PERENNIAL FUEL, FEED, AND CEREAL: HIGH DIVERSITY PERENNIALS FOR BIOFUEL AND INTERMEDIATE WHEATGRASS FOR GRAIN AND FORAGE

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ABSTRACT

Perennials may counteract negative effects of annual agriculture, such as carbon release, water pollution, and erosion and systems are being developed that supply fuel, feed, and cereal. One source of fuel is perennial cellulosic ethanol, and one source of feed and cereal is intermediate wheatgrass. Regarding cellulosic ethanol, markets do not currently exist, making adoption of these systems largely a matter of carbon storage benefit. Regarding intermediate wheatgrass, little is known about its nitrogen balance and reproductive morphology, complicating long-term management.

In the perennial cellulosic ethanol system, I measured aboveground biomass, change in total soil C, soil microbial biomass, and extracellular enzyme activity with and without nitrogen in four treatments ranging from 1-24 planted species at four sites across Minnesota. I found no overall trends, possibly due to variation across sites or due to hands-off management. Over time, soil carbon increased in the shallower depths at one site and decreased in the deeper depths at two sites, possibly due to priming.

I measured plant, tiller, and rhizome densities in plants from sown seed, vegetative propagation, or seed shatter at four sampling times in 1 year old and 2 year old intermediate wheatgrass stands. Tiller density was similar in both stands, but rhizome and propagule densities were greater in the 2 year old stand. Likely, tiller replacement and death rates are equal, but vegetative propagation increases between years, increasing plant population, possibly leading to competition and affecting long-term yield.

Also in intermediate wheatgrass, I measured nitrogen in shoot, root, and grain tissue along with soil mineral and mineralized nitrogen in three nitrogen treatments (80 kg N ha^{-1} in spring, 40-40 kg N ha^{-1} in spring and summer, and unfertilized control) at four sampling times in 1 year old and 2 year old stands. The spring treatment had greater root nitrogen, but it also had greater lodging. The late fall sampling had the greatest soil nitrogen, and since soil mineral N was low at that time there was likely an influx of

organic nitrogen, likely due to root turnover.

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PREFACE

Annual row crop systems, such as corn, wheat, and soybeans, dominate agricultural land globally. By their nature, these systems leave soil uncovered for a portion of the year, failing to maximize photosynthetic capture and leaving the soil vulnerable to erosion. While the soil is bare, there are no living roots present to assimilate excess agrochemicals and reactive nutrients, leaving subsurface and surface water vulnerable to nonpoint source pollution. In between annual crop cycles, many producers choose to implement tillage. Tillage has the benefit of exposing a greater surface area of soil to solar radiation, allowing surface soil to dry and warm quickly for enhanced seedbed conditions. However, exposing a greater surface area of soil to the oxygen also allows more microbes consume organic matter and release carbon dioxide into the atmosphere. The loss of organic matter is detrimental because it promotes cation exchange capacity, nutrient storage, water holding capacity, and both microbial and plant life.

Perennial systems are known to counteract the negative consequences of annual row crop production. Because perennial plants are on the landscape for the entire year, they can utilize sunlight that is available outside the annual growing season, fixing carbon for a greater portion of the year. The deep root systems of perennial crops allow them to store a substantial amount of carbon belowground, hold soil in place and resist erosion, and effectively capture soluble nutrients like nitrate, which could otherwise pollute the groundwater. Since perennials live year after year, their continued production does not require tillage. Tillage can be implemented before and after perennial production, and strip tillage can be implemented during perennial production, but there are fewer opportunities to implement tillage in a perennial system than in an annual rotation. In

general, perennial production leads to greater soil organic matter, higher soil microbial biomass, greater water infiltration, less erosion, and greater overall soil health.

Most cropland in the United States is covered with annual crops. The most economically important perennial crop in the U.S. is alfalfa, although pasture, fruit production, nut production, and asparagus are also economically important perennials. Cellulosic biofuel production using crops such as switchgrass has been one option discussed to increase perennials on the landscape, however the current political and economic environment does not incentivize cellulosic biofuel production. Some horticultural crops, such as asparagus, ginger, fruits, and nuts are perennial. New crops are also being domesticated that are perennial analogues to annual row crops, such as intermediate wheatgrass, perennial rice, perennial flax, and a perennial sunflower. These crops have the potential to offer consumers an alternative to products made with annual row crops.

As there are not currently market-ready perennial analogues for most annual crops, perennials cannot yet replace annual row crop systems. Moreover, where there are perennial alternatives, grain yields are typically much lower than their annual counterparts, meaning more land would need to be converted to agriculture to maintain the same output. However, perennials can be strategically placed on the landscape to maximize their benefit. For example, karst soil is particularly ineffective at filtering out pollutants, so adopting perennials in karst regions may protect the underlying groundwater from soluble nutrients and chemicals. Similarly, planting perennial buffers along riparian areas may prevent sediment based pollution, such as phosphorus, to surface waters. There are also marginal lands not well suited for annual production, for

example steep hillsides prone to erosion or low-lying areas prone to flooding. While some of these lands may be currently planted to annual crops, their yields may be much lower and/or the externalities of production on such land may be much greater. Substituting perennials for annuals on sloped land will reduce erosion by holding soil in place and capturing soil transported in runoff. Adopting perennials on low lying land will increase infiltration and evapotranspiration, minimizing the effects of flood. Moreover, many perennial species are drought and flood tolerant, likely due to their large root systems capable of accessing and storing resources deeper down in the soil profile. Because of their suitability for marginal lands, perennials present an option for farming agricultural land that might not otherwise be in production, reducing the need to intensify production. Lastly, with premiums on grass fed meat and dairy, substituting annual row crop based feed for perennial grassland based feed is economically viable for many ranchers and graziers.

As new perennial crops are developed, several challenges have emerged as especially difficult or unique to perennial systems. With no annual tillage to kill weeds, other strategies need to be developed in perennial systems. Likewise, disease management is especially crucial without a fallow season to reduce pathogen pressure. In the same vein, because there is no rotation, perennial monocultures are less temporally diverse than many annual systems. Without spatial or temporal diversity, nutrient cycling may become imbalanced. Over time, yield of some perennial stands start to decline, yielding less grain year after year. Finally, perennials can store nutrients from one season to the next, making it more difficult to manage nutrient availability and ensure efficient use in the system.

For every challenge to perennial production, there is a role research can play in ameliorating said challenge. To combat the lack of temporal diversity in perennial systems, bicultures and polycultures are being explored, adding in spatial diversity. In order to better understand nutrient cycling, researchers can study translocation and deep soil nutrient uptake. Lastly, because much of the benefit of perennials comes from them being long-lived, longevity is key in maximizing perennial benefits. To understand longevity, researchers can study reproduction and growth patterns in perennial stands, looking for clues as to how seed yield is declining by studying the life cycle of the plant.

My research seeks to address 1) the potential benefits of diversity, 2) nitrogen cycling, and 3) reproduction and growth in two perennial systems, ethanol production and intermediate wheatgrass improved for grain production. To explore the potential benefits of diversity in a perennial cellulosic ethanol stand, I sampled soil carbon, soil nitrogen, extracellular enzyme activity, soil microbial biomass, and aboveground biomass production in 12-13 year old stands planted with 1-24 species at four sites across Minnesota. In order to understand nitrogen cycling in improved intermediate wheatgrass, I observed the mineral nitrogen, nitrogen mineralization, deep soil nitrogen, root tissue nitrogen, shoot tissue nitrogen, and grain nitrogen at four sampling times in a one and two year intermediate wheatgrass stand fertilized with 1) 80 kg N ha^{-1} in the spring, 2) 40 kg N ha^{-1} in the spring and 40 kg N ha^{-1} in the summer, or 3) no nitrogen. To understand reproduction and growth, I monitored population along with tiller production, spike production, and rhizome production on plants grown from sown seed, plants originating from rhizomatous tissue, and plants originating from seed uncollected from the previous harvest in a one year old and two year old intermediate wheatgrass stand. The goal of my

research is to ultimately increase the adoption of these systems by furthering our understanding of the benefits of perennials, how to manage them, and how to increase the time in between tillage events.

CHAPTER 1

Species diversity and N fertilization have inconsistent long-term effects on soil carbon in bioenergy cropping systems

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Synopsis

Conversion of agronomic systems from annual to diverse perennial species offers myriad ecosystem services, but soil carbon storage benefits remain inconclusive. Our objectives were to study the long-term effects of species composition and nitrogen (N) fertilization on 1) aboveground biomass yield, 2) change in total soil C, 3) soil microbial biomass C, and 4) extracellular enzyme activity (EEA) from experimental plots established 11 and 12 years prior to sampling. Species mixture treatments included a switchgrass (*Panicum virgatum* L.) monoculture (SG), a four species grass mixture (GM), an eight species legume/grass mixture (LG), and a 24 species high diversity forb/legume/grass mixture (HD). Each species composition plot was grown with and without N fertilizer, without control of weeds or planted species composition. The experiment was replicated at each of four Minnesota locations: Becker, Lamberton, Roseau, and Waseca. There were no consistent ecosystem responses across locations to either species mixture or nitrogen fertilization. Species mixture had limited and inconsistent effects on aboveground biomass yield, which averaged 5.8, 4.8, 5.5, and 5.1 Mg ha^{-1} at[1] Becker, Lamberton, Roseau, and Waseca, respectively. Across all species

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Abbreviations:
SG = switchgrass monoculture
GM = four species grass mixture
LG = eight species legume/grass mixture
HD = 24 species high diversity forb/legume/grass mixture

mixture treatments, N fertilization increased aboveground biomass at Becker, a sandy, nutrient limited soil. The responses of soil carbon dynamics were also not consistent. The change in total soil C from 2013-2017 increased in the shallower depths at Becker while the total soil C decreased in the deeper depths at Becker and Lamberton, perhaps as a result of soil priming from the introduction of deep rooted perennials. Microbial biomass C, cellobiohydrolase activity, and N-acetyl-glucosaminidase activity were unaffected by species mixture treatment, N fertilization, or their interaction at any location. β-glucosidase and phosphatase activities responded to species mixture treatments at Waseca and Roseau, respectively. Our inconsistent findings highlight the importance of considering local soil and climatic conditions when managing for biomass yield or carbon storage. The lack of overarching species mixture effects suggests that diverse polycultures may be as effective at providing biomass yield and soil carbon benefits as monoculture species such as switchgrass, at the same time that diverse polycultures provide myriad other ecosystem services.

1. Introduction

Perennial cropping systems are increasingly studied for their capacity to balance provisioning services – such as biofuel, animal feed, and natural products – with regulating and supporting services – such as carbon sequestration and nutrient cycling (Asbjornsen et al., 2014; Crews & Rumsey, 2017; *FAOSTAT*, 2019; Werling et al., 2014). Currently, only 19% of the world's cropland is in perennial production (Cox, Glover, Van Tassel, Cox, & DeHaan, 2006). Annual crops are only grown for a portion of the year, exposing soils to potential degradation from water and wind. Meanwhile, perennials cover the soil year-round and have greater net primary productivity than annuals (Jarchow & Liebman, 2013) as they capture more photosynthetic radiation over the course of a year (Crews & DeHaan, 2015). This greater net primary productivity of sustained perennial systems translates into a greater capacity to sequester carbon (Crews & Rumsey, 2017; Lal, 2004), foster microbial communities (S. W. Culman et al., 2010), and recycle nutrients (Crews, 2005). Even in small, targeted areas, native perennial systems have effectively delivered multiple benefits to owners of annual cash crop cropland (Schulte et al., 2017).

Carbon storage is a particularly important global goal of agricultural systems as it has the capacity to mitigate climate change and promote soil fertility, further supporting food production and environmental quality (Lal, 2004; Powlson et al., 2011). The increased carbon storage of perennial systems may be maximized by increasing species and functional group diversity in long-lived stands (Fornara & Tilman, 2008). In a 12-year Minnesota study, for example, carbon sequestration was increased from 193% to 522% when two additional functional groups (C_4 grasses and legumes) were added to a

perennial system (Fornara & Tilman, 2008). Additionally, grasslands with a greater number of species from diverse functional groups may have greater net primary productivity (Loreau et al., 2001), which can further support the benefits of carbon sequestration. While the relationship between diversity and productivity has been studied in perennial systems, little work has been done in the context of agricultural land converted from annual, intensely managed production to perennial, low input production. While greater species richness can lead to greater aboveground biomass in unmanaged systems (Loreau et al., 2001), less diverse systems in an agronomically manged context, particularly switchgrass (*Panicum virgatum* L.) monocultures, have been shown to yield similar or more aboveground biomass than higher diversity plantings (Jacob M. Jungers, Clark, et al., 2015; Zilverberg et al., 2014). These findings are particularly relevant to multifunctionality because increased aboveground biomass is not just an indicator of greater provisioning services such as harvestable material – it can also imply greater regulating and supporting services such as carbon storage.

Soil microbial biomass carbon and microbial decomposition activity are more responsive to management than other C pools and can therefore be used as an early indicator of changes in soil organic matter dynamics (Joergensen, Anderson, & Wolters, 1995; Mohammadi, Heidari, Khalesro, & Sohrabi, 2011). Previous work on tallgrass prairie species has not found an effect of species richness on extracellular enzyme activity (EEA; Docherty & Gutknecht, 2019), but it has been shown that 1,4-N-acetylglucosaminidase increases with species richness (Chung, Zak, Reich, & Ellsworth, 2007). Similarly, increasing plant diversity in perennial systems increases soil microbial biomass (Chung et al., 2007; Wardle, Bonner, & Nicholson, 1997). Recent efforts to

synthesize the mechanisms of soil carbon storage also suggest that microbial biomass and extracellular enzyme pools are the key pools of long-term stored organic carbon (Dwivedi et al., 2019; Robertson et al., 2019). Because of this, a broader analysis of species composition effects on microbial biomass and activity across different conditions may provide greater insight regarding when, and whether, species composition leads to greater carbon storage in perennial agricultural systems.

Soil microbial biomass and activity, soil carbon, and biomass productivity are important outcomes in perennial systems. There is evidence that these outcomes may be influenced by diversity level and nitrogen addition. Our objective was to determine the effects of species mixture (i.e. the number of species and functional groups of each seed mixture) and N fertilization on aboveground biomass yield, change in soil C, microbial biomass C, and extracellular enzyme activity 11-12 years after conversion of agricultural soils to a series of native perennial grassland compositions.

2. Materials and Methods

2.1. Site description and experimental design

The experiment was established in 2006 and 2007 for the purpose of understanding biomass yields across species compositions in an agronomic management setting (Mangan et al., 2011). Data used for this study were collected from four of the nine original experimental locations; Roseau, Becker, Lamberton, and Waseca, Minnesota (Table 1, Mangan et al., 2011). Treatments were arranged in a randomized complete block design with split plots and three replications per location. Main plots were fertilized with either 0 or 60 kg N ha^{-1} yr^{-1}, manually broadcast in the form of ammonium nitrate each spring starting in 2008. Sub plot treatments originally established (Mangan et al., 2011) were species mixture treatments seeded as switchgrass monoculture (SG), four species grass mixture (GM), an eight species legume/grass mixture (LG), and a 24 species legume/grass/forb mixture (HD; Table S1). Some species substitutions were made for the northernmost site, Roseau, to accommodate ecosystem and climatic adaptation (Table S1).

2.2. Soil collection

Soil was collected in 2013 using a hydraulic soil probe (Giddings Machine Company, Inc.; Colorado USA) at all sites except Roseau (Jungers et al., 2015). Two cores from each plot, partitioned into 0-15 cm, 15- 30 cm, 30-60 cm, and 60-90 cm increments, were homogenized in the field. Soil was dried at 60°C until constant weight for total C analysis via combustion analyzer.

Soil was collected again in October 2017 for this current analysis using a

hydraulic soil probe to a depth of 90 cm at Becker, Waseca, and Lamberton and to a depth of 60 cm at Roseau, where conditions prohibited deeper collection. Two cores from each plot, partitioned into 0-15 cm, 15-30 cm, 30-60 cm, and 60-90 cm increments, were homogenized in the field. A subsample of each depth was immediately frozen for extracellular enzyme analysis, and a subsample was kept at 4°C until microbial biomass could be determined. A subsample was also dried at 60°C until constant weight for total C analysis. To determine the effect of treatments on change in soil C over time, we subtracted total soil C measured in 2013 (Jungers, et al., 2015) from final total soil C measured in 2017.

2.3. Microbial biomass carbon

Microbial biomass C was determined by direct chloroform extraction using 0.5 M K_2SO_4 (Gregorich, Wen, Voroney, & Kachanoski, 1990; McDaniel & Grandy, 2016). For each soil sample, pairs of 10g +/- 0.05g sieved (4mm) subsamples were weighed out into 70 mL acid washed test tubes. Gravimetric soil moisture was determined on a third 10 g sieved sample. In one sample from each pair, 1.0 mL chloroform was added, followed by the addition of 40 mL 0.5 M K_2SO_4 to both samples of each pair. All samples were then shaken at 250 rpm on an oscillating shaker for four hours. After sediment settled, the supernatant was poured through rinsed #42 filters into acid washed specimen cups, then transferred into labeled, unused scintillation vials. Extracts were immediately frozen until later analysis on a TOC analyzer (TOC-V-CPN; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). Vials containing extracts from the Becker location were broken, and the entire site was extracted again from frozen soil. Although determining soil

microbial biomass from frozen soil is not recommended, it has been shown that there is no difference in microbial biomass determined on frozen versus fresh soil in conditions similar to ours (Winter, Zhang, Tenuta, & Voroney, 1994). Sites were not statistically compared to each other, and all treatments at Becker were treated similarly for within site comparisons.

2.4. Extracellular enzyme analysis

Soil for extracellular enzyme analysis was kept frozen until the day of the assay. To determine enzyme activity, the protocol outlined by German et al. (2011) was followed. In short, 0.5 g +/- 0.01 g soil was added to 50 ml of 50 mM pH 6.0 tris buffer. The soil slurry was sonicated, shaken vigorously, and 200 µl of slurry was added to 96 well plates in eight replicate wells. The activities of β-glucosidase, N-acetyl-glucosaminidase, cellobiohydrolase, and acid phosphatase were determined by adding 50 µl of 4-methylumbelliferone linked enzyme substrates at saturating concentrations based on pre-optimization testing before the actual assay was performed (German et al., 2011). Controls and standards included tris buffer alone (blank), substrate or soil slurry with tris buffer (negative controls), 4-methylumbelliferone with soil slurry (quench standards), and 4-methylumbelliferone with buffer (reference standards). After an hour of incubation at room temperature, 10 µl of NaOH solution was added to each well. Fluorescence was measured a minute after NaOH addition on a microplate fluorometer with excitation filters at 365 nm and 450 nm. Extracellular enzyme activity was calculated as nmol substrate cleaved (as determined by fluorescence) per gram of soil (oven dry equivalent) per hour.

2.5. Aboveground biomass harvest

In October of 2017, aboveground biomass was harvested to a height of 5 cm. Two randomly placed 0.25 m^2 quadrats were used per plot. Plant material was stored at 4°C until separated by species and dried at 60°C until constant moisture before weighing.

2.6. Statistical Analysis

Statistical analysis was conducted in R (R Core Team, 2016) using the nlme package (Pinheiro J., Bates D., DebRoy S., 2019). Species mixture and N treatments were considered fixed effects, and because of the split plot design, N fertilization was also considered a random effect nested within block. Since responses typically varied by location and location interacted with species mixture treatment and/or N fertility in several cases, each location was analyzed separately, and sites were only compared qualitatively to each other. To determine the effect of time on change in total soil C, 2013 values were subtracted from 2017 values. Total soil C change was determined by assessing the significance of the intercept compared to 0.

3. Results

Variability in species mixture treatment effects on aboveground biomass yield, soil C, and soil microbial responses was large across locations. This variability can be attributed to the wide range of climatic conditions (Table 1), soil characteristics, and final plant species composition of treatments in 2017 (Table S2). There were no consistent treatment effects across locations for any response variables (Table 2).

3.1. Aboveground species composition and biomass yield

Of the seeded grasses, switchgrass and big bluestem (*Andropogon gerardii* Vitman) were most consistently present at all locations at the end of the trial. Switchgrass was as present in monoculture and mixtures in variable amounts at all locations. It constituted 70-98%, 36%-98%, and 1-95%, in monocultures, grass mixtures, and legume/ grass mixtures, respectively. Big bluestem was present to a lesser extent. It constituted 1-82% and 0-56% big bluestem in GM and LG treatments. respectively Table S2). Legumes constituted a very small fraction (>1%) of the LG and HD mixtures except at Waseca where wild blue indigo was present in a significant amount (12- 33%.). Likewise, seeded forbs contributed little to yields of HD mixtures except for wild bergamot at Waseca were it contributed 18 and 40% respectively to the -N and +N treatments. Small amounts of invasive forbs and grasses were present in most treatments, but their presence was location dependent. Canadian horseweed made a significant contribution to the GM+N treatment at Lamberton and the LG+N treatment at Waseca, and stiff goldenrod constituted 8% of the LG+N treatment at Lamberton.

There was no effect of species mixture treatment on aboveground biomass yields

at Becker, Lamberton, and Roseau (Table 2), where yields averaged 5.8, 4.8, and 5.5 Mg ha^{-1}, respectively. There was an interaction between species mixture treatment and N fertilization on aboveground biomass yield only at Waseca, because within mixture treatment yields were siliar for the –N and +N treatments, the unfertilized HD treatment produced more than the unfertilized GM (Figure 1, Table 2). Nitrogen fertilization affected aboveground biomass yield only at Becker (Table 2), where N increased yields by 34% averaged across species mixture treatments.

3.2. Final soil fertility and total soil C

Soil pH, K and P levels in 2017 varied strongly by location, with Lamberton generally having the highest nutrient levels. There was a significant range in soil K level- 131, 558, 96, and 202 ppm at Becker, Lamberton, Roseau, and Waseca, respectively. Values for pH were 5.9, 6.2, 6.8, and 6.1 at Becker, Lamberton, Roseau, and Waseca, respectively. Species mixture treatment and the interaction of species mixture treatment with N fertilization had no effect on these soil properties, but soil parameters were different among N fertilization treatments at all locations. In N fertilized plots at Becker, soil pH was lower, while in N fertilized plots at Lamberton, soil pH was greater than in non-fertilized plots (Table 3). In N fertilized plots at both Becker and Lamberton, soil K was greater than non-fertilized plots, while at Roseau and Waseca, soil in fertilized plots was lower in K than in non-fertilized plots (Table 3). At Becker, Roseau, and Waseca, N fertilized plots had lower soil P than non-fertilized plots (Table 2, Table 3).

Total soil C levels and the trends over depth varied by site (Figure 2). At Becker, Lamberton, and Waseca soil C decreased with depth while at Roseau, soil C varied

considerably with depth and was greater at 45 cm depth an at other depths. Species mixture treatments only affected final total soil C at Roseau in the 30-60 cm depth, where GM and LG treatments had greater total soil C than SG (Table 2, Figure S1). N fertilization decreased soil C in the 30-60 cm depth at Roseau (Table 2).

3.3. Change in total soil C from 2013-2017

We only observed significant changes in soil C from 2013-2017 at Becker, the site with the lowest initial C content, and changes in soil C were observed at each depth (Table 2, Figures S2-S4). At the 0-15 cm and 15-30 cm depths in Becker, soil C increased by 0.34% and 0.48% from 2013 – 2017 (Figure 3). At the 30-60 cm depth in Becker and the 60-90 cm depth in Lamberton, total soil C decreased significantly by 0.09% and 0.25% from 2013 – 2017 (Table 2). Species mixture treatment, N fertilization and their interaction affected the change in soil C at 30-60 cm at Becker (Table 2). At the same time, N fertilization increased total soil C at Becker in the 30-60 cm depth across species mixtures. The soil C did not change at any depth at Roseau or Waseca.

