2011), but the results still support the research that states that the soil community is complex. Although some evidence suggests that diversity of the soil community is important, a few soil functions seem to be organism-specific (Bender et al., 2016; Setälä et al., 2005). Most soil biota do not follow the intermediate disturbance hypothesis and maintain a high level of diversity until essentially catastrophic disturbance severely reduces the diversity (Bender et al., 2016; Wardle, 2006). This highlights the crucial need for more research.

Although finding plant-soil feedbacks in the switchgrass-AMF system would have been a clear explanation for some of the resilience of switchgrass, and could have provided further information on the relationship of AMF to other soil community members (Baumgarten, 2020c), the unclear results are not unexpected. Porazinska et al. (2003) found in a review of aboveground-belowground interactions a "lack of clear responses of soil variables to plant community traits" and note that "generalized plant and soil diversity effects are hard to predict." This, too, highlights the crucial need for more research.

At larger scales, some interesting patterns have been identified. Trivedi et al. (2016) found in a meta-analysis that there was higher microbial diversity in agricultural systems in arid and temperate regions as compared to natural areas, and slightly lower but non-significant differences in continental and tropical regions. They explained that this surprising result does not contradict other research (e.g., Wallenstein et al., 2006) that suggests endemic microbes are being lost due to human activity. Fluctuating nutrients in agricultural systems allows greater local diversity but at broad scales agricultural areas are becoming more homogenized (Trivedi et al., 2016). Turner et al. (2019) studied a famous chronosequence and convincingly showed that the microfauna communities of bacteria, archaea and fungi

were decoupled from each other, and responded independently to soil conditions. Yet, in France, soil community members were found to correlate via abundance with vegetation type (meadows vs. forest vs. cropland) with lower abundance in cropland, and also some agricultural methods (i.e. fertilization intensity) caused predictable shifts in some groups of the soil community (Cluzeau et al., 2012). Similar conclusions, i.e. that land use explains soil community patterns, have been supported in other research (e.g., Bainard et al., 2014).

Part of the complexity of the soil system is that interactions occur on the micro- and macro- scale, and understanding and synthesis must be achieved across these vastly different scales. In the field experiment (Baumgarten, 2020b), switchgrass roots and AMF hyphae certainly extended beyond the plot edge, which could confound the results. Jach-Smith and Jackson (2018) noted that: "extensive evidence indicates that hyphal networks can share resources belowground among individual plants." Yet, fungi are treated as microorganisms in many studies, such as those that look at bacteria:fungi ratios. Soil microbes exist in films on the surface of soil solids, and one gram of grassland soil may have essentially 20 m^2 of surface area (Young & Ritz, 2005). Soil C alone is complex and heterogeneous, with spatial variety at all scales from micro (colloids) to macro (landscape) and varying from surface down the profile (Hopkins & Gregorich, 2005). Even at a so-called "basic" level, soil properties are constantly changing because the soil is constantly becoming wetter or drier, and that has physical and chemical implications (Young & Ritz, 2005). For instance, nematodes can move 100 mm in one hour if water film conditions are ideal, but in higher water concentrations (thicker water films), they move between 5 and 25 mm, and if there is no water, they cannot move at all (Young & Ritz, 2005). Even at a more human-size-level, soil measurements are very localized; i.e. Weaver et al. (2004) mapped the pH buffering capacity of three fields in Georgia using 2 x 2 m^2 plots, and they found significant variation

on that small scale. Although the work was not ecologically focused, pH buffering capacity relates to the availability of nutrients in soils and therefore is a measure that would impact the soil community as well as the plant community.

The complexity of scale leads nicely into the topics of this final section: how best to understand this complex environment when micro-scale interactions that clearly can impact large scale patters do not directly seem to drive soil and plant ecological communities.

"Pedology-ecology" and thoughts on nutrient cycling

The main question here is how to synthesize the knowledge that micro-scale interactions are controlled by the atomic structure of clay particles, yet soil texture does not seem to be a direct driver of soil and plant ecological communities. The phrase "pedology-ecology" is used to suggest this disconnect is an area worthy of future research.