3.4. Soil microbial biomass C and extracellular enzymes

Microbial biomass C was not affected by species mixture treatment or N fertilization at any location (Table 2). Microbial biomass C was 993 (SE = 642), 318 (SE = 21), 1078 (SE = 285), and 113 (SE = 92) μg C g^{-1} at Becker, Lamberton, Roseau, and Waseca, respectively. There were limited and inconsistent effects over locations, species mixture treatment, and N fertilization on EEA (Table S3). At Waseca, the grass mixture had higher β-glucosidase activity than any other mixture (Figure 4, Table 2). At Roseau,

phosphatase activity was greater in the high diversity treatment than the grass mixture treatment (Figure 4, Table 2). There were no species mixture treatment or N fertilization effects on EEA at Becker and Lamberton.

4. Discussion

Across measurements of carbon sequestration in this experiment, from aboveground biomass yield to soil total and microbial biomass carbon, we found inconsistent responses to plant species mixture and N fertilization treatments, emphasizing the importance of local soil and environmental conditions on carbon related ecosystem services. In this experiment, plant species composition was not manipulated after initial establishment of target species mixture treatments, and final plant species compositions changed differently across sites (Jungers, et al., 2015). Switchgrass maintained stands in monoculture with minimal weed invasion and was the most persistent grass in mixtures with other grasses and forbs. Other grasses, legume and non-legume forb species failed to persist in legume and high diversity mixtures possibly raising issues about the long-term viability of diverse native plant polycultures for prairie restoration and CRP mixtures (Jewett et al., 1996). This variability in plant species composition over time likely introduced variability to total soil C and soil microbial properties. This study highlights how effects of species composition and N fertilization can be mediated by soil type and climate, and that predictions of grassland biomass yield, total soil C change, and soil microbial properties should not be generalized across environments.

4.1. Aboveground biomass yield

Overall, aboveground biomass yield did not respond to species mixture treatment or N fertilization. The lack of an overall response to N fertilization contrasts with previous research results that N fertilization ranging from 56 kg ha^{-1} to 100 kg N ha^{-1}

stimulates prairie aboveground biomass productivity in a range of locations (Jarchow & Liebman, 2013; Owensby, Hyde, Anderson, Owensby, & Hyde, 1970; Seastedt, Briggs, & Gibson, 1991). However, the response of prairie aboveground biomass productivity to N fertilization can be more limited in N abundant situations, and it has been hypothesized that N limitation is a key driver of how strongly a prairie responds to N fertilization (Seastedt et al., 1991; Wagle & Gowda, 2018). Similarly, there may have failed to be a response to N fertilization at sites where P was limiting. This could explain why only Becker – a nutrient poor, sandy soil – showed a productivity response to N fertilization in our study. As Becker had the lowest concentration of soil N at the surface (Table S4), perhaps a response to N occurred due to N limitation. Becker also had a lower organic matter content, which is also related to stronger responses to N fertility (Bu et al., 2015). Roseau also had low soil N (Table S4), however that site received very little rainfall in the summer of 2017 (Table 1), and response to N fertilization has been shown to be moisture dependent as well (Hooper & Johnson, 1999; Kordbacheh, Jarchow, English, & Liebman, 2019). In summary, both underlying soil characteristics and within season precipitation may determine the responsiveness of a given plant species mixture to N fertilization.

Although decades of research have shown that plant community productivity increases with diversity in natural plant systems, this relationship is inconsistent in agricultural systems. Specifically, there are many reports that perennial monocultures are more productive than high diversity perennial systems (Griffith, Epplin, Fuhlendorf, & Gillen, 2011; Johnson, Kiniry, Sanchez, Polley, & Fay, 2010; Landis et al., 2018; Sanford et al., 2016; Zilverberg et al., 2014), but others have found polycultures and more diverse

species mixtures are at least as productive as monocultures (Ilya Gelfand et al., 2013; Jacob M. Jungers, Clark, et al., 2015; Tilman, Hill, & Lehman, 2006), if not more productive (Jarchow, Liebman, Rawat, & Anex, 2012; Picasso, Brummer, Liebman, Dixon, & Wilsey, 2008). At most locations in our study, switchgrass and, to a lesser extent, big bluestem – both highly productive C_4 grasses – dominated most plant species mixtures in this experiment (Table S2). Their high productivity could have compensated for any increase in productivity associated with increased diversity. Lastly, it has also been found that C_4 grass mixtures with legumes are similarly productive as high diversity mixtures (L. R. DeHaan, Weisberg, Tilman, & Fornara, 2010), corroborating our findings that the legume/grass mixture had similar yields as other treatments.

In addition to finding higher diversity plantings to be of equal productivity to lower diversity plantings, there was one instance of a higher diversity planting yielding more than a lower diversity planting. At Waseca, the unfertilized high diversity mixture had higher aboveground biomass yield than the unfertilized grass mixture (79% C_4 grass) (Figure 1), demonstrating that high diversity, low input plantings have the potential to produce more aboveground biomass than low diversity, C_4 dominated stands, even in an agricultural context. It is possible that this was due to the presence of large forbs such as compass plant at Waseca (33% average in HD treatments), which were absent from other sites (Table S2). However, it was more often observed that high diversity plantings produced similar yields compared to the switchgrass monoculture, the four species grass mixture, or the eight species legume/grass mixture. Not only can higher diversity plantings have yields equal or greater than lower diversity plantings, they can suppress pests, provide pollinator forage, support bird population, and decrease greenhouse gas

emissions (Landis et al., 2018) and those benefits should also be considered by agricultural land managers.

4.2. Final soil fertility

It is likely that K levels were sufficient at most sites; however, P may have been limiting at some sites. Observed soil K levels spanned the medium (81-120 ppm) to very high (161 + ppm) range (Kaiser and Rosen, 2018). Soil P levels of less than 10 ppm, such as those observed at Roseau and Waseca, are considered insufficient for most plant species, although native grasses are noted for their adaptation to low soil P through mycorrhizal associations (Schultz, Miller, Jastrow, Rivetta, & Bever, 2001). While K and P addition has not been shown to affect prairie aboveground biomass yield, these nutrients are removed through harvest (Jacob M. Jungers, Sheaffer, & Lamb, 2015). Therefore, varying yields in previous years may have affected final K and P measurements. It is possible that removal was also compensated by mineralization.

4.3. Change in total soil C from 2013-2017

Overall, total soil carbon was lost or remained the same over time, and was unaffected by N fertilization or species mixture treatment. The loss of soil carbon from the 30-60 cm depth at Becker and the 30-60 cm depth at Lamberton observed may not have been biologically significant. Specifically, while statistically significant, a soil carbon decrease of 0.09% to 0.25%, such as that observed at the 30-60 cm depth at Becker and the 30-60 cm depth at Lamberton, is unlikely to be biologically significant. Assuming a bulk density of 1.5 (USDA-NRCS, n.d.), 0.09% soil C is 13.5 g C ha^{-1}. There is a linear relationship between carbon addition and prairie aboveground biomass, with

10 g C ha^{-1} leading to an increase in aboveground biomass yield of between approximately 0.015 and 1 kg ha^{-1}, depending on the year (Blumenthal, Jordan, & Russelle 2003). Thus, the observed soil carbon decrease would have led to a decrease in biomass productivity of less than 1 kg ha^{-1}.

Losses in deep soil (30-90cm) were observed across sites and treatments in this study (Figure 3). While is well established that perennial grasslands sequester soil C after 10-35 years (D. Fornara & Tilman, 2012; Hernández, Esch, Alster, McKone, & Camill, 2013; Kelly & Tieszen, 2004), there is evidence that, on agricultural soil below 30 cm, short and long-term high diversity perennial plantings do not store carbon, even when considerable carbon is stored throughout the overall profile as a whole (David, McIsaac, Darmody, & Omonode, 2009; Steinbeiss, Temperton, & Gleixner, 2008). Moreover, recent work suggests that perennials and cover crops do not store more carbon than annuals below 25 cm (Ye & Hall, 2019). Soil inorganic carbon release through priming and climate change could be driving the discrepancy between deep soil carbon in annual cropping systems and perennial plantings. Specifically, the conversion from conventional annual production to high diversity perennial plantings may release deep soil inorganic carbon (Cihacek & Ulmer, 2010; Jelinski & Kucharik, 2009). At the same time, soil carbon can be lost from the 0-15 cm depth at a rate of 0.6% yr^{-1}, regardless of land use, due to a warming climate (Bellamy, Loveland, Bradley, Lark, & Kirk 2005), so it is possible deep carbon is being lost as well. Removing carbon via harvest will likely exacerbate those losses. Thus, the lack of deep carbon storage observed in this study was likely a result of priming, climate change, and the removal of carbon through harvest.

The only location where we observed significant responses in change of total soil

C from species mixture treatment was Becker, the most nutrient limited site with comparatively low initial soil carbon. Our finding that the switchgrass monoculture lost more soil C at 30-60 cm depth than the grass mixture and the legume/grass mixture at Becker is contrary to previous findings that greater warm season grass cover results in greater soil C storage potential (Knops & Tilman, 2000; Spiesman, Kummel, & Jackson, 2018). It could be that at Becker, switchgrass primed the soil C loss more than other species. Previous findings for *Phragmites australis*, an invasive wetland grass, suggest that some grasses are uniquely suited for priming deep soil organic matter in certain situations (Bernal, Megonigal, & Mozdzer, 2017). In that case, 35% of CO_2 respired by *Phragmites australis* in a native system was from soil organic matter priming in the 40-80 cm depth, while only 9% of said CO_2 was from the 0-40 cm depth (Bernal, Megonigal, & Mozdzer, 2017), demonstrating that some grasses are uniquely suited for priming deep soil organic matter. Although soil organic C under switchgrass increases by 2.9 Mg ha^{-1} yr^{-1} in the top 120 cm as a whole (Liebig, Schmer, Vogel, & Mitchell, 2008), subsoil under a grass such as switchgrass may be more vulnerable to soil organic C losses and less likely to store carbon than topsoil (Bernal et al., 2016; Jia et al., 2017; McGowan, Nicoloso, Diop, Roozeboom, & Rice, 2019). At the 30-60 cm depth, switchgrass lost 9.7 Mg C ha^{-1} in one year (Sainju, Allen, Lenssen, & Mikha, 2017). Research is needed to confirm or refute the long-term capacity of switchgrass to affect subsoil C.

The increase in total soil C caused by N fertilization observed at 30-60 cm in Becker is partially in line with previous observations regarding N fertilization and soil organic C. While N fertilization has increased soil organic C by 4.8% in one case (Zhou,

Wang, Zheng, Jiang, & Luo, 2017), this effect was not seen below 30 cm (i.e. Forstner et al., 2019). This could be due in part to the fact that N has been shown to modulate carbon priming caused by the addition of soil organic C (W. Zhang, Wang, & Wang, 2013).

4.4. Soil microbial biomass C

Soil microbial biomass C did not respond to species mixture treatment or N fertilization. The lack of species mixture treatment and N fertilization is contrary to previous findings that higher diversity plantings lead to greater soil microbial biomass C (Zak, Holmes, White, Peacock, & Tilman, 2007). The biomass or relative abundance of different plant functional groups, however, could be more important than plant diversity alone in affecting soil microbial biomass C (Klopf, Baer, Bach, & Six, 2017). For example, grasses may foster more microbial biomass than other species mixtures (Liang et al., 2016; D. Wang, Lebauer, & Dietze, 2010). It has been suggested that changes in microbial biomass due to increased diversity are at least in part due to accompanying changes in above and belowground productivity (Klopf et al., 2017). Local environmental conditions, especially soil temperature and moisture, are also strong drivers of microbial growth and community composition and so care should be taken in interpreting results across our study locations. For example, we expected greater soil microbial biomass at Waseca, where fertile, heavy soils are dominant. However, conditions were dry at Waseca when sampling occurred, which likely lowered soil microbial biomass (Wardle, 1992). Given this caveat, previous results at our study sites demonstrated that lower diversity plantings had greater root biomass than higher diversity plantings (Jacob M. Jungers, Eckberg, et al., 2017). Thus, the increased microbial biomass associated with the

greater root biomass, aboveground productivity, and higher concentration of grasses in the lower diversity treatments likely balanced out the increased microbial biomass associated with the greater species richness of the higher diversity treatments.

4.5. Extracellular enzymes

β-glucosidase activity can be influenced by several factors including fertilization (Ajwa, Dell, & Rice, 1999; Bach & Hofmockel, 2015), environmental conditions (Gutknecht, Henry, & Balser, 2010), living roots, and presence of a green manure (Jain, Jat, Meena, & Chakraborty, 2018). However, we found no evidence of increased β-glucosidase activity due to N fertilization. It has been found that enzyme responses to fertilization can be relatively ephemeral in nature (Bach & Hofmockel, 2015), and perhaps our late fall sampling precluded the observation of any differences between fertilized and unfertilized plots.

β-glucosidase activity may have been higher in the relatively lower yielding grass mixture plots due to lack of adequate labile carbon. Greater β-glucosidase activity is associated with more recalcitrant biomass (Caldwell, 2006), which is common in grass dominated communities with high tissue C:N ratio. It is not clear why β-glucosidase activity was different between the GM and SG treatments given that both were largely dominated by switchgrass. It is also unclear why this response was only present at Waseca, one of our most fertile, high organic matter locations. Variability in species composition in previous years may have resulted in legacy effects on soil organic matter composition, which could have manifested into differences in enzyme activity at the time of harvest.

Phosphatase activity could have been greater in the Roseau high diversity

treatment due to the abundance of golden Alexander (*Zizia aurea* L.; S3), a forb which responds well to high phosphorus soil conditions (Ehrenreich & Aikman, 1963; Howe, 1999) and therefore may have high phosphorus demands. Increased plant demand for phosphorus is correlated with increased phosphatase activity (Caldwell, 2006; Dormaar & Willms, 2000; Hewins, Broadbent, Carlyle, & Bork, 2016; Jain et al., 2018). Golden Alexander was particularly abundant in the high diversity treatment at Roseau compared to other sites (Table S2), explaining why the response was observed at Roseau alone.

5. Conclusion

Our study harvested aboveground biomass annually, distinguishing it from ecological studies of soil C in diverse native prairies. Contrary to previous studies, we found no consistent responses across sites. As botanical composition, total soil C, and climate varied by location, it is not surprising that no consistent responses were seen across all four sites. Similarly, by mimicking realistic management practices, such as not hand weeding or replanting absent species, we may have increased variability, making it more difficult to detect differences between treatments. Species mixture treatments varying in diversity had mostly similar aboveground biomass, total soil C, soil microbial biomass, and nutrient cycling as indicated by extracellular enzyme activity. With no cost to productivity, high diversity perennial species mixtures have other documented ecosystem service benefits, such as pollinator habitat, nutrient cycling, and biodiversity and therefore may be multifunctional on agricultural land.

N fertilization also did not consistently affect aboveground biomass, soil C, soil microbial biomass, or extracellular enzyme activity in these mature perennial mixture plantings. In the sandy soil at Becker, N fertilization did increase aboveground biomass, yet a decline in soil C was also observed at this site. We found no evidence to support the practice of fertilizing mature perennial plantings and, given that N fertilizer can have adverse ecosystem effects, this practice likely has greater costs than benefits in this context.

There is still much to be learned about how N fertilization and productivity affect deep soil C priming in long-lived perennial stands. Long-lived stands pose a unique challenge to multi-site research, as differences in soil properties, such as soil carbon, over

time could cause notable differences in outcomes, such as aboveground biomass yield or soil microbial biomass. For example, soil carbon and aboveground biomass yield form a virtuous cycle, with additional soil carbon leading to greater aboveground biomass yield and therefore greater carbon inputs (Blumenthal et al., 2003). Future work should also focus on to what degree perennial species, such as switchgrass, prime the release of deep soil inorganic carbon, how that affects overall carbon budgets, and the role that succession plays in these dynamics.

Ultimately, soil and region-specific best management practices need to be developed for high diversity native plantings, in order to better derive their ecosystem services. When conducting diversity studies, differing planting regimes should be investigated as plant identity may play a role in whether increasing diversity increases productivity and carbon storage. Based on our findings, there is little evidence to suggest significant soil carbon storage occurs over five years of perennial adoption, therefore, strategies to increase soil carbon storage should focus on long-lived perennial stands.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1. Monthly precipitation and mean temperature for 2017 growing season and 30 year average

	April	May	June	July	Aug	Sept	Oct
	2017 precipitation						
	cm						
Becker	9.2	14.1	6.5	4.1	11.0	5.1	17.1
Lamberton	7.7	15.2	6.9	10.2	12.5	5.4	15.0
Roseau	0.9	0.3	2.7	0.1	0.0	9.8	0.2
Waseca	7.2	13.0	10.6	16.7	9.9	5.1	10.5
	30 yr mean precipitation						
	cm						
Becker	6.5	7.5	10.6	8.4	9.6	8.8	6.3
Lamberton	7.5	8.3	10.6	9.5	9.3	8.4	5.2
Roseau	3.3	7.3	11.2	9.6	8.0	7.0	5.7
Waseca	8.2	10.0	11.9	11.2	12.1	9.3	6.8
	2017 mean temperature						
	ºC						
Becker	7.8	12.7	19.0	21.2	18.1	16.9	8.4
Lamberton	8.4	13.6	20.7	22.4	18.8	17.7	9.4
Roseau	4.9	11.0	16.8	19.9	17.2	14.0	6.9
Waseca	9.5	14.3	21.2	23.1	19.1	17.7	9.8
	30 yr mean temperature						
	ºC						
Becker	6.9	13.6	18.7	21.3	19.9	14.8	7.6
Lamberton	7.2	14.6	20.1	22.1	20.6	15.8	8.6
Roseau	4.0	11.2	16.8	19.5	18.4	12.9	5.6
Waseca	7.8	14.8	20.3	22.2	21.0	16.3	9.0

Table 2. ANOVA significance results by site for aboveground biomass (AB), microbial biomass carbon (MBC), final soil properties at 0-15 cm, final total soil Carbon at four depths, change total soil carbon from 2013 to 2017 at four depths, and the activity of four hydrolytic enzymes at 0-15 cm

			Soil properties			Final total soil C				Change in total soil C '13 - '17					
Factor	AB	MBC	pH	P	K	0-15 cm	15-30 cm	30-60 cm	60-90 cm	0-15 cm	15-30 cm	30-60 cm	60-90 cm	B	C
Becker															
Species mixture											.	*			
N fertilization	*		**	*	*		.					*			
Mixture* N												*			
Lamberton															
Species mixture															
N fertilization				*	*										
Mixture* N															
Roseau†‡															
Species mixture								*	N/A	N/A	N/A	N/A	N/A		
N fertilization					*			*	N/A	N/A	N/A	N/A	N/A		
Mixture* N									N/A	N/A	N/A	N/A	N/A		
Waseca															
Species mixture	.								.					**	
N fertilization				*	*	**	.								
Mixture* N	*			*											

Table 3. Final soil pH, K, and P by site and nitrogen fertilization

Site	N addition	pH	SE	K ppm	SE	P ppm	SE
	kg N ha^{-1} yr^{-1}			ppm		ppm	
Becker	0	6.02	0.04	117.4	3.9	12.18	0.88
	60	5.78	0.07	145.2	9.6	9.75	0.62
Lamberton	0	6.08	0.06	513.3	21.6	27.92	1.21
	60	6.26	0.07	602.4	41.3	39.17	3.41
Roseau	0	6.96	0.13	101.8	4.2	2.42	0.19
	60	6.73	0.10	90.2	2.7	2.08	0.08
Waseca	0	6.12	0.06	220.2	9.6	9.50	1.02
	60	6.13	0.09	183.3	8.5	6.58	0.51

Supplemental table 1. Species planted by species mixture treatment

Type	Common name	Latin name	SG[†‡]	GM[†]	LG[†]	HD[†]	GM[‡]	LG[‡]	HD[‡]
Grass	Switchgrass	*Panicum virgatum*	X	X	X	X	X	X	X
Grass	Big bluestem	*Andropogon gerardii*		X	X	X	X	X	X
Grass	Indiangrass	*Sorghastrum nutans*		X	X	X	X	X	X
Grass	Canada wild rye	*Elymus canadensis*		X	X	X			X
Grass	Virginia wild rye	*Elymus virginicus*				X	X	X	X
Grass	Little bluestem	*Schizachyrium scoparium*				X			X
Grass	Slender Wheatgrass	*Elymus trachycaulum*				X			X
Grass	Sideoats grama	*Bouteloua curtipendula*				X			X
Legume	Leadplant	*Amorpha canescens*			X	X		X	X
Legume	Canada milk vetch	*Astragalus canadensis*			X	X		X	X
Legume	Wild blue indigo	*Baptisia australis*			X	X			
Legume	Purple prairie Clover	*Petalostemum purpurea*			X	X		X	X
Legume	Showy tick trefoil	*Desmodium canadense*				X		X	X
Legume	Partridge pea	*Chamaecrista fasciculata*				X			
Legume	Bush clover	*Lespedeza capitata*				X			
Legume	Perennial lupine	*Lupinus perennis*				X			
Legume	American licorice	*Glycerryza lepidota*							X
Legume	Pale pea	*Lathyrus ochroleucus*							X
Legume	White prairie clover	*Petalostemum candidum*							X
Legume	American vetch	*Vicia americana*							X
Forb	Stiff goldenrod	*Solidago rigida*				X			X
Forb	Maximillian sunflower	*Helianthus maximilianii*				X			X
Forb	Yellow coneflower	*Ratibida pinnata*				X			X
Forb	Butterfly milkweed	*Asclepias tuberosa*				X			X
Forb	Cup plant	*Silphium perfoliatum*				X			
Forb	Wild bergamot	*Monarda fistulosa*				X			X
Forb	Rough blazingstar	*Liatris aspera*				X			
Forb	Golden Alexander	*Zizia aurea*				X			
Forb	Purple coneflower	*Echinacea pallida*							X
Forb	Northern bedstraw	*Galium boreale*							X
Forb	Black-eyed susan	*Rudbeckia hirta*							X

[†]*Becker, Lamberton, and Waseca*
[‡]*Roseau*
X *denotes species was planted in designated species richness treatment*
SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture

Supplemental table 2. Proportion of total biomass of 24 most prevalent species[†] collected in multiple plots by location and species mixture treatment/ nitrogen fertilization combination

	Legume					Grass																
	Alfalfa	Canada milkvetch	Leadplant	Showy tick trefoil	Wild blue indigo	Big bluestem	Canada wild rye	Indiangrass	Kentucky bluegrass	Little bluestem	Sideoats grama	Switchgrass	Virginia wild rye	Canada thistle	Common ragweed	Compass plant	Dandelion	Golden Alexander	Canadian horseweed	Morning glory	Wild bergamot	Yellow coneflower
Becker																						
SG -N				0.02								0.85										0.13
SG +N												0.91							0.01			0.05
GM -N						0.22						0.57										0.06
GM +N						0.01						0.95										0.03
LG -N			0.05			0.08						0.65										0.05
LG +N			0.01									0.95										0.04
HD -N				0.08						0.35											0.01	0.03
HD +N						0.04				0.12		0.72									0.08	0.03
Lamberton																						
SG -N							0.03				0.03	0.70	0.01	0.04					0.02			
SG +N											0.04	0.78			0.05					0.01		
GM -N						0.01	0.02				0.01	0.58							0.25			
GM +N						0.12					0.16	0.36	0.01		0.07		0.02			0.01	0.01	0.01
LG -N					0.02						0.01	0.77	0.01	0.05					0.01		0.01	0.01
LG +N		0.01			0.04						0.13	0.38	0.01		0.01		0.01					0.02
HD -N												0.02	0.05	0.08	0.01			0.21		0.01	0.28	0.08

HD +N						0.01	0.03				0.19	0.08	0.09	0.01	0.01		0.01	0.06		0.01	0.29	0.07
Roseau																						
SG -N						0.06		0.01				0.73	0.13									
SG +N	0.01					0.07						0.78	0.12									
GM -N						0.46		0.08				0.07	0.02									
GM +N						0.82						0.16	0.02									
LG -N	0.01			0.02		0.36						0.11	0.15									
LG +N				0.01		0.56						0.18	0.01									0.04
HD -N	0.05																	0.32				0.12
HD +N		0.02		0.04		0.01							0.02					0.47			0.06	
Waseca																						
SG -N												0.98										
SG +N												0.97						0.01				0.01
GM -N						0.01						0.78										0.05
GM +N						0.01						0.98										
LG -N					0.33							0.28						0.01				0.01
LG +N			0.01		0.24	0.01						0.33							0.35			
HD -N					0.26											0.44		0.02			0.18	0.01
HD +N					0.12				0.02							0.22		0.01			0.40	

Supplemental table 3. Extracellular enzyme activity by site

	β-glucosidase		Cellobiohydrolase		N-acetylglucosaminidase		Phosphatase	
	mean	SE	mean	SE	mean	SE	mean	SE
	---------- umol g^{-1} hr^{-1} ----------							
Becker	35.8	3.9	29.3	4.1	40.2	4.4	50.0	6.8
Lamberton	58.4	5.0	66.8	5.0	62.0	5.1	40.7	5.5
Roseau	53.1	6.9	51.5	5.8	40.6	5.5	48.2	5.1
Waseca	46.7	5.8	46.5	5.2	51.3	6.6	55.1	5.1

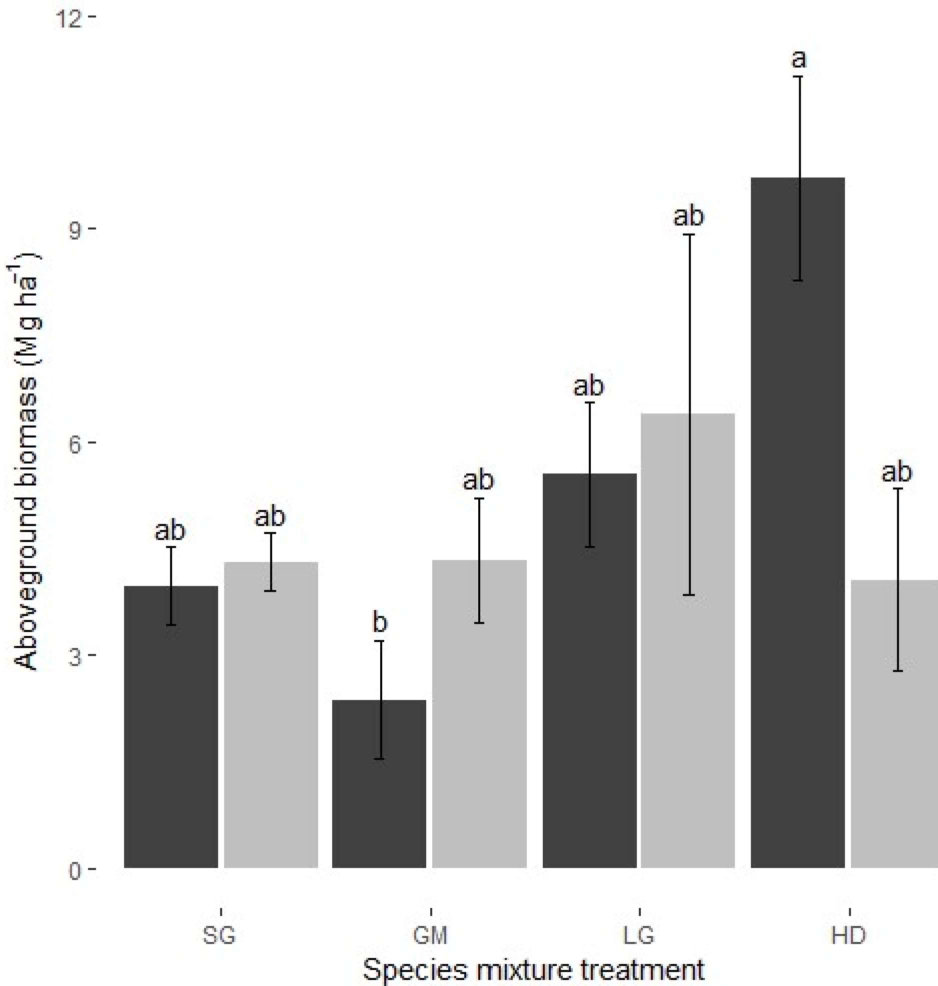
Supplemental table 4. Total soil nitrogen by depth and location

	0-15 cm		15-30 cm		30-60 cm		60-90 cm	
	N	SE	N	SE	N	SE	N	SE
	-----------------	-----------------	-----------------	-----------------	%	-----------------	-----------------	-----------------
Becker	0.103	0.003	0.109	0.004	0.076	0.006	0.047	0.004
Lamberton	0.226	0.004	0.19	0.005	0.156	0.041	0.073	0.006
Roseau	0.121	0.004	0.078	0.003	0.045	0.002	N/A	N/A
Waseca	0.228	0.009	0.205	0.006	0.138	0.006	0.093	0.008

N/A = No soil was collected

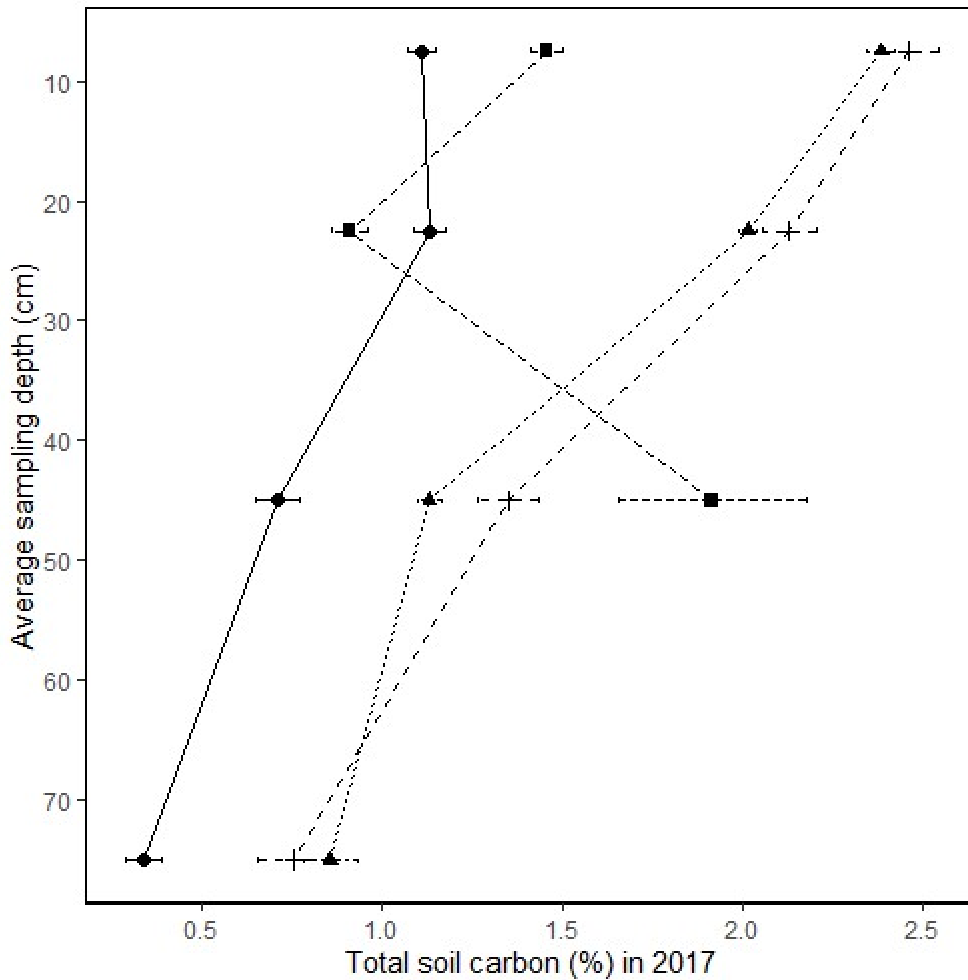
Figures

Figure 1. Total aboveground biomass at Waseca, MN by species mixture and N addition



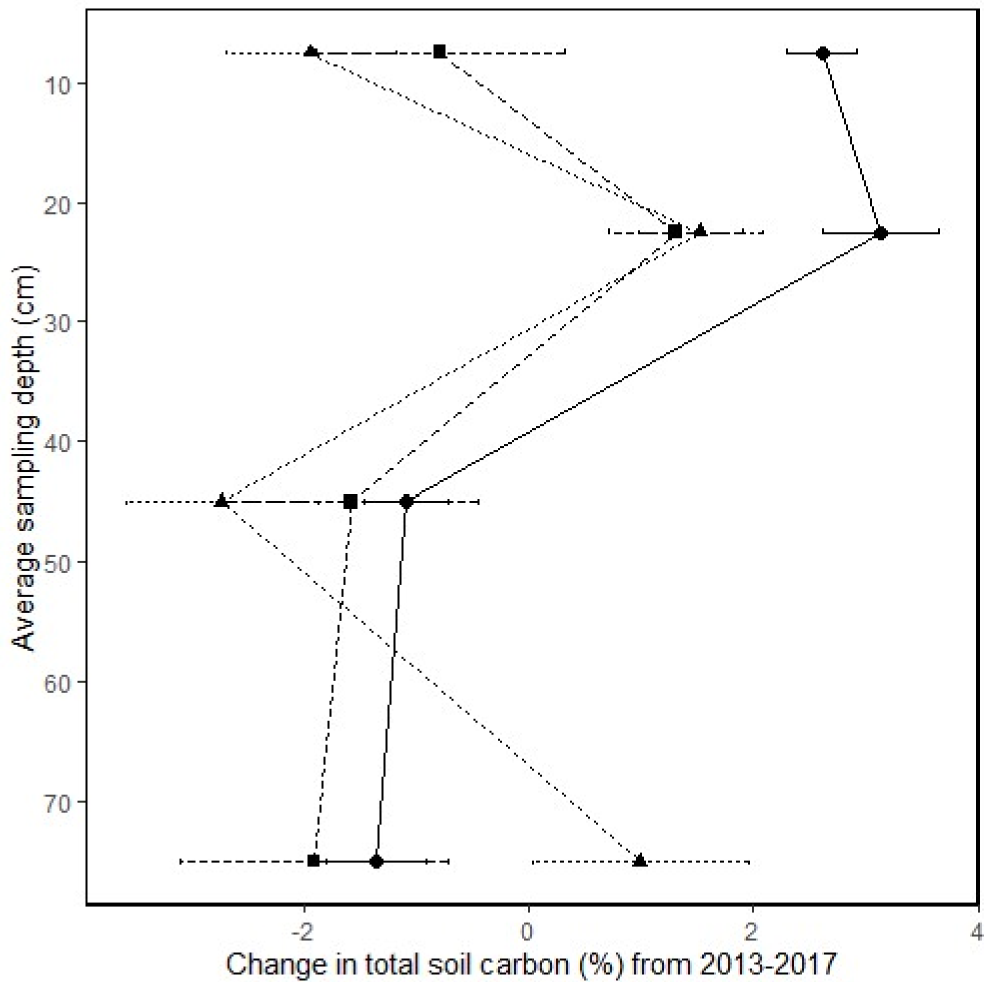
Within sites, species mixture by N addition combinations with the same or no letter are not different according to Tukey's HSD.
Error bars represent standard error of the mean.
SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture.
Dark gray bars represent unfertilized plots and light gray bars represent yearly fertilization at 60 kg N ha^{-1}.

Figure 2. Final total soil carbon by location.



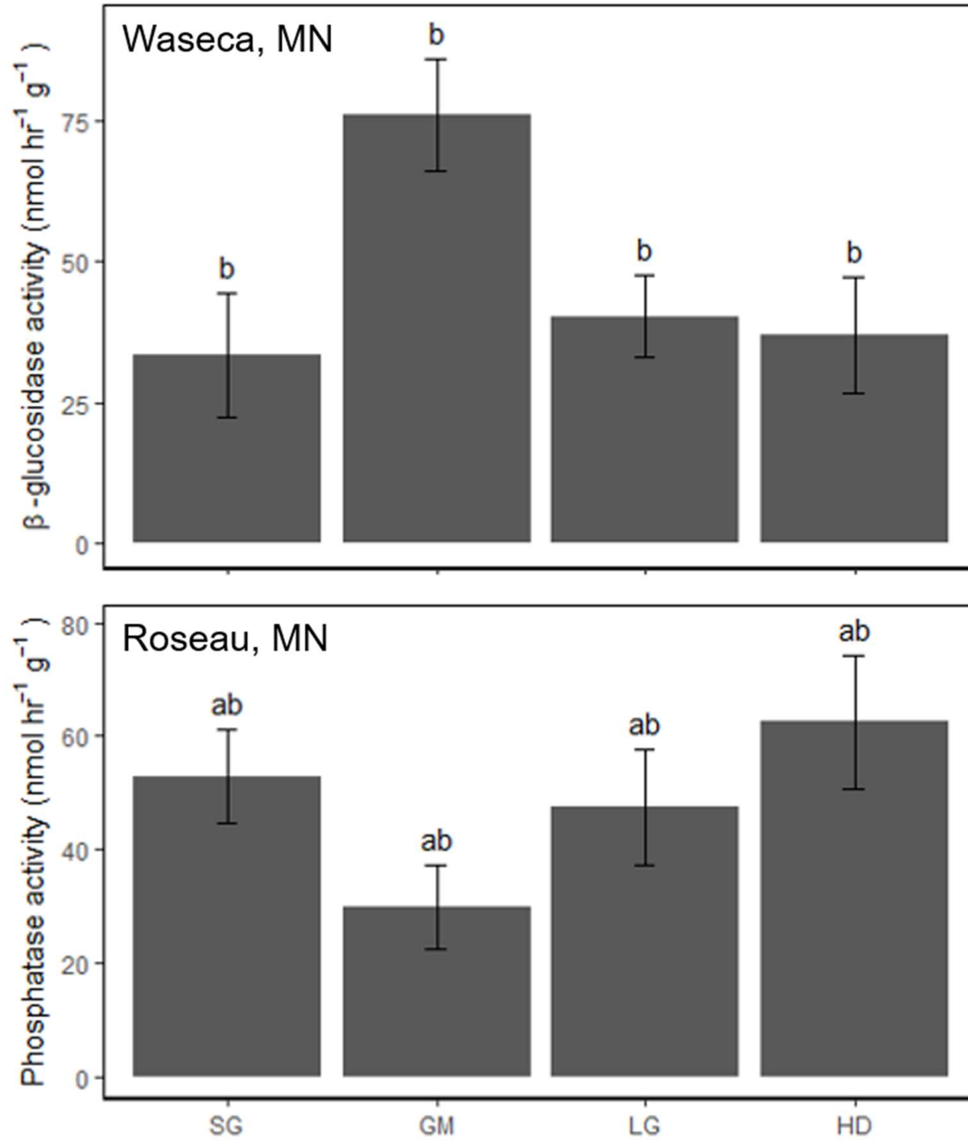
Circles with a solid line represent Becker, triangles with a dotted line represent Lamberton, squares with a short dashed line represent Roseau, and crosses with a long dashed line represent Waseca.
Points on figure represent average sampling depth for depth increments 0-15 cm, 15-30 cm, 30-60 cm, and 60-90 cm. Although sites are presented together in this figure for visualization of results, statistics were performed separately for each site.

Figure 3. Change in total soil carbon from 2013 to 2017 by location



Circles represent Becker, triangles represent Lamberton, squares represent Waseca. Points on figure represent average sampling depth for depth increments 0-15 cm, 15-30 cm, 30-60 cm, and 60-90 cm. Although sites are presented together in this figure for visualization of results, statistics were performed separately for each site.

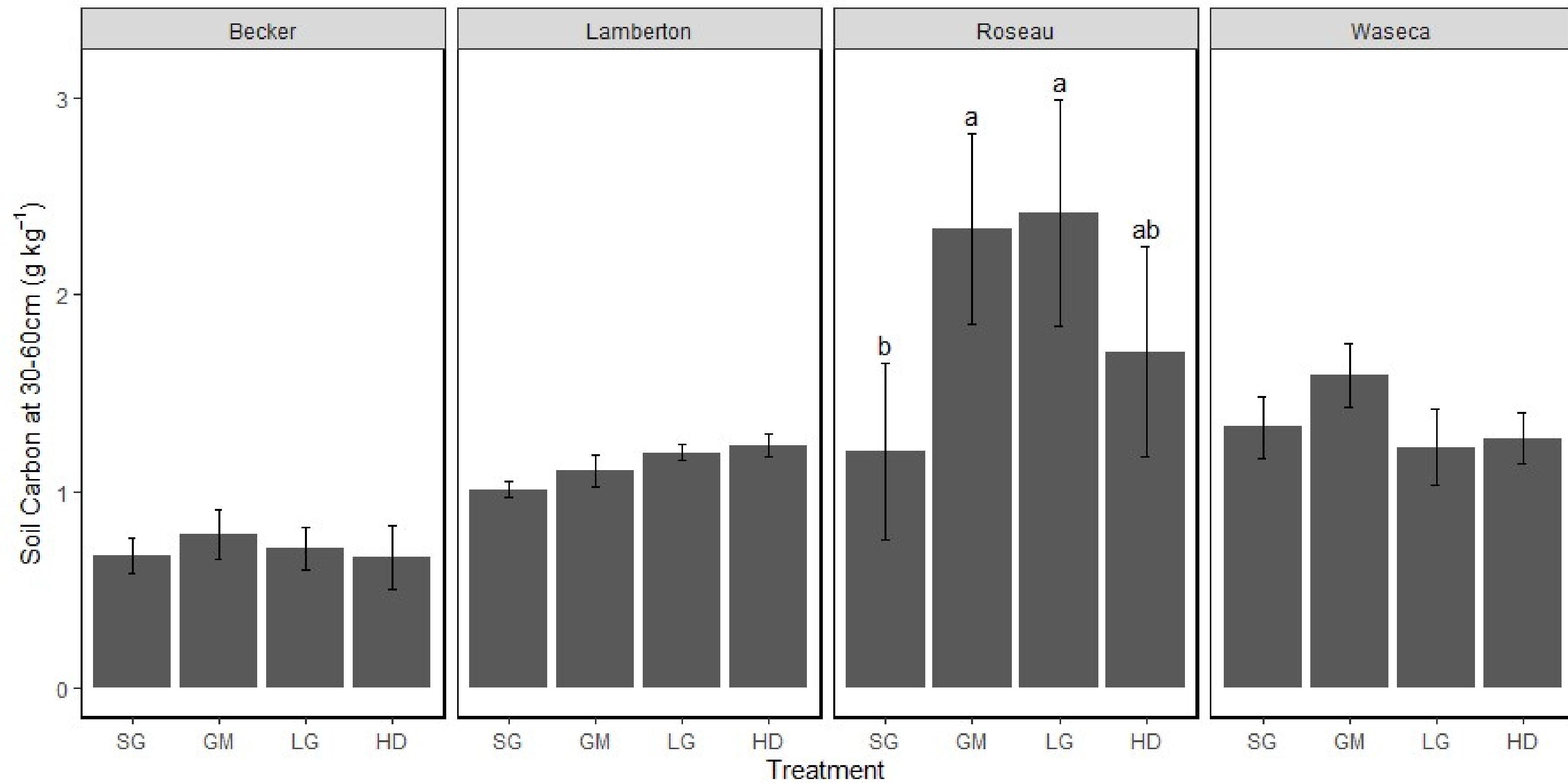
Figure 4. β-glucosidase at Waseca, MN and phosphatase activity at Roseau, MN



Within plots, species mixtures with the same or no letter are not different according to Tukey's HSD at $P < 0.05$.

SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture.

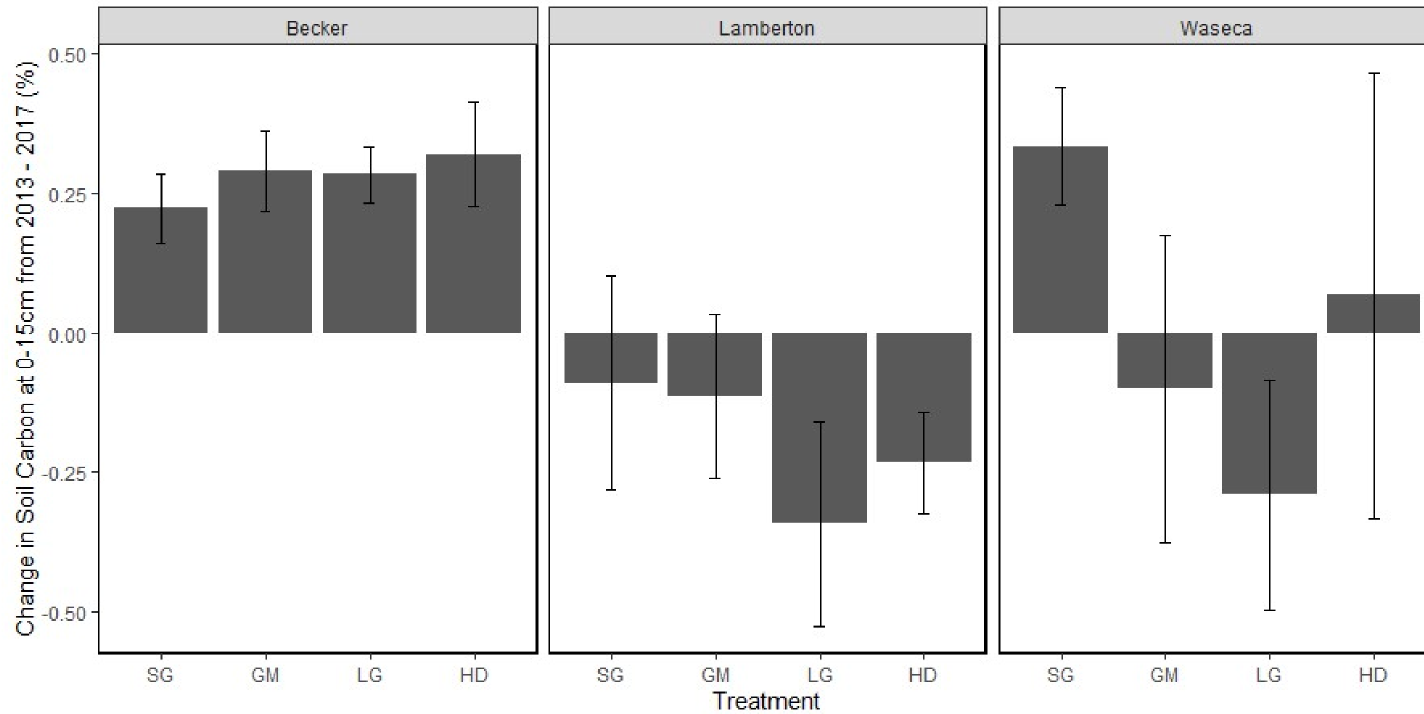
Supplemental figure 1. Soil total carbon at 30-60 cm in 2017 at four sites in Minnesota.



Within plots, species mixtures with the same or no letter are not different according to Tukey's HSD at $P < 0.05$.

SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture.