The result that soil type is the most dominant factor in the three-soil experiment (Baumgarten, 2020d) could be interpreted to mean that soil pedology is more important than has previously been assumed. Yet, that contradicts some vast field experiments that conclude that soil type does not drive soil communities (Bainard et al., 2014; Cluzeau et al., 2012). Thus, although soil structure is important, and it seems to be driving reactions in the greenhouse experiment, why does that not match field results? Kulmatiski et al. (2017) highlight some general issues with greenhouse experiments being used to study plant-soil feedbacks, especially soil sterilization. What changes when soil is brought into a manipulated-greenhouse setting? Could soil texture become more important because of the homogenization process? Another ecological plant study manipulated field soil for a greenhouse experiment and found the only factor that related to plant growth was if gravel was integrated into the soil (Perzley, 2018), which could be explained by soil texture

becoming more important after the soil has been manipulated and installed in a greenhouse. One of the first points made in a soil diversity textbook by Young and Ritz (2005) is that "soil is not a bed of aggregates." Does this statement describe the disturbance to the whole soil system when field soil is homogenized and redistributed, that the soil has been disrupted into separate small entities and needs time to reform the interconnected matrix that actually best represents what soil is to the soil community? The results from Chapter 4 (Baumgarten, 2020d) lead to the question: under what conditions do soil texture and pedology matter more than other drivers of plant and soil ecological communities?

As previously referenced (Baumgarten 2020a, d), the interplay of the cycles of major nutrients does inform and structure ecological understanding. It is well proven that if C is added, then N needs of the soil community increase, thus C:N ratios have been commonly incorporated into research (van der Heijden et al., 2008; Weil & Brady, 2017). Similarly, it is now taken as fact that if N is applied to P-limited soil, then plants become more P-limited (Jach-Smith & Jackson, 2018). Coleman et al. (2014) describe part of the relationship of the C- and N- cycles as thus: " […] a positive feedback can occur wherein plants and the soil food web feed off one another's excess and waste. Under this scenario, plants exude excess photosynthate in the form of labile C substrates from their roots, which in turn are utilized by microbes and subsequently as food for microbivorous invertebrates, which release nitrogenous waste that is utilized by plants." Even at the cellular level, interactions are dominated by cycles. Plant roots take up nutrients as cations and anions, and must maintain the proper charge in their cells, so they ultimately release the same charge that they took up, which is the explanation for N fertilizer resulting in soil acidification (Weil & Brady, 2017). Similarly, it is not a debate that soil colloids have an impact on fertilizer efficiency and the

ability of plants to uptake nutrients (Weil & Brady, 2017); any debate is about how the cellular level interactions scale up to applications at a field-level.

The key point is that even in the context of plants utilizing nutrients, it is easy to envision a one-way path rather than a part of a cycle. Is the cyclical nature and the fact that every single action in the ecological world is not isolated but is part of a cycle being minimized? Are commonly used measurements and current statistical analysis capable of fully representing the interconnectedness of nutrient cycling?

Conclusion

I have to take a personal tone and approach to this conclusion.

At the beginning of this process, I thought that certain parts of ecology could be used and studied as a proxy for other environmental factors. However, my current fundamental take away from this work is that nothing can be studied in isolation. From studying the soil community as a complex whole to studying N-cycles in connection to C- and P-cycles, to measuring pH and considering the average rain amount for each site, all of these factors have to be integrated in future research to further our understanding. The future of ecology is in this massive challenge to integrate all of these things that are hard to measure.

My idea of the interconnectedness of nutrient cycling posits that perturbation of the cycles necessarily results in a rebalancing of the system, and the multifaceted effects of this are not being captured fully, which explains some of the varying results found in the literature. This idea of balance and cycling fits nicely with the concept of soil formation. The five factors of soil formation (parent material, climate, biota, topography, time) interact to

create soils that are very specific to their locality. Could not nutrient cycles be similar? I hope to further refine this idea in future research.

I realize that saying, "we are forgetting the cyclical nature of life" is but a different way of flattening the complexity of all of this. Similarly, it downplays the fact that there is no other way to have gotten to this point of knowledge where we currently exist (metaphysically); minimization and generalizations are incredibly necessary to build a foundation of knowledge. However, the main challenge with this conclusion, that the cyclical nature of life must be better understood, is that any attempt to further synthesize the complexity will be an extensive undertaking.

Further directions that I propose range from individual endeavors to those that require collaborations between many. The concrete directions that can be driven by individual effort include more cross-disciplinary collaboration; focusing on soil conservation and balance in agricultural production; and including basic measures of the soil, like pH, in ecological research. The more ambitious and collaborative directions include: increased soil arthropod and nematode expertise; statistics that can better handle multivariate data and sampling through time; and synthesizing the disconnect between the micro- and macro-scales.

The questions I am left with after all this are unending and exciting. But the two challenges that I would address tomorrow (if funding allowed) would be to study the impact of taking soil from field to greenhouse, and ensuring that multiple soil-nutrient measurements are integrated into ecologically focused plant and soil community research endeavors.

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