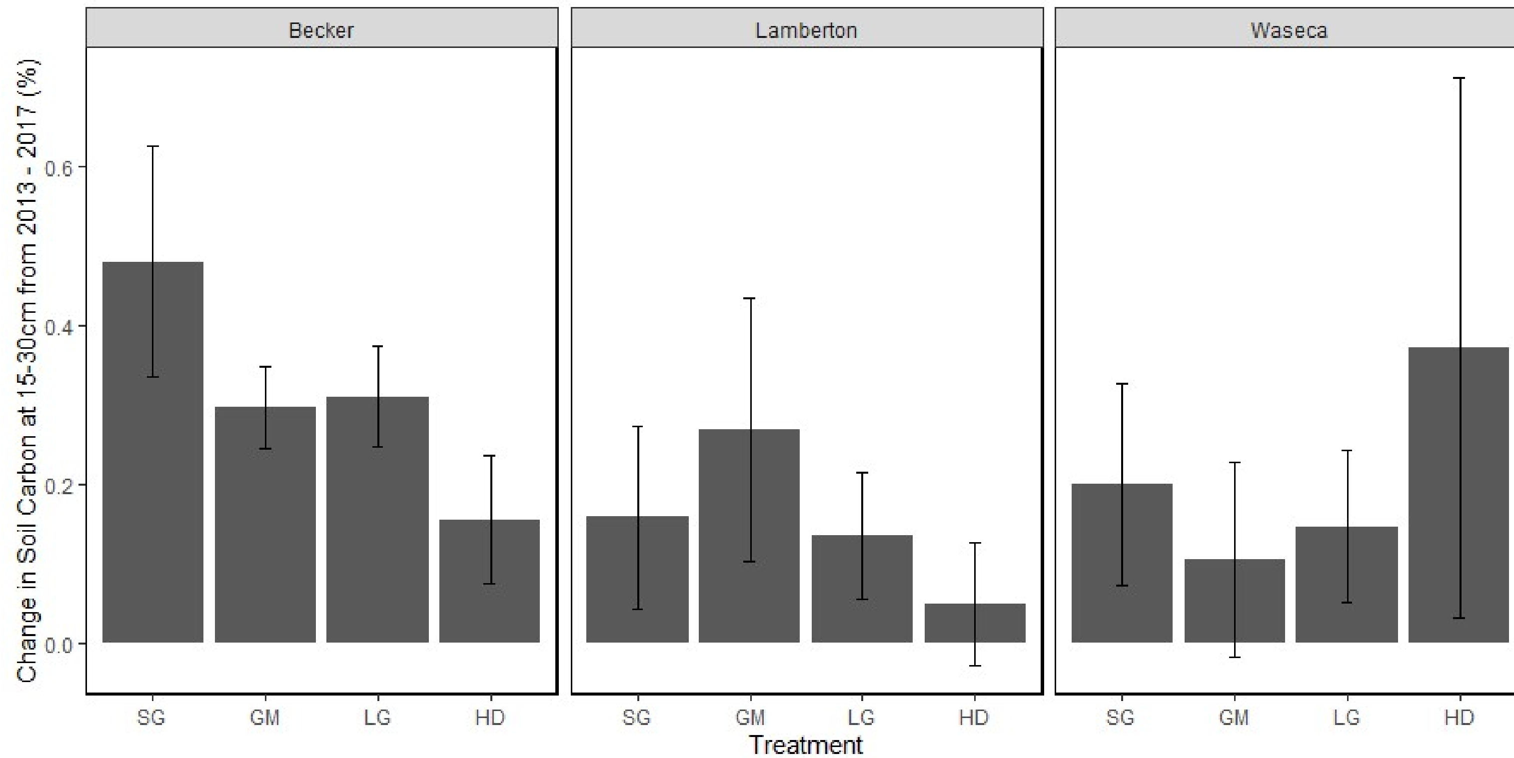
Supplemental figure 2. Change in total soil carbon from 2013 -2017 at 0-15 cm in three sites in Minnesota.



Within plots, species mixtures with the same or no letter are not different according to Tukey's HSD at $P < 0.05$.

SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture.

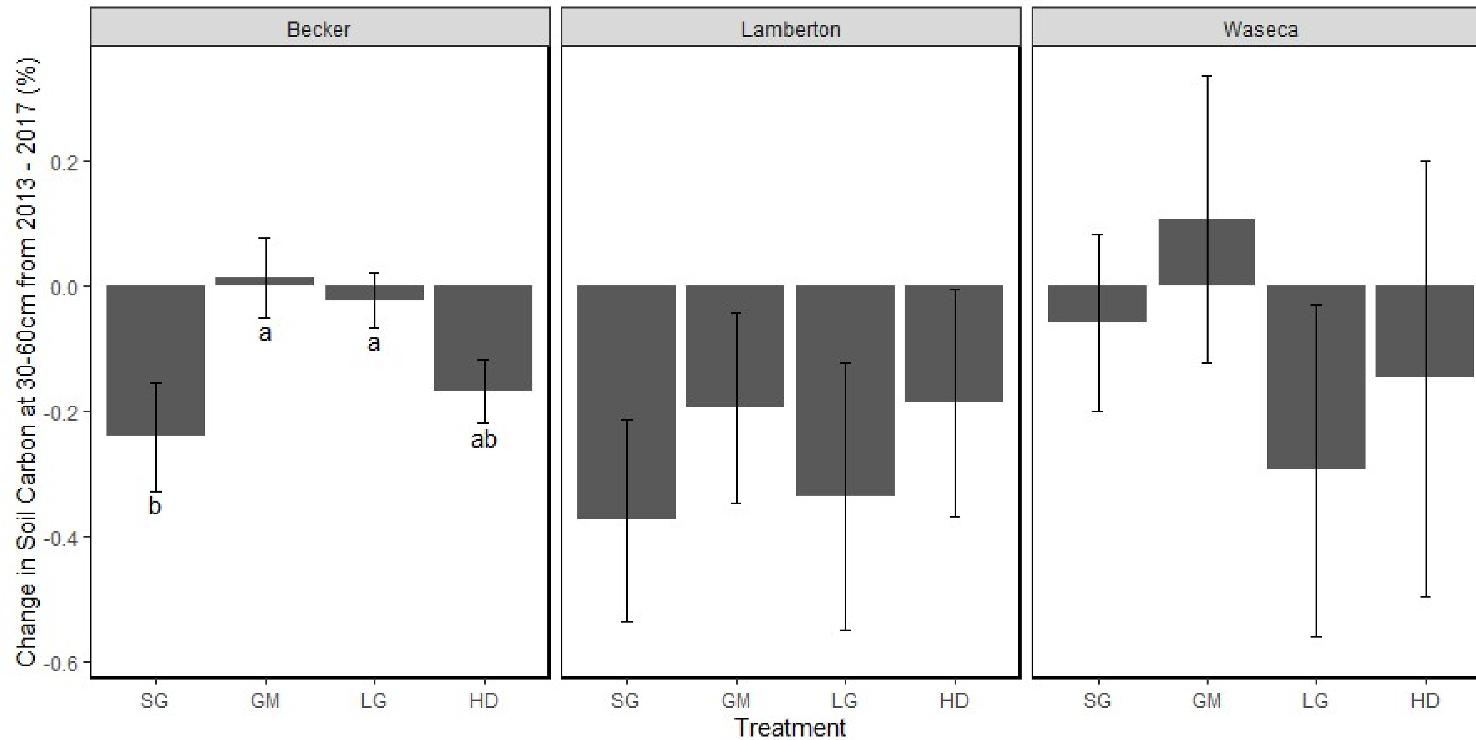
Supplemental figure 3. Change in total soil carbon from 2013 -2017 at 15-30 cm in three sites in Minnesota.



Within plots, species mixtures with the same or no letter are not different according to Tukey's HSD at $P < 0.05$.

SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture.

Supplemental figure 4. Change in total soil carbon from 2013 -2017 at 30-60 cm in three sites in Minnesota.



Within plots, species mixtures with the same or no letter are not different according to Tukey's HSD at $P < 0.05$.

SG = switchgrass monoculture, GM = four species grass mixture, LG = eight species legume/grass mixture, HD = 24 species high diversity forb/legume/grass mixture.

CHAPTER TWO
INTERMEDIATE WHEATGRASS (*Thinopyrum intermedium*) STAND MORPHOLOGY

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SYNOPSIS

Intermediate wheatgrass (*Thinopyrum intermedium*; IWG) is a rhizomatous perennial grass that is being developed for dual use as a grain and forage crop. The characteristics of IWG stand development over time (i.e. stand morphology parameters of tiller production and reproduction through rhizomes versus seed) may affect grain yields as stands age. However there are no detailed observations of these stand morphology parameters related to grain yields in northern climates. To better understand these dynamics and the role of nitrogen fertilization, we conducted two experiments in 1 year old (1 yo) and 2 year old (2 yo) IWG stands to: 1) describe population and stand development characteristics by measuring plant population density (plants m^{-2}), tiller density (tillers m^{-2}), rhizome density (rhizomes m^{-2}), and seed propagation (seed propagules m^{-2}) at four times during the growing season (spring regrowth, anthesis, harvest, and fall regrowth) and 2) correlate population and stand morphology characteristics with grain and biomass yield under three nitrogen fertilization treatments. In both experiments, we classified individual plants as original (from sown seed), vegetative propagule (asexually produced), or seed propagule (from shattered seed). The number of tillers and rhizomes from all individual plants were also recorded. Overall, our findings suggest that differing population, tiller, and rhizome dynamics vary across plant type (original plant, vegetative propagule, or seed propagule), and that a positive association exists between both grain and biomass yield and spike density. Our findings also suggest that there is minimal influence of nitrogen fertilization on relationships between stand morphology and grain yield. With regard to population dynamics, original plant density decreased from anthesis to harvest in the 1 yo stand and remained the same over time in the 2 yo stand. Moreover, original plant density was positively correlated with grain and biomass yield in the 1 yo stand, suggesting that maintaining original population density may bolster yields. Total rhizome density was greatest at harvest in both stand ages, and rhizome and vegetative propagule densities were positively correlated with grain yield in 1 yo IWG, implying no trade-offs between seed production, aboveground vegetative growth, and asexual reproduction within new stands. Lastly, total spike density predicted grain and biomass yield in both stands, indicating that managing IWG stands for increased spike density may optimize grain and forage yields in dual use IWG production. This paper is the first to describe stand morphology in improved IWG, and these findings may be used to advance both agronomic and genetic research into this recently domesticated crop.

Terms used in this paper

Individual plant- One or more stems originating from a single crown
Original plant- An individual plant originating from sown seed

Main stem- A stem from seed or rhizomatous growth
Tiller- A grass stems arising from axillary buds on a main stem. In this paper, all primary, secondary, and tertiary tillers are included as one group
Spike- a reproductive stem (contains flowers and/or seeds) either from a main stem (either from the original plant or from a vegetative propagule) or tiller
Stems on an individual plant- The sum total of the main stem plus the tillers on an individual plant
Vegetative propagule- An individual plant originating from the underground rhizome of another individual
Seed propagule- An individual plant originating from unsown seed
Total stems- the total stems counted, including tillers and main stems from original plants, seed propagules, and vegetative propagules
Stem recruitment- The process by which a grass stand creates new stems, either through increasing stand population or by increasing the number of stems per individual plant
Stand morphology- the growth characteristics which together allow one to interpret stand development, including the number and biomass of main stems, tillers, vegetative propagules, seed propagules, and number of spikes produced
Population density- the number of individual plants per unit area

INTRODUCTION

Intermediate wheatgrass (*Thinopyrum intermedium* (Host) Buckworth & D.R. Dewey) is a rhizomatous cool-season perennial grass originating from Eurasia, and has since been introduced around the world for use in pastures and as a forage crop (Hanson, 1972). In the US, cultivars have been in development since mid-20th century to improve forage yield and quality of intermediate wheatgrass (Hanson, 1972; Ogle, St John, Tober, & Jensen, 2011), and more recently, researchers have been breeding intermediate wheatgrass for use as a grain crop (Bajgain, Zhang, & Anderson, 2019; L. DeHaan, Christians, Crain, & Poland, 2018; Wagoner, 1990; X. Zhang et al., 2017). Intermediate wheatgrass is a promising new crop for many reasons, but there are gaps in fundamental knowledge about its growth patterns and agronomic management that need to be addressed.

Compared with annual crops, perennial crops show potential to increase profitability and provide ecosystem services (Crews & Rumsey, 2017; LaCanne & Lundgren, 2018; M. R. Ryan et al., 2018). Intermediate wheatgrass shows promise as a dual-use perennial crop for concurrent grain and forage production. Grain harvested from improved intermediate wheatgrass genotypes – trademarked Kernza™ by The Land Institute – is suitable for many human food applications (Marti, Bock, Pagani, Ismail, & Seetharaman, 2016; Rahardjo et al., 2018; M. R. Ryan et al., 2018). Its deep root system has the potential to provide multiple environmental benefits, such as reduced soil erosion, carbon sequestration, and improved water quality (Steve W. Culman, Snapp, Ollenburger, Basso, & DeHaan, 2013; Jacob M. Jungers, DeHaan, Mulla, Sheaffer, & Wyse, 2019; Pugliese, Culman, & Sprunger, 2019; M. R. Ryan et al., 2018).

Advances in breeding and genetics research have resulted in increased intermediate wheatgrass seed size and yield over time, leading to an increase in the profitability of intermediate wheatgrass grain production (L. DeHaan et al., 2018). However, one of the challenges of intermediate wheatgrass cropping systems is that after relatively high first year grain yields, there is a marked decrease in yields in subsequent years, limiting its viability as a long-lived perennial grain crop (Hunter, Jungers, Sheaffer, & Culman, 2020; Jacob M. Jungers, DeHaan, Betts, Sheaffer, & Wyse, 2017; Tautges, Jungers, Dehaan, Wyse, & Sheaffer, 2018). There is evidence that increased stem production is related to this yield decline (Hunter et al., 2020), and a more in depth exploration of the relationships between intermediate wheatgrass stand development and grain yields will better inform future breeding, agronomic, and production efforts to achieve the promise of this novel crop.

While it is known that intermediate wheatgrass biomass is positively correlated with grain production when planted in breeding spaced plots (individual plants with 1 m of space between each plant), that relationship has become weaker as the crop has been improved for grain yield (Cattani & Asselin, 2018), and little is known about the relationship between biomass and grain yield in swards. One mechanism by which stands increase their photosynthetic potential, and thereby yield, is stem recruitment- the process by which a grass stand creates new stems via sexual propagation, vegetative propagation, or aboveground vegetative growth (Lemaire, Da Silva, Agnusdei, Wade, & Hodgson, 2009). While stem recruitment has been studied in some detail on caespitose grass species, stem recruitment in rhizomatous grass species such as intermediate wheatgrass remains understudied (Dalgleish, Kula, Hartnett, & Sandercock, 2008; Mueller &

Richards, 1986; Ott & Hartnett, 2012).

In many perennial grasses, stem recruitment occurs predominantly through aboveground vegetative growth and vegetative propagation- few seedlings establish, and the seedlings that do establish quickly die (Benson & Hartnett, 2006; Jónsdóttir, 1991). Sexual propagation can be challenging in established grasslands, as light conditions for seedling establishment are reduced by litter and grassland canopy density (Jensen & Gutekunst, 2003). Seedlings establishing in perennial grasslands often remain small, subsequently producing few tillers, shorter rhizomes, and less biomass, unless establishing in a large areas of disturbance where light and resources are abundant (G. X. Liu & Han, 2007; Rafael Otfinowski & Kenkel, 2010). Since sexual propagation is a negligible source of stem recruitment in perennial grassland swards, tiller replacement is dependent on species' ability to either form active meristems that become tillers on plant crowns, or to produce vegetative propagules from rhizomes (Maschinski & Whitham, 1989). Whether a stem is recruited as a vegetative propagule or a tiller affects how the individual plant can access the resources needed for growth and development (Tomlinson, Dominy, Hearne, & O'Connor, 2007). Specifically, the recruitment of tillers on the crown- a growth pattern in which little horizontal spread occurs- is less suited to acquiring nutrients and water than a rhizomatous growth pattern, in which horizontal spread is maximized (Hutchings & de Kroon, 1994). One advantage of rhizomatous growth is that in-tact rhizomes connecting vegetative propagules to originating plants increases the mass of invading shoots, making rhizomes essential for invasion (R. Otfinowski & Kenkel, 2008). Since plants are constrained to a basic architecture based on their genetic background (Bangerth, 1989; Brouwer, 1962; Hutchings & de Kroon, 1994;

Tomlinson et al., 2007; White, 1937), the degree to which stems are asexually recruited on crowns versus rhizomes may vary by species.

Although rhizome production may increase nutrient and water acquisition in a sward, the accompanying asexual propagation and related resource allocation may lead to increased plant density, adversely affecting seed production and thus grain yield. In tall fescue, another cool-season perennial grass crop, higher population density led to lower seed yield and spike density - an important component of seed yield (Chastain, Garbacik, Young, & Silberstein, 2002). Within rhizomatous grasses, growth strategies vary considerably among species (Judziewicz, Clark, Londono, & Stern, 1999), so research is needed to better understand tiller and rhizome development specifically in improved intermediate wheatgrass grown for grain and forage production.

Here, we quantified the morphology of 1 year old (1 yo) and 2 year old (2 yo) intermediate wheatgrass stands at different phenological stages across the growing season in order to better understand how intermediate wheatgrass stands develop over time. Based on these development characteristics, our second objective was to determine how these stand morphology characteristics (tiller, rhizome, and seedling growth) relate to yield parameters under different nitrogen management strategies. We seek to inform plant breeding and agronomic management practices that may raise overall yields and ameliorate yield decline. Because tiller density is affected by growing season (Bertin, 1988), we used two identically managed fields of different stand ages, in close proximity to one another, to better isolate the effects of age. We address each of the two objectives with a separate experiment. In experiment 1, we quantified the effects of phenological stage on population density (number of individual plants per meter squared), stem density

(number of stems per meter squared), and rhizome density (number of rhizomes per meter squared) for individual plants that originated from sown seed (original plants), from rhizomatous tissue (vegetative propagules), and from shattered seed (seed propagules) in both the 1 yo and 2 yo intermediate wheatgrass stands. In experiment 2, we explored if total spike density, total rhizome density, total plant density, original plant density, vegetative propagule tiller density, and total stem density at harvest were correlated with grain and biomass yield in both the 1 yo and 2 yo stands across three nitrogen fertilization treatments.

MATERIALS AND METHODS

Location and Experimental Design

The two experiments were conducted on a Tallula silt loam (Coarse-silty, mixed, superactive, mesic Typic Hapludoll) at the University of Minnesota Rosemount Research and Outreach Center (44°41'05"N 93°04'13"W). An improved population of intermediate wheatgrass from the fifth cycle of breeding for increased grain yield by The Land Institute (Salina, KS) was used. The previous crop of soybean (*Glycine max*) was terminated prior to seedbed preparation, after which intermediate wheatgrass was planted with a cone seeder in 15 cm rows in September of 2016 (2 yo) and 2017 (1 yo). Both experiment 1 and experiment 2 were conducted within the same 1 yo and 2 yo fields, and data collection for both experiments occurred in 2018. In experiment 2, three nitrogen fertilization treatments were applied using urea: 1) 80 kg N ha^{-1} in early May, 2) 40-40 kg N ha^{-1} in early May and mid-June, and 3) an unfertilized control. Early May was chosen for the earliest fertilization due to abundant late snow cover. Plots were arranged in a randomized complete block design with one replication per block and four blocks per field.

Vegetative Tissue Sampling and Quantification of Morphological Components

All sampling occurred at four timepoints chosen to represent key growth stages: spring regrowth, anthesis onset, harvest, and fall regrowth- after frost (Table 1). In experiment 1, we sampled from the 80 kg N ha^{-1} nitrogen fertilization treatment because it best represents the agronomically optimum nitrate rate (Jacob M. Jungers, DeHaan, et al., 2017). In experiment 2, we sampled from the late August harvest timepoint from all three nitrogen fertilization treatments to better understand the relationship between

nitrogen fertilization strategies, morphological parameters, and yield.

Plant sampling for the purpose of counting and identifying components of stand morphology was conducted by first marking three 15 cm by 33 cm (0.05 m^2) quadrats at predetermined sampling locations in each plot. Each marked area was then excavated to a depth of 20 cm. Excess soil was gently removed from the belowground biomass, taking care to keep individual plants in-tact. All plant matter was stored at 4° C until sorted.

Excavated plants were classified as either "original" (originating from sown seed), "vegetative propagule" (originating from a rhizome), or "seed propagule" (originating from seed shatter) based on the above- and belowground morphology. Original plants were designated based on 1) the absence of any inward growing rhizome and 2) the presence of more advanced development than would be expected from a seed propagule. Vegetative propagules were identified by the presence of a (primary or secondary) rhizome growing upward through the center of the crown of the plant. Rhizome direction was determined by its position in the crown and the orientation of the sheath. Like tillers, rhizomes have sheaths that open away from the crown. Rhizomes were distinguished from tillers by their color, transparency, and direction of growth. Seed propagules were nearly always identifiable based on the presence of a lemma and palea at the base of the plant, however any individual plant that was missing an inward rhizome and was too small to be an original plant was considered a seed propagule. Visual characteristics of seed propagules include few (typically no) tillers, etiolation, and an absence of secondary tillering. Seed propagules were expected to be observed at all timepoints in the 2 yo stand and at harvest and fall regrowth of the 1 yo stand. Original plant, vegetative propagule, and seed propagule density were calculated as the number of

individual plants per square meter.

After classification, the total number of stems (including tillers and the main stem), rhizomes, and spikes (reproductive stems) of a plant were counted. In experiment 2, spikes and rhizomes were not categorized by plant type (original plant, vegetative propagule, or seed propagule). Rhizomes were counted by cleaning belowground material and identifying any belowground structure with sheaths. A rhizome was counted as belonging to an individual plant if it originated from the crown of said individual plant. Only primary rhizomes were counted for the purposes of this experiment. Any tiller connecting to the crown of a plant was categorized as belonging to that individual plant. Tiller, rhizome, and spike density was calculated as the number of tillers, rhizomes, or spikes per square meter. Aboveground biomass was collected at soil level, removed from grain (at harvest), and dried at 35° C until constant weight.

At harvest, grain yield was measured, along with a suite of other potentially explanatory factors. Plant height was measured from the soil surface to the tallest structure of five plants per plot. Lodging was assessed on a 1-10 scale, where 1 = all plants in the plot were fully upright, 5 = most plants were at a 45° angle, and 10 = all plants were lying down. Grain was dried at 35° C until constant weight, threshed from mature spikes, and weighed to determine grain yield. Harvest index was calculated as grain yield divided by biomass yield plus grain yield.

Statistical Analysis

Statistical analysis was conducted in R (R Core Team, 2019). In experiment 1, a generalized linear mixed model (glmer) with a Poisson distribution was used for the

count variables, including population, stem, and rhizome densities. All other response variables had a Gaussian distribution and were analyzed with the nlme package (Pinheiro J., Bates D., DebRoy S., 2019). Sampling time was considered a fixed effect and block was considered a random effect. Due to evident differences between the fields and statistical interactions between stand age and sampling time for most response variables in a preliminary statistical analysis, each stand age was analyzed separately for this final analysis. For *post hoc* analysis, the emmeans package (Lenth, Singmann, Love, Buerkner, & Herve, 2019) was used. Means were separated using Tukey's HSD.

In experiment 2, a linear model was used to explore the relationship between the regressors total spike density, total rhizome density, total plant density, original plant density, vegetative propagule stem density, and total tiller density with the response variables grain yield and biomass yield at harvest. These relationships were explored separately for the 1 yo and 2 yo stands. Total stem density was regressed with total rhizome density and total spike density, and total spike density was also regressed with total rhizome density. Fields were analyzed separately due to interactions between stand age and both total stem density and vegetative propagule stem density. Nitrogen fertilization treatment was preliminarily included in the linear models, but was found to have no effect in any linear model, so the models were simplified to one regressor. ANOVAs were also used to analyze the effect of nitrogen fertilization treatment on grain and aboveground biomass yield.

RESULTS

Growing conditions during the study were suitable for intermediate wheatgrass production. Mean temperature was slightly higher than the 30 year average from May to September, and cumulative precipitation by month was higher in September than average, however these rains occurred after harvest (Table 2). No notable diseases, pests, or weather phenomenon adversely affected growing conditions in 2018. A minor amount of weed pressure was observed- mainly from warm season grasses in the 2 yo stand and broadleaves in the 1 yo stand, but this pressure was not significant in either case.

Stand morphology characteristics- experiment 1

The way in which the density of individual plants in each of the two populations changed during the growing season depended on plant type and the stand age. Total plant density in the 1 yo stand was greater at harvest and fall regrowth than at spring regrowth (Table 3; Figure 1D). While original plant density in the 1 yo stand was greater earlier in the season (spring regrowth and anthesis) than later in the season (harvest and fall regrowth), vegetative propagule density in the 1 yo stand was greater later in the season (Table 3; Figure 1A&B). Conversely, total plant density declined throughout the growing season in the 2 yo stand, as did seed propagule density (Table 3; Figure 2D). Vegetative propagule density did not change over the growing season in the 2 yo stand.

Stem density varied by plant type in both stand ages. In the 1 yo stand, total stem density increased throughout the growing season, and the pattern of increase was similar to that observed for original plant stem density (Table 3; Figure 3A&D). Similarly, original and total stem densities in the 2 yo stand were greater at fall regrowth than at

anthesis and harvest (Table 3, Figure 4D). Vegetative propagule stem density peaked at harvest in the 1 yo stand, while in the 2 yo stand vegetative propagule stem density peaked at fall regrowth (Table 3, Figures 3B and 4B). Although there was no change in seed propagule stem density in the 1 yo stand, seed propagule stem density declined in the 2 yo stand from anthesis through fall regrowth (Table 3, Figure 4C).

Total rhizome density and original plant rhizome density fluctuated over time in both stand ages (Table 3). No rhizomes were observed originating from seed propagules, and few rhizomes were observed originating from vegetative propagules. In the 1 yo stand, original rhizome density was highest at spring regrowth, then substantially decreased at anthesis (Figure 5A). Between anthesis and harvest, both stands gained rhizomes, and both stands lost rhizomes between harvest and fall regrowth (Figures 5C and 6C). The longest rhizome observed during sampling was 74 cm long, suggesting that growth through vegetative reproduction could span as many as six 15 cm rows.

Grain yield averaged 410 (SE 162) kg ha^{-1} in the 1 yo stand and 189 (SE 154) kg ha^{-1} in the 2 yo stand. Biomass yield averaged 12.4 (SE = 0.7) Mg ha^{-1} in the 1 yo stand and 10.8 (SE = 1.9) Mg ha^{-1} in the 2 yo stand. Harvest index was 0.032 (SE = 0.006) in the 1 yo stand and 0.034 (SE = 0.016) in the 2 yo stand.

Correlations- experiment 2

For both stand ages, total spike density was positively related to grain and biomass yields. In the 1 yo stand only, grain yield was also correlated with the densities of other stand morphology parameters (total rhizomes, total plants, original plants, vegetative propagule tillers, and total tillers) and biomass yield was also correlated with

total tiller density. (Table 4).

Total stem density, total rhizome density, and total spike density were significantly correlated to each other ($P < 0.05$) in the 1 yo stand but not in the 2 yo stand. Specifically, total stem density was positively associated with both total spike density ($R^2 = 0.87$; slope = 0.26) and total rhizome density ($R^2 = 0.51$, slope = 0.05). Similarly, total spike density and total rhizome density were positively correlated ($R^2 = 0.63$, slope = 3.2).

DISCUSSION

In experiment 1, population, tiller, and rhizome dynamics were affected by plant type, and in experiment 2, a positive correlation was observed between spike density and yield. Different trends were observed in the 1 yo field and the 2 yo field. At spring regrowth in early May, the two fields looked very different, as the 2 yo intermediate wheatgrass plants were more mature and robust than the 1 yo plants and the 2 yo field had crop residue left from senesced fall regrowth from the previous growing season. By anthesis onset in mid-June, the two fields looked similar in their growth and development.

Plant density throughout the growing season

The original plant density decline in between anthesis and harvest in the 1 yo stand (Figure 1A) suggests a surprising loss of population between anthesis and seed fill. This thinning process could have occurred as light became a limiting resource during canopy closure near the time of anthesis. However, during this time between anthesis and harvest, original plants were replaced by vegetative propagules, and total plant density remained stable after spring regrowth throughout the season in the 1 yo stand.

Total plant density was much greater at spring regrowth in the 2 yo stand, but declined throughout the year. This suggests that the turnover in plant types and subsequent changes in total plant density continues through two years of production. We did not identify a stable equilibrium density for intermediate wheatgrass. Moreover, as vegetative propagule distance from the originating plant is decreased by competition (Cheplick & Gutierrez, 2000), decreasing plant density throughout the year is likely to lead to vegetative propagules that venture further from their originating plants.

After the increase in vegetative propagule density between anthesis and harvest in the 1 yo stand, vegetative propagule density remained stable over time in the 2 yo stand (Figure 2B), possibly due to bud bank dynamics. Juvenile rhizomes are more likely to produce buds, and thus stems, than older rhizomes (Ott & Hartnett, 2015). Thus, it follows that the 1 yo stand may have given rise to more juvenile rhizomes through the course of the growing season than the 2 yo stand. Regardless, our findings that vegetative propagule density increased between anthesis and harvest is contrary to previous evidence in Kentucky bluegrass (*Poa pratensis* L.) that short days lead to vegetative propagule formation and longer days with higher temperature lead to rhizome formation and elongation (Aamlid, 1992). Given our finding that vegetative propagule stem density was positively associated with grain yield in the 1 yo stand, understanding vegetative propagule population dynamics might allow agronomists, geneticists, and breeders to better improve first year grain yield.

Stem and tiller density

We found that original plant stem densities and total stem densities (sum of original plant, vegetative propagule, and seed propagule stem densities) increased during the growing season in both stand ages, but in different ways. The 1 yo intermediate wheatgrass recruited vegetative propagule stems between anthesis and harvest, and recruited new stems on original plants as tillers throughout the growing season including after harvest (Figure 3A&B). This indicates that first year intermediate wheatgrass recruits stems during seed fill, possibly diverting resources away from grain production. We also observed a decline in vegetative propagule density between harvest and fall

regrowth in the 1 yo stand. This decline indicates that vegetative propagule stem death exceeded replacement during that time. As stem death is not affected by defoliation (Scheneiter & Améndola, 2012), the harvest event unlikely contributed directly to this decline, although harvest may have affected stem death by altering light or other resource conditions. There is evidence that the death of clonal fragments, such as vegetative propagule stems, is due to shading (Tomlinson et al., 2007). Thus, post-harvest residue may have shaded out vegetative propagule meristems, leading to stem death.

In contrast with the 1 yo stand, the 2 yo stand recruited new stems only during fall regrowth after harvest both on original plants and vegetative propagules (Figure 4A&B). The lack of mid-season recruitment of stems in the 2 yo stand could be due to increased competition from vegetative propagules, as stem recruitment is lower when more neighboring plants are present (Phillips, Tainton, Reusch, & Cumming, 1996). We also observed a decline in seed propagule stems at each timepoint, suggesting that seed propagules may not effectively compete with the more robust original and vegetative propagule stems for resources as intermediate wheatgrass stands develop over multiple years.

Rhizome density

In both stand ages, rhizome density decreased between harvest and fall regrowth, and in the 1 yo stand, rhizome density decreased between spring regrowth and anthesis. The rhizomes identified in the 1 yo stand at spring regrowth were short, transparent, hairless, and growing belowground. Either these rhizomes died back during tillering, or more likely, they were recruited as stems. It has been established that tissue senescence

typically provides resources that can be reallocated to areas of growth or storage (Vries, 1989). Given the degree of stem recruitment observed post-harvest, it could be that carbohydrates and nitrogen were reallocated from rhizomes to stems between harvest and fall regrowth, resulting in rhizome dieback to support stem production. Contrarily, if harvest resulted in rhizome death for other reasons, it may adversely affect the ability of intermediate wheatgrass to access nitrogen for fall regrowth. This is because rhizomes aide in resource acquisition (Hutchings & de Kroon, 1994) and mid-season soil nitrogen levels are low in this system (Dobbratz, 2020).

Notably, rhizome production occurred concurrently with seed production, as rhizome density increased between anthesis and harvest in the 1 yo stand, suggesting that intermediate wheatgrass invests simultaneously in sexual and asexual reproduction. This concurrent sexual and asexual reproduction may have been atypical, as our grain yields were lower than previous studies (Frahm et al., 2018; Jacob M. Jungers, DeHaan, et al., 2017; Tautges et al., 2018). There is evidence that, in cool season perennial grasses, when the cost of seed production is high, rhizome production is favored (Reekie, 1991). As resource allocation patterns are determined by genetic factors (Reekie, 1991), producing intermediate wheatgrass that preferentially invests in sexual over asexual propagation will likely require breeding and genetic tools.

Correlations with biomass and grain yield- experiment 2

While stem density was positively associated with spike density in the 1 yo stand, other yield components that were not measured, for example seed size and number of seeds per spike, may have been impacted. Seed production, aboveground vegetative

growth, and asexual reproduction all occured concurrently in 1 yo intermediate wheatgrass. This is contrary to previous stand morphology assessments in cool season grasses, where either negative relationships (J. T. Zhang et al., 2014) or no relationships (Didyk, 1998) between aboveground and belowground growth have been observed. Light availability may explain this discrepancy in findings, based on evidence that stubble removal, allowing more light to penetrate a developing canopy, is associated with increases in both spike production and rhizome development (Meints, Chastain, Young, Banowetz, & Garbacik, 2001). The lack of observed concurrence of aboveground vegetative growth and seed production in the 2 yo stand suggests the beginning of stand decline, when biomass yields remain stable or increase but grain yields diminish (Jacob M. Jungers, DeHaan, et al., 2017).

Total spike density, total stem density, and biomass yield were all positively correlated in 1 yo intermediate wheatgrass, indicating that there was no tradeoff between sexual reproduction and aboveground vegetative growth. This is in line with other research on intermediate wheatgrass grown in spaced plots that found a positive correlation between biomass and seed yield (Cattani & Asselin, 2018), indicating that the increased competition of the sward environment did not adversely affect the concurrence of aboveground vegetative growth and seed production. In another cool season perennial grass, narrow row spacing was associated with greater spike density (M. Wang et al., 2017), suggesting a positive relationship between population density and spike production.

Total spike density was a consistent predictor of grain and biomass yield across 1 yo and 2 yo intermediate wheatgrass, suggesting that managing and breeding for fertile,

or spike bearing, stems will benefit both aspects of dual use intermediate wheatgrass. Our findings agree with evidence that spike density is an important predictor of seed yield in perennial grasses (M. Wang et al., 2017; Young, Youngberg, & Chilcote, 1996). However, we found no evidence of biotic or abiotic competition between fertile and non-fertile stems, unlike previous work (Vleugels, Rijckaert, & Gislum, 2017). In fact, in the 1 yo stand, spike density and stem density were positively correlated. Spike production has been shown to increase with hay removal in another cool season perennial grass (Meints et al., 2001). Best management practices for managing intermediate wheatgrass as a dual use crop for both grazing and grain production will likely need to consider these tradeoffs between fertile and infertile stem production of future intermediate wheatgrass varieties as they develop.

Whereas intermediate wheatgrass grain and biomass yields have been shown to respond to nitrogen fertilization in the past (Jacob M. Jungers, DeHaan, et al., 2017), we did not find a significant effect of nitrogen fertilization treatment on yield in any model. Similarly, the nitrogen by stand morphology parameter interaction was only significant in two models out of 26, indicating that treatment did not interact with most stand morphology parameters to affect either biomass or grain yield in either field. Since this intermediate wheatgrass was produced after soybeans, it is possible that there was abundant mineralizable soil nitrogen, meeting the needs of intermediate wheatgrass, thus allowing the control to produce yields similar to the fertilized treatments. Given the interest in intermediate wheatgrass for mitigating nitrate pollution (Jacob M. Jungers et al., 2019), it is interesting that stand development was not impacted by these different nitrogen fertilization strategies.

CONCLUSION

Original plants and vegetative propagules exhibited different patterns with regard to changes over time in their population, stem, and rhizome densities. Original plant density declined after anthesis of the first year, while vegetative propagule density steadily increased throughout the growing season. Although the number of original plants declined, original plant stem density increased steadily intra-annually, while vegetative propagule stem density fluctuated intra-annually in the 1 yo stand. In the 2 yo stand, vegetative propagule population did not increase intra-annually, which could be a response to canopy closure and changes in light quantity. Shade is known to inhibit bud formation on rhizomes (Tomlinson et al., 2007), so the overall greater stem density – especially at green up – may have limited vegetative propagule development.

A positive relationship between seed production, aboveground vegetative growth, and asexual reproduction in 1 yo intermediate wheatgrass was observed, which could have promising implications for producers looking to intermediate wheatgrass as a dual use grain and forage system. While the relationship diminished in 2 yo intermediate wheatgrass, there were no negative associations observed, suggesting that managing for grain yields need not reduce biomass yields. In both stands, spike density predicted both grain and biomass grain yield. Therefore, managing for spike density is a viable strategy for improving seed production in years one and two of intermediate wheatgrass production.

TABLES

Table 1. Stage index, date, day of year (DOY), and growing degree days (GDDs) at four sampling times in both 1 year old and 2 year old intermediate wheatgrass

Sampling time	**Stage index**	**Date**	**DOY**	**GDDs**
	1 year old stand			
Spring regrowth	1.3	7-May	127	493
Anthesis	3.7	14-Jun	164	1443
Harvest	4.9	21-Aug	231	3339
Fall regrowth	1.5	23-Oct	293	4523
	2 year old stand			
Spring regrowth	1.3	2-May	122	373
Anthesis	3.7	12-Jun	162	1391
Harvest	4.9	13-Aug	223	3109
Fall regrowth	1.5	29-Oct	299	4589

Table 2. 30 year normal (30 yr) and 2018 average temperature and monthly rainfall for growing season in Rosemount, MN

	May	June	July	Aug	Sept	Oct
	------------ mm ------------					
30 yr	103.0	120.0	114.0	120.0	92.0	73.0
2018	109.0	154.0	111.0	99.0	157.0	91.0
	------------ ºC ------------					
30 yr	14.3	19.6	21.9	20.7	16.0	8.9
2018	18.6	21.4	22.0	21.1	17.4	6.2

Table 3. Results of one-way analysis of variance to test for significant sampling time effects on whole plant, average stem weight (Avg stem), rhizome weight, total plant density, original plant density, vegetative propagule density, seed propagule density, total stem density, original stem density, vegetative propagule tiller density, seed propagule tiller density, total rhizome density, original rhizome density, and vegetative propagule density

		1 year old Sampling Time	2 year old Sampling Time
Mass	Whole plant	***	*
	Avg stem	***	***
	Rhizome	NS	**
Density	Total plant	*	**
	Original plant	***	NS
	Vegetative propagule	***	NS
	Seed propagule	NS	***
	Total stem	***	***
	Original stem	***	***
	Vegetative propagule tiller	***	***
	Seed propagule tiller	NS	***
	Total rhizome	***	***
	Original rhizome	***	**
	Vegetative propagule	NS	NS

NS = P > 0.05; . = 0.05 < P < 0.10; * = 0.01<P<0.05; **=0.001<P<0.01; *** P<0.001

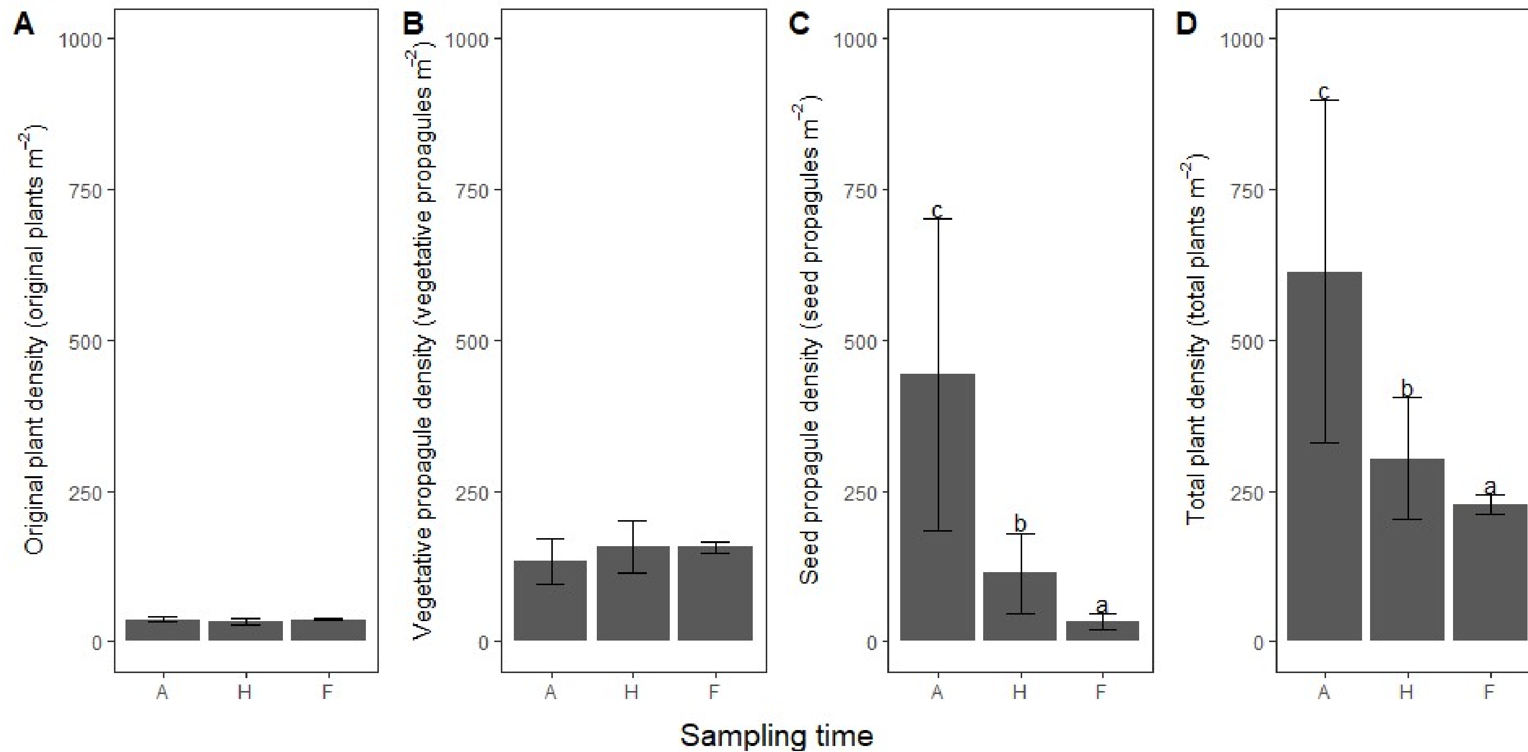
Table 4. Results of one factor regression analysis to test for significant slope of total spikes, total rhizomes, total plants, original plants, vegetative propagule stems, total stems, and seed propagule stems (2 year old stand only) on grain yield and biomass yield in a 1 year old and 2 year old intermediate wheatgrass stand

			Response			
Stand age (yrs)	**Sampling Time**	**Regressor**	**Grain yield**		**Biomass yield**	
			slope	**R^2**	**slope**	**R^2**
1	Harvest	Total spikes	0.69	0.7	0.12	0.44
1	Harvest	Total rhizomes	2.31	0.5	NA	NA
1	Harvest	Total plants	1.34	0.34	NS	NS
1	Harvest	Original plants	3.06	0.46	NA	NA
1	Harvest	Vegetative propagule stems	0.47	0.35	NS	NS
1	Harvest	Total stems	0.17	0.6	0.04	0.66
2	Harvest	Total spikes	1.33	0.44	0.12	0.58
2	Harvest	Total rhizomes	NS	NS	NS	NS
2	Harvest	Total plants	NS	NS	NS	NS
2	Harvest	Original plants	NS	NS	NS	NS
2	Harvest	Vegetative propagule stems	NS	NS	NS	NS
2	Harvest	Total stems	NS	NS	NS	NS
2	Harvest	Seed propagule stems	NS	NS	NS	NS

$P<0.05$
NA= model not run

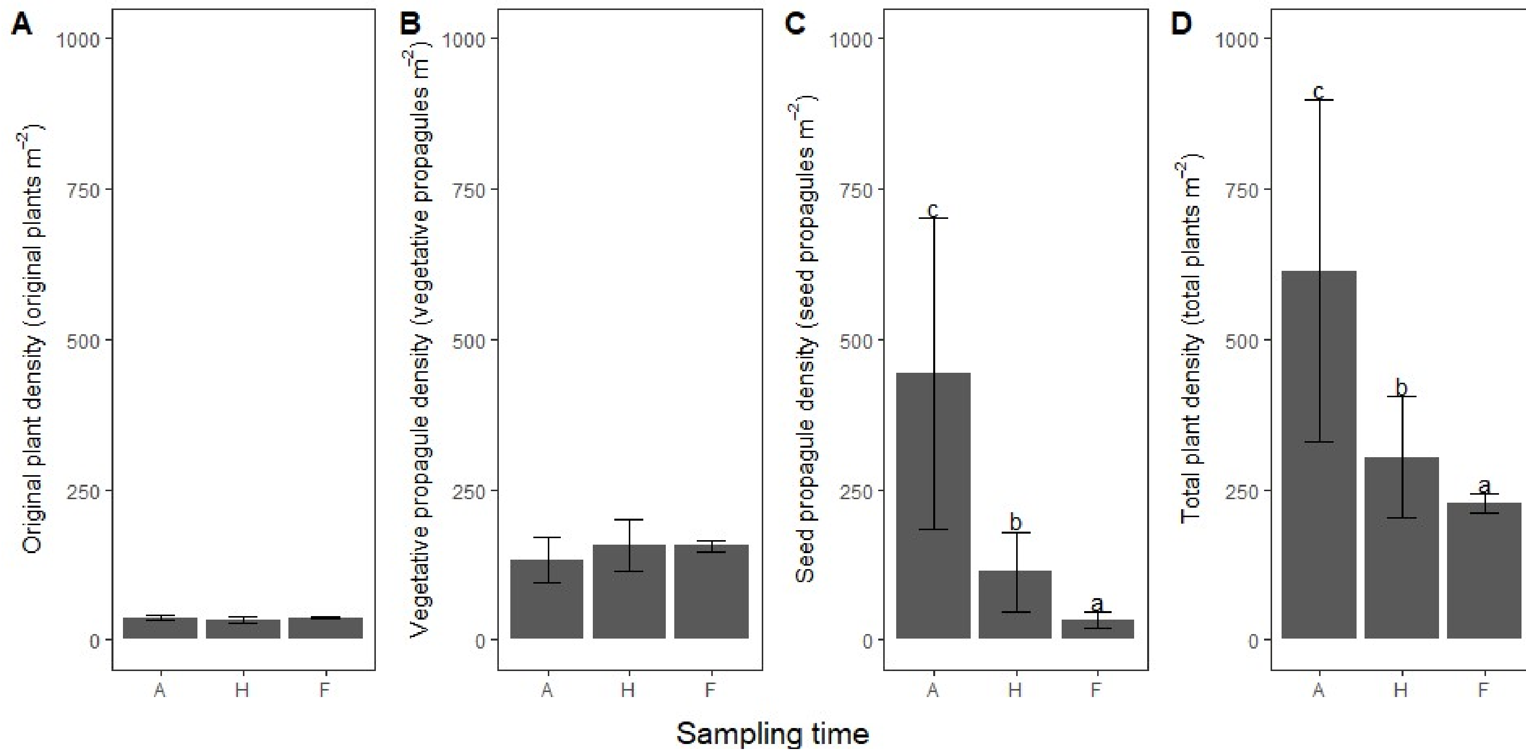
FIGURES

Figure 1. Density of original plants, vegetative propagules, seed propagules, and total plants in a 1 year old intermediate wheatgrass stand



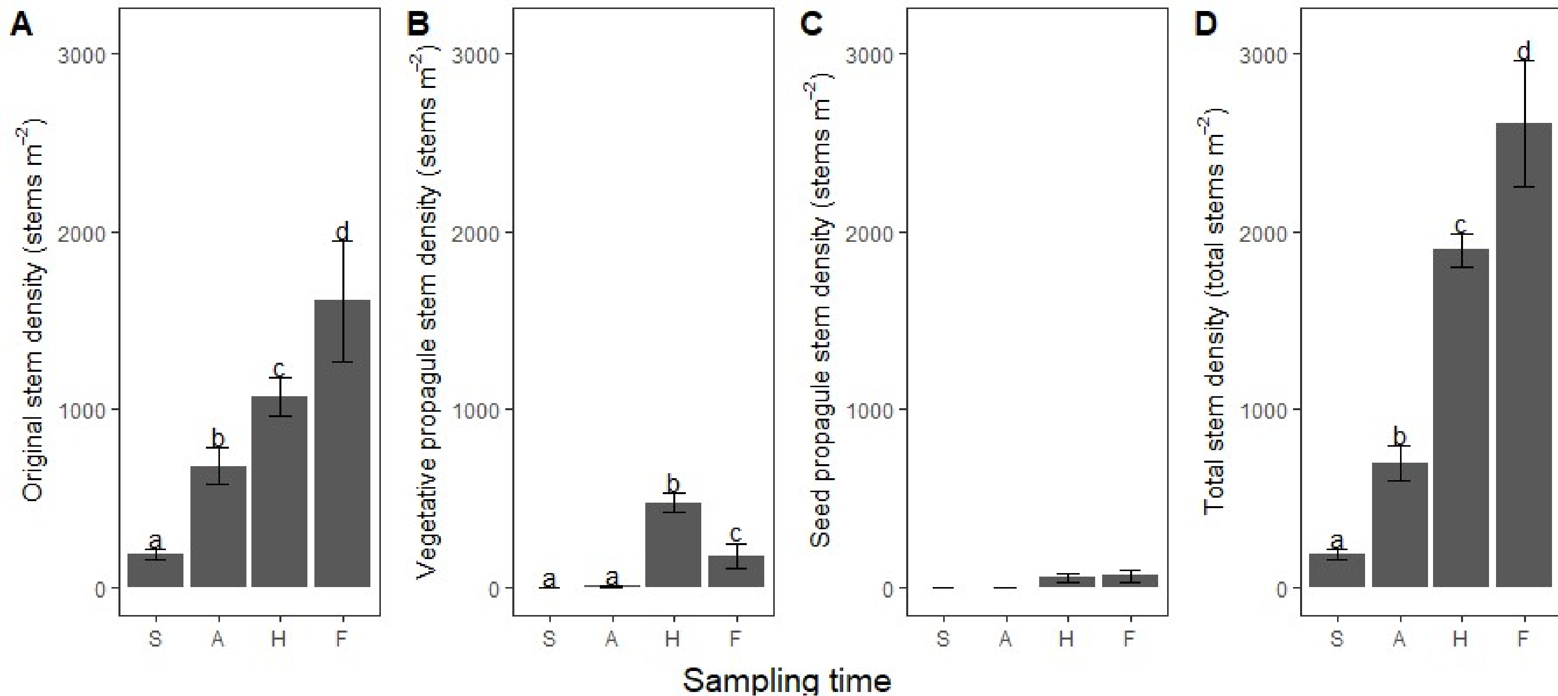
S= spring regrowth (early May), A= anthesis onset (mid-June), H= harvest (late August), and F= fall regrowth (late October). Error bars represent standard error of the mean. Within graphs, means with the same or no letters are not different as per Tukey's HSD based on a generalized linear model using a log-link function.

Figure 2. Density of original plants, vegetative propagules, seed propagules, and total plants in a 2 year old intermediate wheatgrass stand



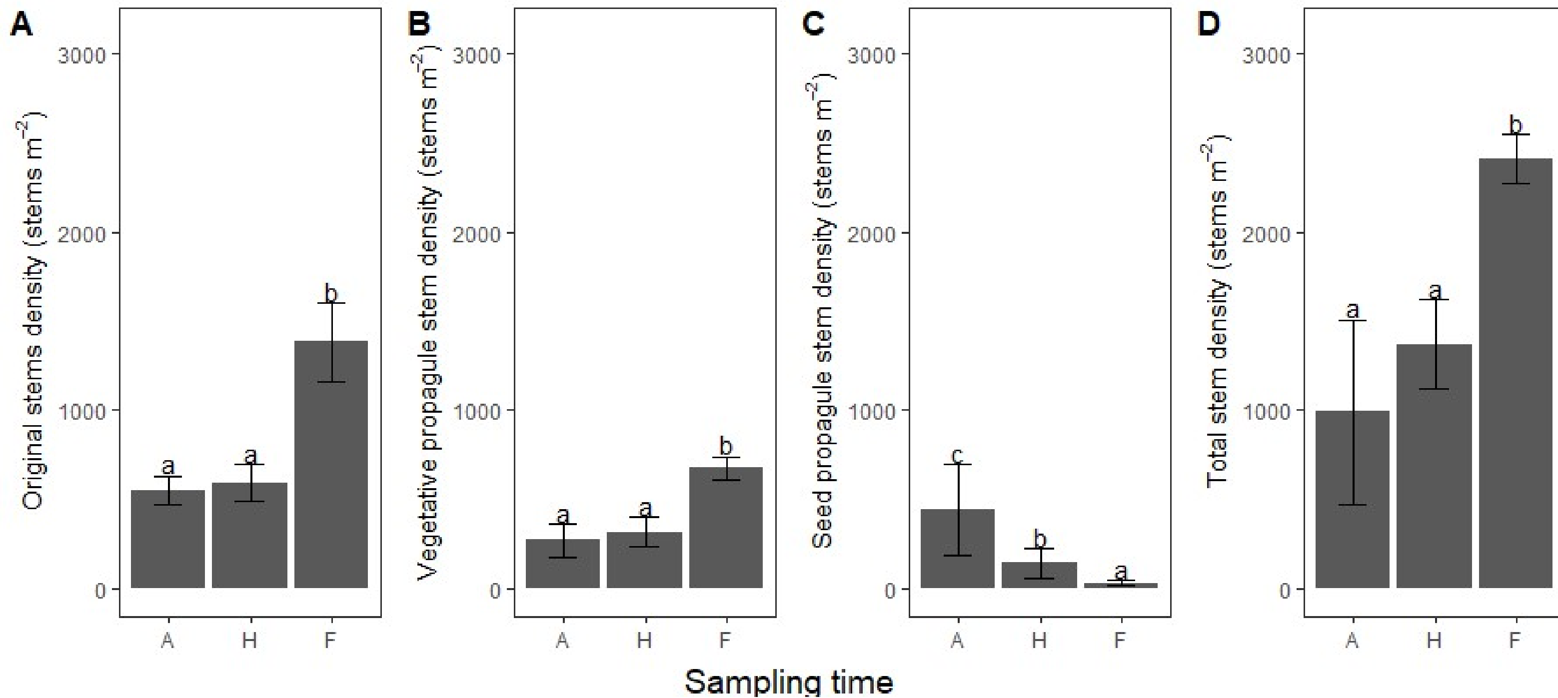
A= anthesis onset (mid-June), H= harvest (late August), and F= fall regrowth (late October). Error bars represent standard error of the mean. Within graphs, means with the same or no letters are not different as per Tukey's HSD based on a generalized linear model using a log-link function.

Figure 3. Density of original stems, vegetative propagule stems, seed propagule stems, and total stems in a 1 year old intermediate wheatgrass stand.



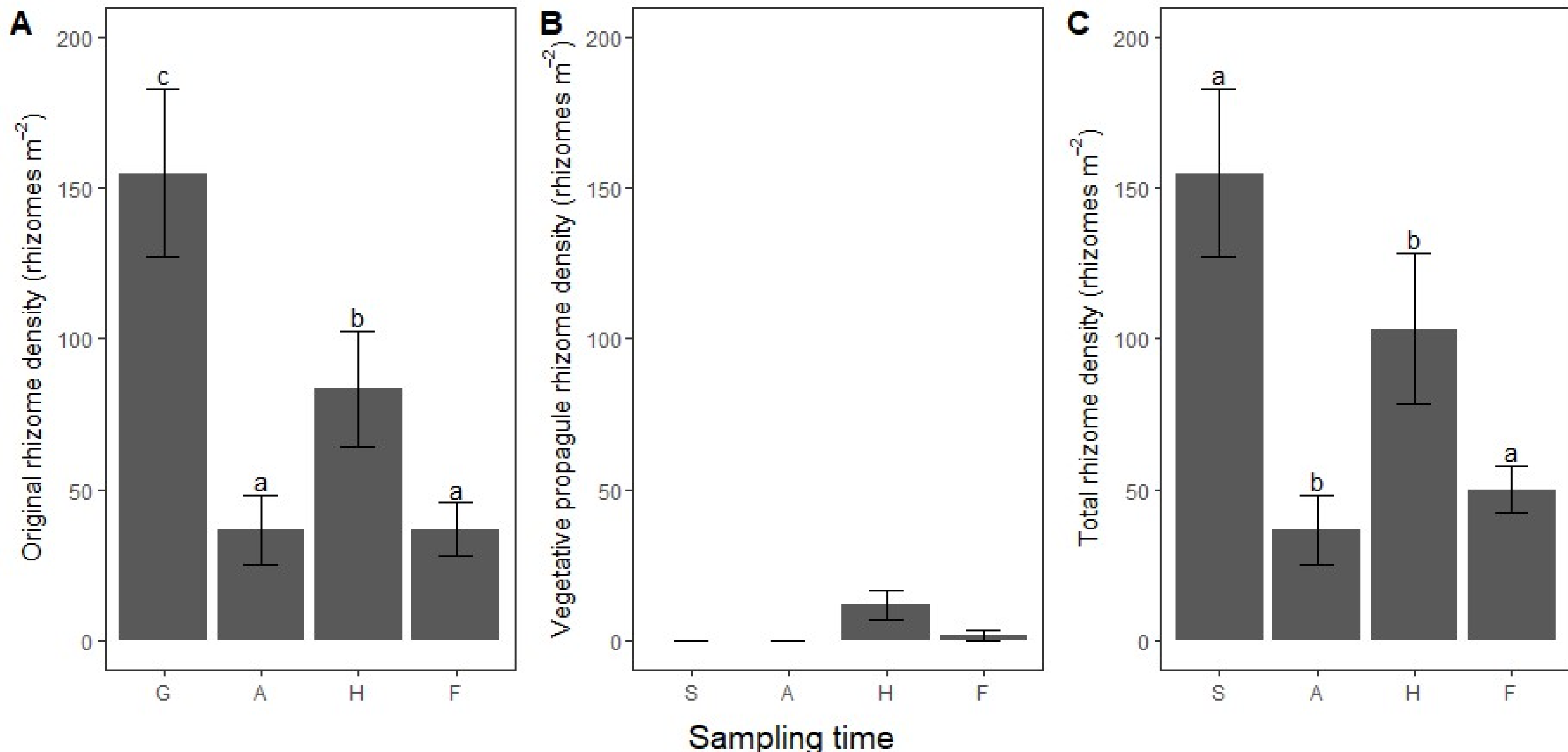
S= spring regrowth (early May), A= anthesis onset (mid-June), H= harvest (late August), and F= fall regrowth (late October). Error bars represent standard error of the mean. Within graphs, means with the same or no letters are not different as per Tukey's HSD based on a generalized linear model using a log-link function.

Figure 4. Density of original stems, vegetative propagule stems, seed propagule stems, and total stems in a 2 year old intermediate wheatgrass stand.



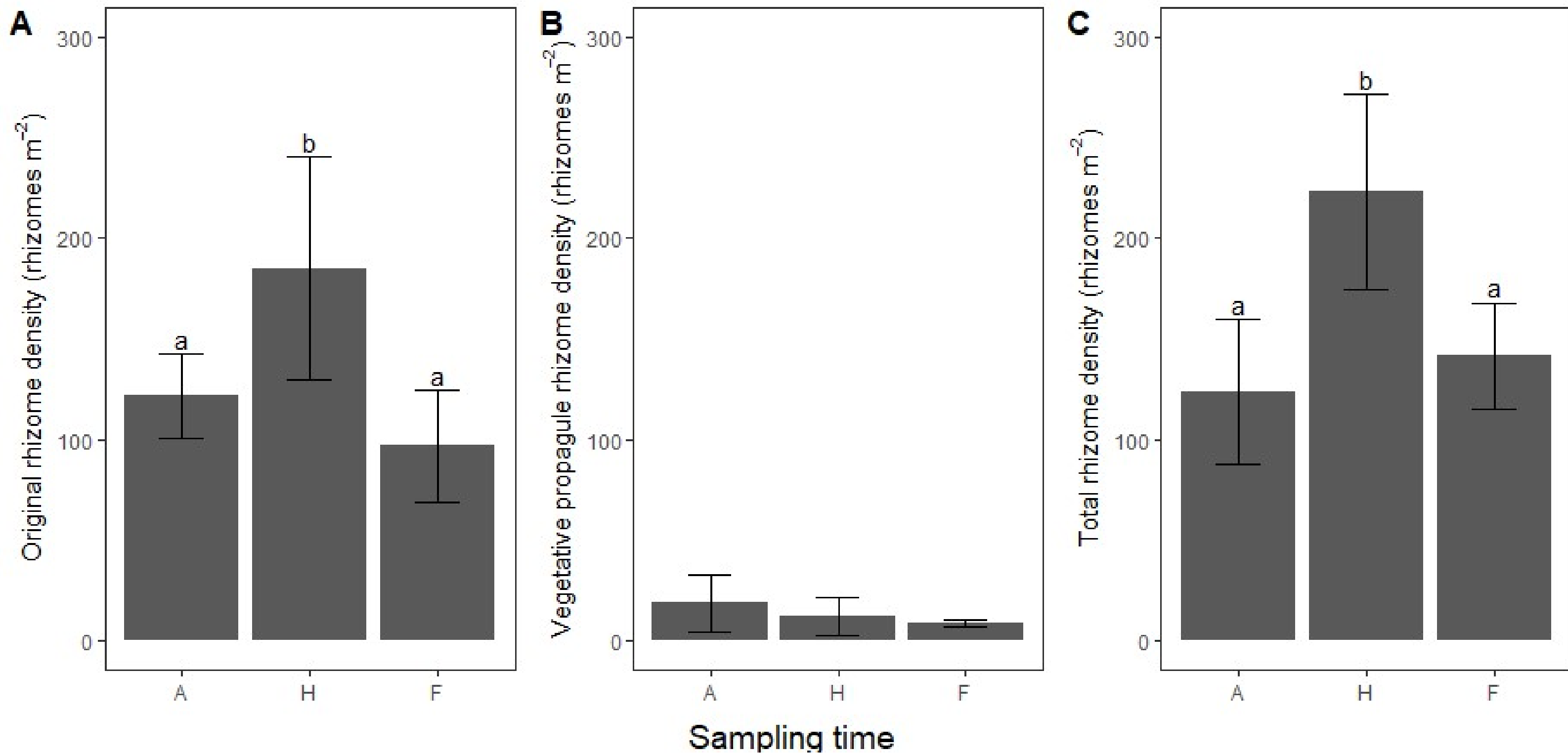
A= anthesis onset (mid-June), H= harvest (late August), and F= fall regrowth (late October). Error bars represent standard error of the mean. Within graphs, means with the same or no letters are not different as per Tukey's HSD based on a generalized linear model using a log-link function.

Figure 5. Density of original rhizomes, vegetative propagule rhizomes, and total rhizomes in a 1 year old intermediate wheatgrass stand.



Sampling was to 20 cm depth, capturing the entire crown of each individual plant. S= spring regrowth (early May), A= anthesis onset (mid-June), H= harvest (late August), and F= fall regrowth (late October). Error bars represent standard error of the mean. Within graphs, means with the same or no letters are not different as per Tukey's HSD based on a generalized linear model using a log-link function.

Figure 6. Density of original rhizomes, vegetative propagule rhizomes, and total rhizomes in a 2 year old intermediate wheatgrass stand.



Sampling was to 20 cm depth, capturing the entire crown of each individual plant. A= anthesis onset (mid-June), H= harvest (late August), and F= fall regrowth (late October). Error bars represent standard error of the mean. Within graphs, means with the same or no letters are not different as per Tukey's HSD based on a generalized linear model using a log-link function.

CHAPTER THREE
INTRA-ANNUAL SOIL AND PLANT NITROGEN DYNAMICS IN ONE AND TWO YEAR INTERMEDIATE WHEATGRASS (Thinopyrum intermedium)

Dobbratz, Michelle; Jungers, Jacob; Sheaffer, Craig; Grossman, Julie; Gutknecht, Jessica

SYNOPSIS

Intermediate wheatgrass (*Thinopyrum intermedium*; IWG) is a newly domesticated perennial grass being explored as a grain and forage crop. Little is known about nitrogen dynamics in an IWG agroecosystem. Here, we describe the nitrogen content and concentration in IWG shoot, root, and grain tissue at spring regrowth (early May), anthesis (mid-June), harvest (late August), and fall regrowth (late October) in three nitrogen fertilization treatments in 1 and 2 year old IWG stands. The three fertilization treatments were 1) 80 kg N ha^{-1} applied at spring regrowth (spring), 2) 40-40 kg N ha^{-1} applied at spring regrowth and anthesis (split), and 3) an unfertilized control. In all plots, we monitored soil for NO_3^--N, NH_4^+-N, net mineralized NO_3^--N, and net mineralized NH_4^+-N in the upper 15 cm of the soil and total nitrogen throughout the profile. Shoot biomass was similar at harvest and fall regrowth in the 1 year old stand, indicating IWG regrowth was substantial and plentiful for fall grazing. Overall, our results show that fertilization treatment and soil mineral nitrogen affects whole plant nitrogen concentration, but fertilization treatment does not affect biomass. In the both stands, shoot and root nitrogen concentration declined at harvest, suggesting that surplus nitrogen was allocated to grain or that growth during that time period led to dry matter dilution. Fertilization did not have a lasting effect on soil mineral nitrogen levels, net mineralization, or total soil nitrogen, suggesting that in an IWG system, soil nitrogen dynamics are buffered from nitrogen fertilization regimes. Fall regrowth had the lowest overall mineral nitrogen levels, but the highest total soil nitrogen levels, suggesting a large influx of organic nitrogen between harvest and fall regrowth. This, along with a loss of total root nitrogen in the 2 year old control treatment, suggests root dieback. Since there was no accompanying loss of root biomass in the 1 year old stand, any root dieback that occurred in that stand between harvest and fall regrowth was matched by growth. This large influx of organic nitrogen in the fall may mineralize over the winter to provide available nitrogen for the subsequent year of growth. Based on this first nitrogen dynamics study of IWG, the crop is well suited to both produce grain and forage as well as protect subsurface water from leached nitrate.

INTRODUCTION

Perennial grass crops have the capacity to take up large amounts of nitrogen (Lasisi, Akinremi, Tenuta, & Cattani, 2018), affecting both their production as crops and their use as environmental regulators. For example, perennial crops can reduce nitrate leaching (Jungers, DeHaan, Mulla, Sheaffer, & Wyse, 2019) and nitrous oxide emissions (I. Gelfand, Scherbak, Millar, Kravchenko, & Robertson, 2016) when compared with annuals. As excess nitrate can leach into groundwater, causing concerns for human health (Ward et al., 2018), strategies must be developed that can deliver mineral nitrogen to crops without residual soil nitrate. While perennials are known to reduce off-target nitrogen export through their capacity to take up large quantities of nitrogen, they can also have high agronomically optimal nitrogen rates (AONR), which are accompanied by nutrient loss (McGowan, Min, Williams, & Rice, 2018).

Intermediate wheatgrass [*Thinopyrum intermedium* (Host) Barkworth and D.R. Dewey] is a cool-season grass being improved as the world's first widely cultivated perennial grain crop and shows promise as a dual use grain and forage crop (Ryan et al., 2018). As a dual use crop, the AONR of intermediate wheatgrass is 61 to 96 kg ha^{-1} depending on the stand age (Jungers, DeHaan, Betts, Sheaffer, & Wyse, 2017). While intermediate wheatgrass nitrogen demands increase with age, grain yields are known to decline with age (Jungers et al., 2017). Under intermediate wheatgrass, nitrate leaching was reduced by greater than 86% compared with annual wheat production (Steve W. Culman et al., 2013). Relatedly, intermediate wheatgrass has 12-16 times the root nitrogen content as annual wheat (Sainju et al., 2017). Despite the existing body of work on intermediate wheatgrass, little is known about how nitrogen addition affects its

nitrogen uptake or nitrogen partitioning. Likewise, no work has been done on the soil nitrogen mineralization under this crop. Intermediate wheatgrass shows potential as a crop that can be fertilized according to agronomically optimal nitrate rates without allowing nitrate to leach into the groundwater (Jungers et al., 2019). However, in order to establish best management practices that balance high yields with reduced off-target nitrogen transport, it is essential to understand the connection between the dynamics of soil mineral nitrogen and those of plant growth and partitioning.

One strategy that might increase the nitrogen use efficiency of perennials is fertilizer split application. There is evidence that splitting nitrogen can increase seed yield and harvest index in cool season grasses (Koeritz, Watkins, & Ehlke, 2013; Vleugels et al., 2017), and in general, split nitrogen applications are recommended for the production of cool season grass seed (Dodds, Carter, Meyer, & Haas, 1987; Horton et al., 1990). A better understanding of the effects of splitting nitrogen in intermediate wheatgrass on grain yield, forage yield, and nitrogen dynamics might allow researchers and producers to more efficiently and effectively manage the crop.

In addition to studying the effects of splitting nitrogen application, understanding the overall nitrogen dynamics in perennial grasses could illuminate pathways for agronomic and genetic improvement. While a detailed study on intra-annual nitrogen dynamics has been conducted on switchgrass (*Panicum virgatum*, L.), a warm season perennial (Garten et al., 2010) and a nitrogen budget has been conducted on a cool season perennial wheatgrass (Redmann & van Kessel, 1992), neither of these accounted for seed. Seed represents a large portion of nitrogen storage in grass crops and is the primary economic output in intermediate wheatgrass.

Our objective was to understand the effect of nitrogen addition on plant and soil nitrogen dynamics in 1 and 2 year old intermediate wheatgrass fertilized with three nitrogen treatments. To that end, we sampled roots, shoots, grain, and soil at four timepoints under three nitrogen treatments throughout the growing season. To better understand the plant/soil interface, we also correlated plant and soil parameters.

MATERIALS AND METHODS

Experimental design and location

Research plots were located 24 miles south of St. Paul, MN at the Rosemount Research and Outreach Center (44°41'05"N 93°04'13"W). Intermediate wheatgrass was sown in 15 cm rows in two nearby fields. The 1 year old stand was sown in September of 2017 and the 2 year old stand was sown in September of 2016. In both cases, the soil was a Tallula silt loam (Coarse-silty, mixed, superactive, mesic Typic Hapludoll) and the previous crop was the soybean (*Glycine max* L.) phase of a corn (*Zea mays* L)-soy rotation. In the 2018 growing season, there were no climactic abnormalities that would have affected intermediate wheatgrass production (Table S1).

A randomized complete block design with four replicates was used in both fields. The three fertilization treatments were 1) 80 kg N ha^{-1} urea in early May (spring), 2), 40 kg N ha^{-1} urea in early May plus 40 kg N ha^{-1} urea in mid-June (split), and 3) no fertilization (control). Due to heavy snowfall in April 2018, fertilization took place in early May. The anthesis fertilizer application occurred in mid-June. Fertilization always took place after sampling for plant tissue.

Mineral soil sampling and lab analysis

Soil sampling occurred at spring regrowth, anthesis, harvest, and fall regrowth (Table 1). To collect soil for mineral nitrogen and *in-situ* net nitrogen mineralization analysis, sharpened 5 cm diameter by 17 cm tall PVC cores were used (Raison, Connell, & Khanna, 1987). *In-situ* cores had four evenly spaced 0.5 cm holes drilled in the top to allow oxygen exchange. In each plot, two cores were driven to 15 cm 15 cm apart. One

core was immediately removed, and the soil was placed in a cooler. The remaining core was left *in-situ*, capped, until the next sampling event. At the subsequent sampling event, *in-situ* cores were removed, and the soil placed in a cooler prior to mineral nitrogen analysis. The difference between the mineral nitrogen in the *in-situ* cores after incubation and the mineral nitrogen in the core removed at the initial time was considered to be the net nitrogen mineralization (Raison et al., 1987). Fertilization took place before capping *in-situ* cores.

Once removed from the field, soil for mineral nitrogen analysis was kept at 4° C and nitrogen was extracted within 7 days. First, 4.0g +/- 0.05g of wet soil was placed in an acid washed or sterile 50 mL centrifuge tube. Then, 20 mL 2M KCl was added to each tube. Tubes were shaken for 1 hour at 200 rpm, then centrifuged for 5 min at speed at 2500 rpm. Aliquot was then filtered through pre-rinsed Whatman #42 filter paper into an acid washed 50 mL beaker before being stored in a scintillation vial at -20° C.

Soil NH_4^+-N was determined using a well plate assay modified from Sinsabaugh, Reynolds, & Long (2000). In brief, 100 µL pre-prepared standards and soil extracts were transferred into a 96 well plate. Then, 40 µL ammonia salicylate was added to each well, converting ammonia to ammonium. Exactly 3 minutes after beginning to add the ammonia salicylate to the well plate, 40 µL ammonia cyanurate was added to the well plate. Once ammonia cyanurate was added to each well, the plate was incubated in the dark for 20 minutes. After incubation, the plate was read at 630 nm using a BioTek Synergy HT microplate spectrophotometer (BioTek, Winooski, VT).

Soil NO_3^--N was determined using a well plate assay modified from Doane and Horwath (2003). In brief, 100 µL pre-prepared standards and soil extracts were

transferred into a 96 well plate. Then, 1 mL vanadium cocktail solution was added to each tube. Tubes were covered and incubated overnight. Between 15 and 15 and a half hours later, 200 μL was transferred from each tube into the well of a 96 well plate. The plate was then read at 540 nm using a BioTek Synergy HT microplate spectrophotometer (BioTek, Winooski, VT).

Total soil nitrogen and roots

Soil and roots were collected at spring regrowth, anthesis, harvest, and fall regrowth (Table 1) using a hydraulic soil probe (Giddings Machine Company, Inc.; Colorado USA) to the beginning of the gravel layer, 80-102 cm deep. Four 5 cm cores were randomly taken from each plot- two for bulk soil and two for roots. Cores were partitioned into 0-15 cm, 15- 30 cm, 30-45 cm, 45-60 cm, and 60 cm + increments. Root cores were stored at -20° C until further analysis. Soil cores were dried at 35° C until constant weight, then contracted for total nitrogen analysis via dry combustion (Brookside Laboratories, New Bremen, OH). Percent total nitrogen is reported on a per mass basis. Root mass was separated from soil via a hydropneumatic elutriation system (Smucker, McBurney, & Srivastava, 1982), which forces water through soil being held by a series of mesh screens until only biomass remains. Samples were cleaned by hand to remove above ground tissue, rhizomes, and sand. Roots were then dried at 35° C until constant weight. Dry weights were recorded for each sample. As samples were too small to grind, roots were combined across depths before being ground to pass through a 1-mm screen. Finally, the total nitrogen of the tissue was determined using a combustion analyzer. Roots collected at anthesis were not processed due to resource constraints.

Aboveground plant tissue and growth staging

Intermediate wheatgrass aboveground plant tissue was collected at spring regrowth, anthesis, harvest, and fall regrowth (Table 1) by hand sampling three 33 cm by 15 cm (0.05 m^2) quadrats centered on the row. Plants were harvested at the soil surface, cleaned, and separated into weeds and intermediate wheatgrass tissue. Tissue was dried at 35° C until constant mass and recorded. Then the intermediate wheatgrass tissue was ground to pass through a 1-mm screen before being analyzed for total nitrogen on a combustion analyzer. No aboveground tissue was collected at spring regrowth in the 2 year old stand.

The growth stage index of intermediate wheatgrass plants was determined by following the procedures of Moore & Moser (1995). Briefly, 1.0-1.9 is the tillering stage, in which leaves appear. Anthesis occurs at stage 3.7, and harvest occurs at stage 3.9. Our observations were validated by Jungers et al. (2018) who have previously correlated growing degree days with growth stage in intermediate wheatgrass.

Grain yield, net nitrogen uptake, lodging, and heights

Lodging and grain yield were measured at harvest (late August). Grain was harvested by hand from three 33 cm by 15 cm quadrats centered on the row, then dried at 35° C until constant weight. Dry weights were recorded. Then, grain was ground to pass through a 1-mm screen. Finally, ground grain was run through a combustion analyzer to determine total N. Net uptake was calculated as the nitrogen content of the roots, shoots, and grain at harvest minus the nitrogen content of the roots and shoots at spring regrowth.

Lodging was observed on a whole plot basis by multiplying the percent of stems that were lodged by the degree to which they were lodged, on a 0-100% scale. Percentages were then converted to a 10 point scale, where 10% = 1 and 90% = 9. The same individual rated each observation. Heights from the soil to the tip of the inflorescence were taken as the average of 5 randomly selected plants from at least 1 m from the border of the plot.

Statistical analysis and calculations

All ANOVAs and ANCOVAs were conducted in R (R Core Team, 2019) using the nlme package (Pinheiro J., Bates D., DebRoy S., 2019). Nitrogen fertilization treatment and sample timing were considered fixed effects, and block was considered a random effect. When analyzing total soil nitrogen, average sampling depth was considered a continuous variable. Because the two stand ages were visually different and a preliminary analysis found significant interactions were evident between stand age and several other variables, each stand age was analyzed separately. *Post hoc* analysis was conducted using the emmeans package (Lenth et al., 2019), separating means using Tukey's HSD. Correlations between plant and soil parameters were conducted using linear models (lm).

Growing degree day summation commenced at the first day of 2018 after five consecutive days above 0° C. Daily maximum temperature was added with daily minimum temperature and divided by two to obtain average daily temperature. The number of growing degree days on any given day is the sum of any previous daily

average temperature above 0° C, beginning on the fifth consecutive day of the year when the average temperature was above 0° C.

RESULTS

Significant treatment effects

In the 1 year old stand, fertilization treatment affected shoot nitrogen concentration, soil NO_3^--N, soil NH_4^+-N, and lodging (Table 2; Table 3; P=0.011). Fertilization treatment interacted with sampling time to affect soil NO_3^--N, soil NH_4^+-N, and net mineralized NO_3^--N in the 1 year old stand (Table 3). In the 2 year old stand, fertilization treatment affected whole plant nitrogen content, root nitrogen concentration, grain nitrogen concentration, net nitrogen uptake. and net mineralized NO_3^--N from spring regrowth to harvest (Table 4; Table 3). Fertilization treatment interacted with sampling time to affect soil NO_3^--N and soil total N in the 2 year old stand (Table 4; Table 3).

Nitrogen content and concentration

Root, shoot, and grain nitrogen content were not affected by fertilization treatment in either stand age, but whole plant nitrogen was affected by treatment in the 2 year old stand (Table 2; Table 4). In the 1 year old stand, shoot nitrogen content was greater at anthesis, harvest, and fall regrowth than at spring regrowth (Table 5). Similarly, in the 1 year old stand, whole plant nitrogen content was greater at harvest and fall regrowth than at spring regrowth (Table 5). In the 2 year old stand, root nitrogen content was greater at harvest than at spring regrowth and fall regrowth (Table 5). Also in the 2 year old stand, whole plant nitrogen content was greater in the control treatment than in the spring or split fertilization treatments.

Intermediate wheatgrass tissue nitrogen concentration varied by fertilization treatment and sampling time (Table 2; Table 4). In the 1 year old stand, intermediate wheatgrass shoot tissue in the spring fertilization treatment had greater nitrogen concentration than intermediate wheatgrass shoot tissue in the control and spring fertilization treatments. Overall, in the 1 year old stand shoot tissue had greater nitrogen concentration at spring regrowth and anthesis than at harvest and fall regrowth (Table 5). Similarly, in the 1 year old stand root tissue had greater nitrogen concentration at spring regrowth than at harvest and fall regrowth (Table 5). Also in the 1 year old stand, whole plant nitrogen concentration was greatest at spring regrowth, followed by fall regrowth, followed by harvest (Table 5). With regards to the 2 year old stand, the root tissue lost nitrogen concentration between spring regrowth and harvest (Table 5). Lastly, the shoot tissue nitrogen concentration in the 2 year old stand was greater at anthesis and fall regrowth than at harvest (Table 5).

Above- and belowground biomass and grain yield

Fertilization treatment did not affect above- or belowground biomass in either stand age (Table 2; Table 4). However, sampling time affected shoot biomass, root biomass, and whole plant biomass in at least one stand age (Table 2; Table 4). In the 1 year old stand, shoot biomass was greatest at harvest and fall regrowth and lowest at spring regrowth (Table 5). Similarly, in the 1 year old stand, whole plant biomass was greater at harvest and fall regrowth than at spring regrowth (Table 5). In the 2 year old stand, root biomass was greater at harvest than at spring regrowth and fall regrowth (Table 5). Also in the 2 year old stand, whole plant biomass in the control treatment was

greater at harvest than fall regrowth. There was no treatment effect on grain yield in the 1 year old stand, but lodging was greater in the spring fertilization treatment than in the control treatment (Table 5; $P = 0.011$).

Total soil nitrogen

Nitrogen fertilization treatment was not a significant factor in total soil nitrogen, but there were significant differences across sampling times (1 year old stand $P < 0.001$, 2 year old stand $P = 0.032$), depth (1 year old stand $P < 0.001$, 2 year old stand $P < 0.001$), and in the 2 year old stand, the interaction of fertilization treatment with sampling time ($P = 0.034$). In both fields, more soil nitrogen was observed at fall regrowth than earlier in the season (Figure 1). This effect was most pronounced in the 1 year old field, when the soil nitrogen at fall regrowth was nearly than twice as much as all other timepoints at most depths (Figure 1).

Soil mineral nitrogen

NO_3^--N decreased over time in both fields (Table 2; Figure 3). At spring regrowth in the 1 year old field, the spring and split fertilization treatments had greater NO_3^--N than the control treatment (Figure 3). In both the control and spring fertilization treatments in the 1 year old field, the NO_3^--N concentration decreased between spring regrowth and anthesis and then leveled off for the rest of the season (Figure 3). In the 2 year old stand, nearly every fertilization treatment by sampling time combination had statistically similar concentrations of NO_3^--N (Figure 3).

NH_4^+-N was affected by fertilization treatment, sampling time, and their interaction in the 1 year old field and affected by sampling time only in the 2 year old field (Table 3). At spring regrowth in the 1 year old field, the split and spring fertilization treatments had a greater concentration of NH_4^+-N than the control treatment (Figure 2). Averaged across fertilization treatments in the 2 year old field, there was a greater concentration of NH_4^+-N at spring regrowth than at harvest and fall regrowth (Figure 2).

Net mineralized nitrogen

Fertilization treatment did not affect net mineralized NO_3^--N or NH_4^+-N in either stand age (Table 3). Sampling time and fertilization treatment by sampling time interaction effected net mineralized NO_3^--N in both stand ages (Table 3). Within the spring fertilization treatment in the 1 year old stand, net mineralized NO_3^--N was greater from harvest to fall regrowth than from spring regrowth to anthesis (Figure 4). In the 2 year old stand, net mineralized NO_3^--N was greater in the split fertilization treatment from spring regrowth to anthesis than in the control treatment during the same time (Figure 4). The net mineralized NO_3^--N was also greater from spring regrowth to anthesis than from anthesis to harvest in the 2 year old split fertilization treatment (Figure 4). With regard to net mineralized NH_4^+-N, there was no effect of treatment, sampling time, or interaction.

Plant/ soil correlations

In the 1 year old stand, NH_4^+-N concentration was negatively correlated with grain yield (R^2 = 0.38, slope = -133), grain nitrogen concentration (R^2 = 0.34, slope = -

0.42), and grain nitrogen content ($R^2 = 0.35$, slope = 0.07). In the 2 year old stand, NO_3^--N had a weak negative correlation with root nitrate concentration ($R^2 = 0.09$, slope = -0.007). No other correlations between soil mineral nitrogen or net mineralized nitrogen and tissue biomass, yield, nitrogen content, or nitrogen concentration were statistically significant.

DISCUSSION

Intermediate wheatgrass is a new crop that has demonstrated potential for reducing nitrogen losses in managed agroecosystems. Here, we sought to understand the dynamics of nitrogen in intermediate wheatgrass systems. Our findings have implications for those planting intermediate wheatgrass for environmental benefits as well as those looking for best management practices for the dual use crop.

Plant tissue

Patterns of above- and belowground biomass development show suitability for dual use with grazing systems or multifunctional agroecosystems. Shoot biomass was comparable at fall regrowth and harvest in the 1 year old stand, demonstrating potential for regrowth that could be grazed. Likewise, root biomass was comparable at harvest and at fall regrowth in the 1 year old stand. The persistence of root productivity post-harvest coupled with evidence that defoliating intermediate wheatgrass increases root biomass (Pugliese et al., 2019) suggests there is little to no tradeoff between the agronomic productivity of intermediate wheatgrass and its ability to maintain large root systems in 1 year old intermediate wheatgrass. This is important because larger root systems are more capable of protecting subsurface water and sequestering carbon (Lal & Augustin, 2012;

Sullivan, Jiang, & Hull, 2000). Thus, our findings suggest that intermediate wheatgrass is well suited for dual use systems and that harvest of intermediate wheatgrass does not reduce the mass of its nitrate capturing root systems. However, the lower root biomass observed at fall regrowth in the 2 year old intermediate wheatgrass stand suggests there is a limit to the post-harvest growth of intermediate wheatgrass roots.

Even in the 1 year old stand, where comparable root biomass at harvest and fall regrowth suggests that either root growth stagnates post-harvest, or that root growth is matched by root dieback, there was likely some degree of dieback during that time period. Given that intermediate wheatgrass roots are known to grow in response to defoliation (Pugliese et al., 2019), it is likely some degree of dieback occurred during the same time period, and it is clear root dieback occurred in the 2 year old stand. Root dieback is a known result of defoliation in other cool season perennial grasses (Huang & Gao, 2000; X. Liu & Huang, 2002). Additionally, average total soil nitrogen more than doubled between harvest and fall regrowth in the 1 year old stand. Given that mineral nitrogen did not also increase during those time periods, the increased soil nitrogen was likely organic.

Our findings suggest that intermediate wheatgrass may be storing nitrogen in root tissue between spring regrowth and harvest, but not between harvest and fall regrowth. Intermediate wheatgrass root tissue had greater nitrogen concentration in the spring fertilization treatment than in the control treatment in the 1 year old stand, suggesting intermediate wheatgrass stores abundant nitrogen by enriching root tissue. Our results are similar to previous findings in a perennial system where fertilization increased root nitrogen content (Lemus, Parrish, & Abaye, 2008). No increase in root nitrogen

concentration was observed between harvest and fall regrowth, suggesting that retranslocation is not taking place during that time period. This is contrary to previous findings that perennial grasses translocate nutrients to belowground tissue prior to senescence (Smith, Monson, & Anderson, 1997). Harvest timing might also be related to root nitrogen translocation in perennial cropping systems, where more nitrogen may be translocated with later harvest dates (Wayman, Bowden, & Mitchell, 2014). Current intermediate wheatgrass varieties are harvested relatively early to avoid seed shatter. Perhaps later maturing varieties of intermediate wheatgrass could be explored for their potential to retain nitrogen and reduce fertilizer demands.

Intermediate wheatgrass shoot tissue nitrogen concentration declined between anthesis and harvest in both stands, suggesting that either nitrogen was translocated to the grain during that time (Sanchez-Bragado, Serret, & Araus, 2017) or that the decline was a result of dry matter dilution. Root nitrogen concentration also declined between spring regrowth and harvest in the 1 year old stand and the 2 year old stand, likely a result of dry matter dilution or translocation. In another perennial wheatgrass, it was found that 40% of nitrogen stored before fertilization treatment was translocated to vegetative tissue, regardless of fertilization treatment (Redmann & van Kessel, 1992). Taken with our findings that both stands lost nitrogen concentration between spring regrowth and anthesis, this suggests that early season root nitrogen may have been translocated to aboveground sinks.

The net nitrogen uptake from spring regrowth to harvest averaged 6.6 g N m^{-2} in 1 year old intermediate wheatgrass and 9.6 g N m^{-2} in 2 year old intermediate wheatgrass. This is in line with current fertilizer recommendations of 6.1 to 9.6 g N m^{-2}, assuming

little to no off-target nutrient transport (Jacob M. Jungers et al., 2019; Jacob M. Jungers, DeHaan, et al., 2017). Because the control did not have different net uptake values than the split and spring fertilization treatments, intermediate wheatgrass is likely capable of meeting its nitrogen demand from native soil mineral nitrogen alone.

Soil

The greater mineral nitrogen concentration in the spring fertilization treatment at spring regrowth and in the split fertilization treatment at spring regrowth and anthesis was due to having added fertilizer to the soil before sampling. The observed greater concentration of mineral nitrogen early in the season is typical, as nitrogen is mineralized over the winter and in the early spring (Malhi & Nyborg, 2010; M. C. Ryan, Kachanoski, & Gillham, 2000). Due to the greater concentration of mineral N at the spring regrowth sampling time, immobilization prevailed in between spring regrowth and anthesis. The net mineralization between harvest and fall regrowth suggests an influx of organic nitrogen, possibly from harvest residue or root dieback. In other species, defoliation has been shown to reduce root dry matter (Biswell & Weaver, 1933). If root dieback did occur, regrowth also occurred, since the total root nitrogen was not different at harvest and fall regrowth. In that case, soil nitrogen could have been from rhizodeposition, which is known to occur alongside root development (Shamoot, McDonald, & Batholomew, 1968). More work is needed to understand the implications of this nitrogen influx on nitrogen mineralization, in particular if mineralized nitrogen is taken up by intermediate wheatgrass or if it is leached during the freeze/ thaw cycles of the off season.

Since the amount of mineral nitrogen at fall regrowth and harvest were statistically similar, it can be inferred that the NO_3^--N produced by mineralization was taken up by intermediate wheatgrass. Alternatively, NO_3^--N produced by mineralization may have leached deeper into the soil profile. Given that intermediate wheatgrass is known to prevent leaching of nitrate below the root layer (Steve W. Culman et al., 2013; Jacob M. Jungers et al., 2019), it is likely that NO_3^--N was absorbed by intermediate wheatgrass. Furthermore, the lack of effect of fertilization treatment on NO_3^--N at harvest and fall regrowth implies that intermediate wheatgrass readily absorbed surplus mineral nitrogen.

Net mineralized NO_3^--N was greater, or at least less negative, in the 1 year old split fertilization treatment than the 1 year old spring fertilization treatment in between spring regrowth and anthesis. The greater nitrate immobilization during this time period can be explained by the greater amount of urea applied at spring regrowth in the 1 year old spring fertilization treatment. Because soil mineralization cores were capped after treatment application, soil in split fertilization treatment plots had a greater concentration of nitrate at anthesis.

Plant/ soil correlations

Soil NH_4^+-N was associated with several aboveground plant parameters, while nitrate was only associated with root nitrogen concentration. This suggests that intermediate wheatgrass mobilizes nitrate and ammonium differently. Although previous work has found that urea increases intermediate wheatgrass biomass yield relative to ammonium nitrate (Sneva, 1973), we found no evidence that increased soil ammonium

leads to greater shoot biomass in intermediate wheatgrass. There is evidence that, relative to ammonium, nitrate application increases winter survivability in intermediate wheatgrass (Lawrence, 1963), contradicting our observation that nitrate application decreases root nitrogen investment.

Soil NH_4^+-N affected intermediate wheatgrass nitrogen allocation. Our observation that soil NH_4^+-N was negatively correlated with grain yield, grain nitrogen concentration, and grain nitrogen content is evidence that soil ammonium affects intermediate wheatgrass seed production. As markets for intermediate wheatgrass grain are developed, there may be demand for higher nitrogen grain. Based on these findings, intermediate wheatgrass producers may want to avoid fertilizer forms higher in ammonia/ ammonium, such as manure, to optimize grain quantity and quality.

CONCLUSION

This was the first study to examine the nitrogen dynamics in intermediate wheatgrass, a perennial grain potentially capable of retaining and improving water quality through its nitrogen use, and a crop in need of fertility optimization. Across fertilization treatments, shoot and root tissue nitrogen concentration declined between spring regrowth and harvest, suggesting a reallocation of nitrogen to grain tissue leading up to reproductive maturity. Root nitrogen concentration or content did not increase between harvest and fall regrowth in either field, suggesting that no retranslocation of nitrogen took place during that time period.

Fertilization treatment did not affect soil mineral nitrogen levels or net nitrogen mineralization, beyond the initial spike in mineral nitrogen due to the addition of fertilizer. Mineral nitrogen levels were generally lowest at harvest and fall regrowth, and NO_3^--N mineralization rates were highest between harvest and fall regrowth. Total soil nitrogen was greater at fall regrowth than at other timepoints in the 1 year old stand. Taken together, there is an influx of soil organic nitrogen between harvest and fall regrowth, likely due to root die back or release of nitrogen rich root exudates. However, root biomass remained constant between harvest and fall regrowth in the 1 year old stand, so any root die back was matched by growth. Based on our findings, fertilizing intermediate wheatgrass at the agronomically optimal nitrogen rate does not affect net nitrogen mineralization, demonstrating that even when fertilized, perennial crops have the potential to preserve environmental quality through maintaining or even decreasing soil mineral nitrogen pools and thereby preventing their movement into surrounding waterways.

TABLES

Table 1. Stage index, date, day of year (DOY), and growing degree days (GDDs) at four sampling times in both 1 year old and 2 year old intermediate wheatgrass

Sampling time	Stage index	Date	DOY	GDDs
	1 year old stand			
Spring regrowth	1.3	7-May	127	493
Anthesis	3.7	14-Jun	164	1443
Harvest	4.9	21-Aug	231	3339
Fall regrowth	1.5	23-Oct	293	4523
	2 year old stand			
Spring regrowth	1.3	2-May	122	373
Anthesis	3.7	12-Jun	162	1391
Harvest	4.9	13-Aug	223	3109
Fall regrowth	1.5	29-Oct	299	4589

Table 2. Results of analysis of variance to test for significant nitrogen addition treatment, sampling time and interaction effects on whole plant N content, root N content, shoot N content, whole plant N concentration root N concentration, shoot N concentration, whole plant biomass, root biomass, and shoot biomass in a 1 year old intermediate wheatgrass stand

	N content			**N concentration**			**Biomass**		
Factor	**Whole**	**Root**	**Shoot**	**Whole**	**Root**	**Shoot**	**Whole**	**Root**	**Shoot**
Treatment	NS	NS	NS	NS	NS	*	NS	NS	NS
Sampling time	***	NS	***	***	***	***	***	NS	***
Treatment X sampling time	NS	NS	NS	NS	NS	NS	NS	NS	NS

* = P < 0.05; ** = P < 0.01; *** = P < 0.001; NS= not significant, NA = factor or interaction not included in model

Table 3. Results of analysis of variance to test for significant nitrogen addition treatment, sampling time, and interaction effects on soil NO_3^--N (0-15), soil NH_4^+-N (0-15), soil mineralized NO_3^--N (0-15 cm), and soil mineralized NH_4^+-N (0-15 cm) in 1 year old and 2 year old intermediate wheatgrass stands

	1 year old stand				**2 year old stand**			
	Mineral N		**Mineralized N**		**Mineral N**		**Mineralized N**	
Factor	**NO_3^--N**	**NH_4^+-N**	**NO_3^--N**	**NH_4^+-N**	**NO_3^--N**	**NH_4^+-N**	**NO_3^--N**	**NH_4^+-N**
Treatment	**	**	NS	NS	NS	NS	***	NS
Sampling time	***	***	***	NS	***	*	NS	NS
Treatment X sampling time	**	*	**	NS	*	NS	**	NS

* = P < 0.05; ** = P < 0.01; *** = P < 0.001; NS= not significant, NA = factor or interaction not included in model

Table 4. Results of analysis of variance to test for significant nitrogen addition treatment, sampling time, soil depth, and interaction effects on whole plant N content, root N content, shoot N content, whole plant N concentration root N concentration, shoot N concentration, whole plant biomass, root biomass, shoot biomass, and grain yield in a 2 year old intermediate wheatgrass stand

	N content			**N concentration**			**Biomass**		
Factor	**Whole**	**Root**	**Shoot**	**Whole**	**Root**	**Shoot**	**Whole**	**Root**	**Shoot**
Treatment	***	NS	NS	NS	***	NS	NS	NS	NS
Sampling time	NS	***	NS	NS	***	NS	***	***	NS
Treatment X sampling time	NS	NS	NS	NS	NS	NS	NS	NS	NS

* = P < 0.05; ** = P < 0.01; *** = P < 0.001; NS= not significant, NA = factor or interaction not included in model

Table 5. Whole plant (WP), root, and shoot nitrogen content, nitrogen concentration, and biomass by sampling time and stand age

Sampling Time	N content			N concentration			Biomass		
	WP	Root	Shoot	WP	Root	Shoot	WP	Root	Shoot
	---------- g N m⁻² --- -------			---------- % N ----------			---------- g m⁻² --------- -		
1 year old stand									
Spring regrowth	4.4 a	2.2	2.1 a	1.9 c	1.6 b	2.6 b	230 a	141	89 a
Anthesis	NA	NA	7.5 b	NA	NA	2.5 b	NA	NA	300 b
Harvest	11.0 b	1.8	7.9 b	1.2 a	1.1 a	1.1 a	915 b	166	649 c
Fall regrowth	11.2 b	2.0	9.2 b	1.3 b	1.1 a	1.4 a	836 b	187	711 c
2 year old stand									
Spring regrowth	NA	3.8 a	NA	NA	1.1 b	NA	NA	359 a	NA
Anthesis	NA	NA	9.1	NA	NA	1.4 b	NA	NA	621 ab
Harvest	10.7	6.9 b	7.1	0.8	0.9 a	0.9 a	1549 b	733 b	762 b
Fall regrowth	11.8	4.1 a	6.8	1.2	0.9 a	1.4 b	933 a	448 a	485 a

Within column and stand age, values with the same letters are not different at P = 0.05 as per Tukey's HSD

NA = data not collected

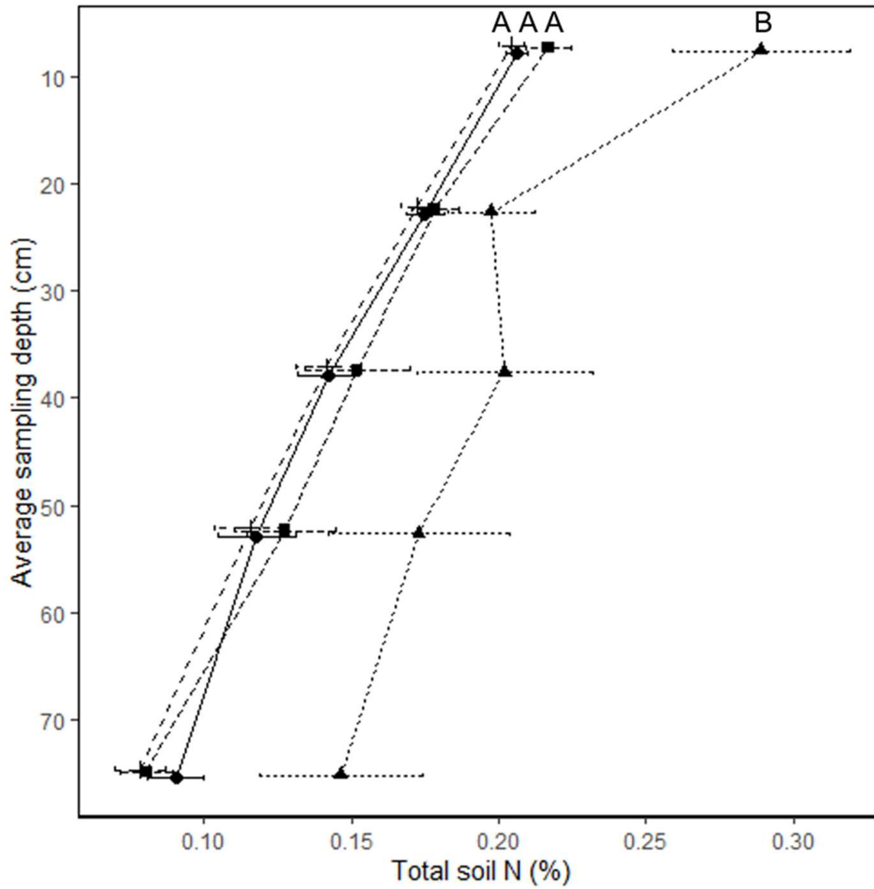
Table 6. Grain nitrogen content, grain nitrogen concentration, grain yield, lodging score, and net uptake between spring regrowth and harvest in 1 year old and 2 year old intermediate wheatgrass stands under three nitrogen application treatments- spring (80 kg N ha^{-1} in early May), split (40 - 40 kg N ha^{-1} in early May and mid-June) and control (unfertilized)

	1 year old stand					2 year old stand				
Treatment	**Grain**			**Lodging**	**Net uptake**	**Grain**			**Lodging**	**Net uptake**
	g N m^{-2}	**% N**	**kg ha^{-1}**	**0-10**	**g N m^{-2}**	**g N m^{-2}**	**% N**	**kg ha^{-1}**	**0-10**	**g N m^{-2}**
Control	1.5	3.2	450	2.8 a	5.5	3.1	3.1	990 b	3.0	13.2
Split	1.0	3.3	300	4.3 ab	5.2	1.2	3.3	350 a	3.0	8.0
Spring	1.4	3.3	410	5.5 b	9.1	0.6	3.3	189 a	2.3	7.5

Within columns, values with the same letters are not different at P = 0.05 as per Tukey's HSD

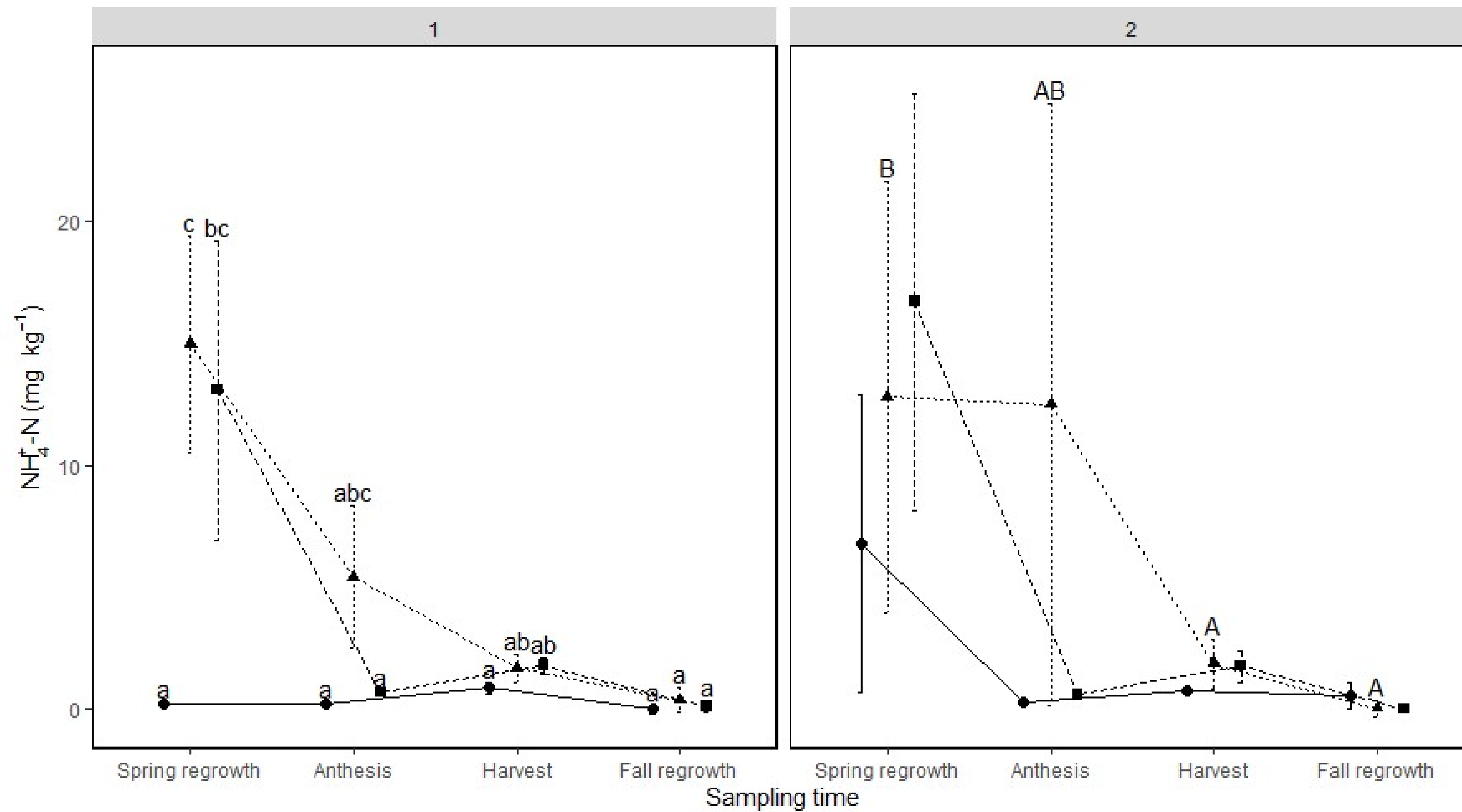
FIGURES

Figure 1. Soil total nitrogen concentration by depth and sampling time in 1 year old intermediate wheatgrass



Sampling depth indicated on Y axis is the center of the depth increment sampled
Sampling times with the same uppercase letter are statistically similar as per Tukey's HSD (P=0.05)
Crosses with long dashed line = spring regrowth, circles with solid line = anthesis, squares with short dashed line = harvest, triangles with dotted line = fall regrowth

Figure 2. Soil NH_4^+-N in 1 year old and 2 year old intermediate wheatgrass fields across sampling times and nitrogen fertilization treatments

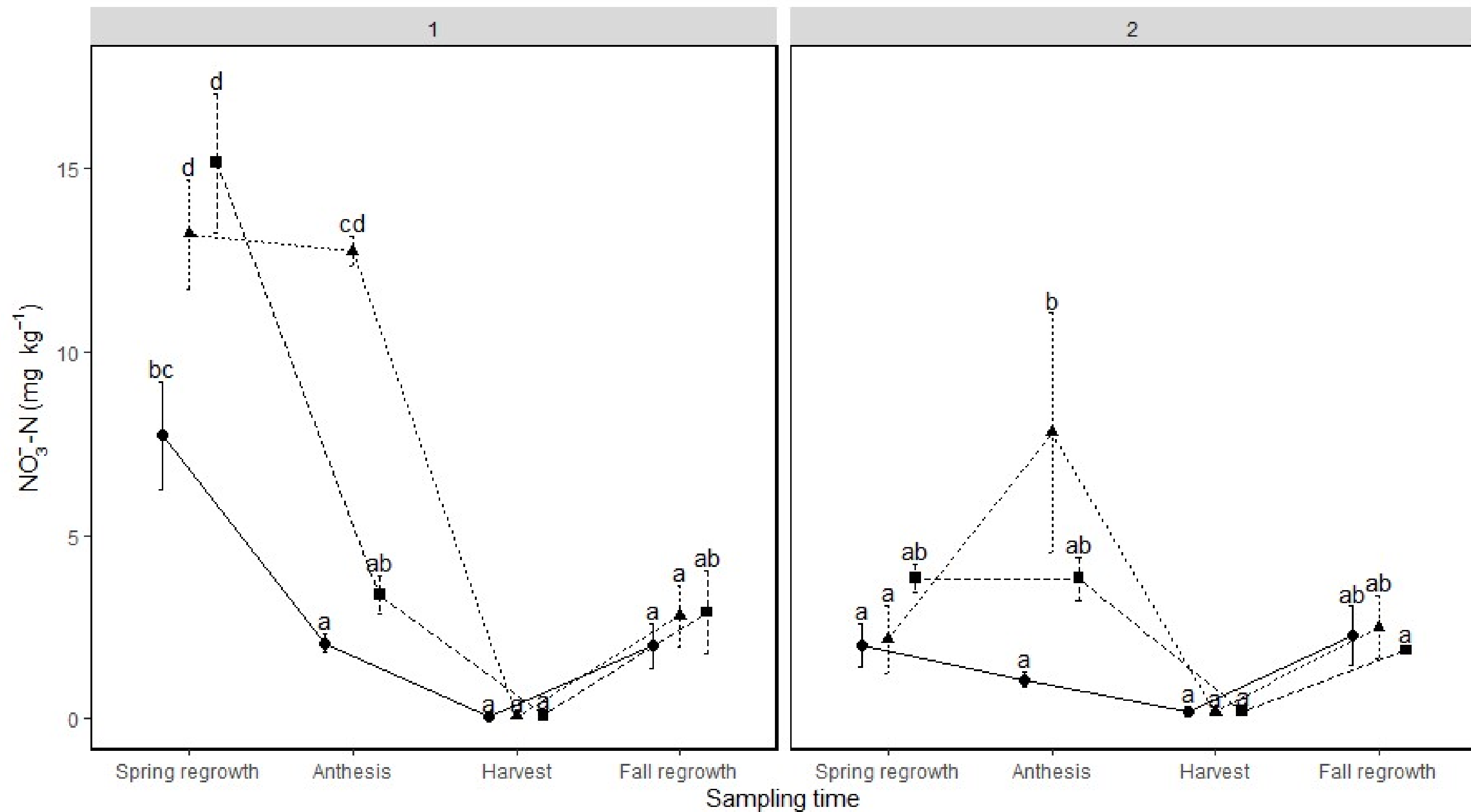


1 = 1 year old field; 2 = 2 year old field

Within a field, treatment by sampling time interactions with the same or no lowercase letter and sampling times with the same or no upper case letter are statistically similar as per Tukey's HSD (P=0.05)

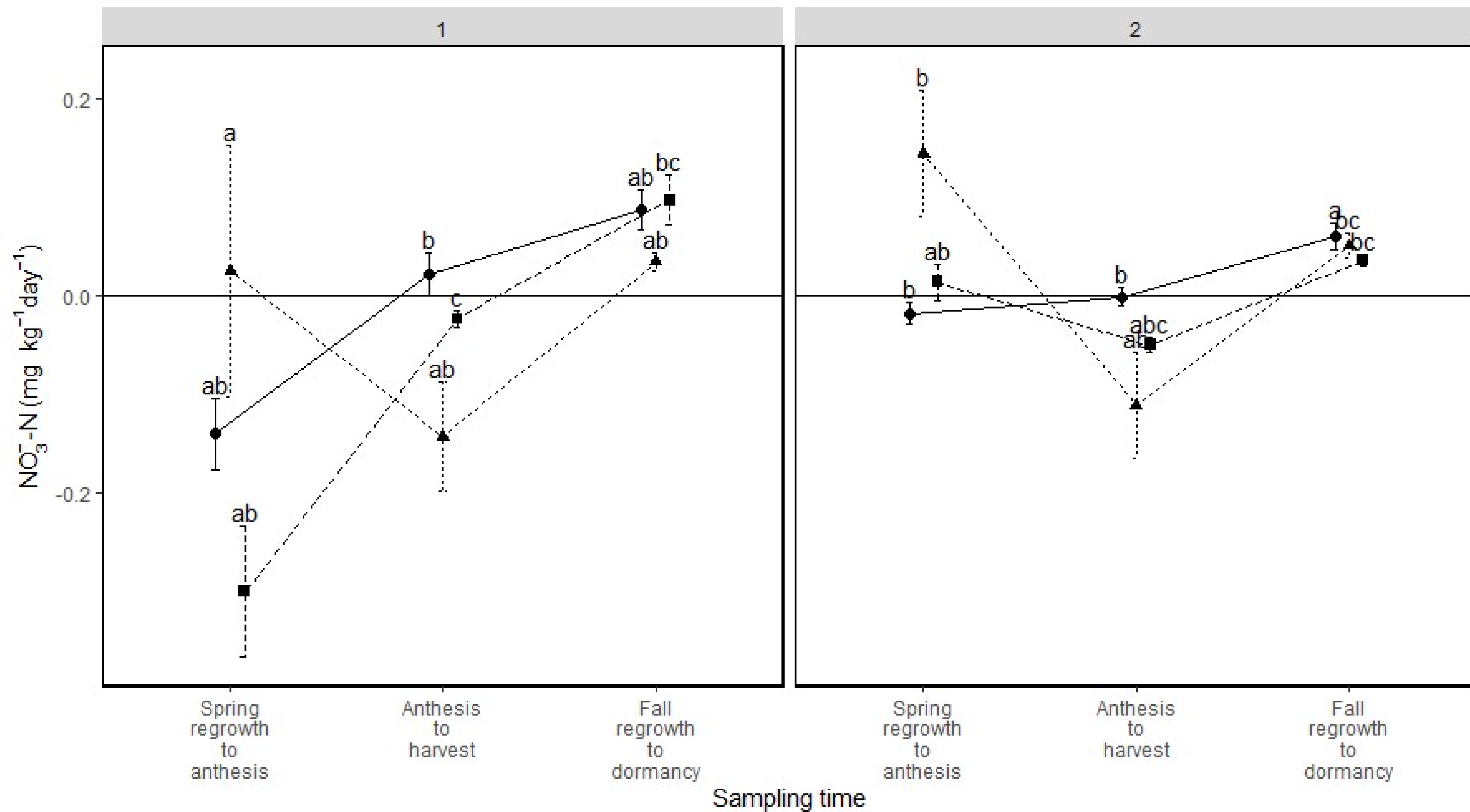
Circles with a solid line = control (0 kg N ha^{-1}), triangles with a dotted line= split (40-40 kg N ha^{-1}), and squares with a dashed line = spring (80 kg N ha^{-1})

Figure 3. Soil NO_3^--N in 1 year old and 2 year old intermediate wheatgrass fields across sampling times and nitrogen fertilization treatments



1 = 1 year old field; 2 = 2 year old field
Within a field, treatment by sampling time interactions with the same or no lowercase letter are statistically similar as per Tukey's HSD (P=0.05)
Circles with a solid line = control (0 kg N ha^{-1}), triangles with a dotted line= split (40-40 kg N ha^{-1}), and squares with a dashed line = spring (80 kg N ha^{-1})

Figure 4. Mineralized NO_3^--N in 1 year old and 2 year old intermediate wheatgrass fields across sampling durations and nitrogen fertilization treatments



Within a field, treatment by sampling time interactions with the same or no lowercase letter are statistically similar as per Tukey's HSD (P=0.05)
Circles with a solid line = control (0 kg N ha^{-1}), triangles with a dotted line= split (40-40 kg N ha^{-1}), and squares with a dashed line = spring (80 kg N ha^{-1})

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APPENDIX ONE- CODE USED IN STATISTICAL ANALYSIS
CODE FOR CHAPTER ONE

Script one

```
## May 14, 2019
## Michelle Dobbratz
## Polyculture biomass ANOVAs
## focusing on response variables:
#### total biomass
#### MBC (microbial biomass carbon)
#### Enzymes B, C, N, and P

## step 1: import file, load packages, arrange data
## step 2: write linear models; test assumptions
## step 3: refine linear models (remove non-sig intxn)
## step 4: export ANOVAs including location in model
## step 5: ANOVAS by location
## step 6: make figures for total biomass & planted richness, functional richness, grass
biomass, and legume biomass
## step 7: regressions & ANCOVAs


## step 1: import file, load packages, arrange data ----
#####
library(XLConnect); library(car); library(alr4); library(Rmisc); library(nlme);
library(ggplot2); library(emmeans)
data17 <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly17.csv")
data17$gm2 <- data17$TotalBiomass*2; data17$lbm2 <- data17$gm2*0.00220462;
data17$lbac <- data17$lbm2 * 4046.86; data17$kgha <- data17$lbac * 1.121
data17$C4gm2 <- data17$Weight.C4*2; data17$C4lbm2 <- data17$C4gm2*0.00220462;
data17$C4lbac <- data17$C4lbm2 * 4046.86; data17$C4kgha <- data17$C4lbac * 1.121
data17$C3gm2 <- data17$Weight.C3*2; data17$C3lbm2 <- data17$C3gm2*0.00220462;
data17$C3lbac <- data17$C3lbm2 * 4046.86; data17$C3kgha <- data17$C3lbac * 1.121
data17$leggm2 <- data17$Weight.Legume*2; data17$leglbm2 <-
data17$leggm2*0.00220462; data17$leglbac <- data17$leglbm2 * 4046.86;
data17$legkgha <- data17$leglbac * 1.121
bio <- data17
bio$trt <- as.factor(bio$trt)
bio$Nfert <- as.factor(bio$Nfert)
bio$B <- as.numeric(bio$B)
bio$C <- as.numeric(bio$C)
bio$N <- as.numeric(bio$N)
bio$P <- as.numeric(bio$P)

SEex <- summarySE(bio, measurevar = "TotalBiomass", groupvars = c("Nfert", "trt",
"loc"))
```

```
write.csv(SEex, file =
"C:/Users/Michelle/Dropbox/WD/polyculture/CSV/biomass_summary.csv")

SEx <- summarySE(bio, measurevar = "MBC", groupvars = "loc", na.rm = T)
write.csv(SEx, file =
"C:/Users/Michelle/Dropbox/WD/polyculture/CSV/mbc_summary.csv")

#####


## step 2: write linear models; test assumptions ----
#####
## checking for outliers in enzymes by analyzing without mixed effects
b <- lm(B ~ trt * Nfert * loc, data = bio)
c <- lm(C ~ trt * Nfert * loc, data = bio)
n <- lm(N ~ trt * Nfert * loc, data = bio)
p <- lm(P ~ trt * Nfert * loc, data = bio)
outlierTest(b)
outlierTest(c)
outlierTest(n)
outlierTest(p)
anova(b)
anova(c)
anova(n)
anova(p)


b <- lme(B ~ trt * Nfert, data = subset(bio, loc =="Becker"),
        random = ~1|Rep/Nfert, na.action = na.omit)
c <- lme(C ~ trt * Nfert, data = subset(bio, loc =="Becker"),
        random = ~1|Rep/Nfert, na.action = na.omit)
n <- lme(N ~ trt * Nfert, data = subset(bio, loc =="Becker"),
        random = ~1|Rep/Nfert, na.action = na.omit)
p <- lme(P ~ trt * Nfert, data = subset(bio, loc =="Becker"),
        random = ~1|Rep/Nfert, na.action = na.omit)
anova(b)
anova(c)
anova(n)
anova(p)


b <- lme(B ~ trt * Nfert, data = subset(bio, loc =="Lamberton"),
        random = ~1|Rep/Nfert, na.action = na.omit)
c <- lme(C ~ trt * Nfert, data = subset(bio, loc =="Lamberton"),
        random = ~1|Rep/Nfert, na.action = na.omit)
n <- lme(N ~ trt * Nfert, data = subset(bio, loc =="Lamberton"),
```

```
        random = ~1|Rep/Nfert, na.action = na.omit)
p <- lme(P ~ trt * Nfert, data = subset(bio, loc =="Lamberton"),
        random = ~1|Rep/Nfert, na.action = na.omit)
anova(b)
anova(c)
anova(n)
anova(p)


b <- lme(B ~ trt * Nfert, data = subset(bio, loc =="Roseau"),
        random = ~1|Rep/Nfert, na.action = na.omit)
c <- lme(C ~ trt * Nfert, data = subset(bio, loc =="Roseau"),
        random = ~1|Rep/Nfert, na.action = na.omit)
n <- lme(N ~ trt * Nfert, data = subset(bio, loc =="Roseau"),
        random = ~1|Rep/Nfert, na.action = na.omit)
p <- lme(P ~ trt * Nfert, data = subset(bio, loc =="Roseau"),
        random = ~1|Rep/Nfert, na.action = na.omit)
anova(b)
anova(c)
anova(n)
anova(p)
m <- emmeans(p, ~trt, cov.reduce = F)
cld(m)

b <- lme(B ~ trt * Nfert, data = subset(bio, loc =="Waseca"),
        random = ~1|Rep/Nfert, na.action = na.omit)
c <- lme(C ~ trt * Nfert, data = subset(bio, loc =="Waseca"),
        random = ~1|Rep/Nfert, na.action = na.omit)
n <- lme(N ~ trt * Nfert, data = subset(bio, loc =="Waseca"),
        random = ~1|Rep/Nfert, na.action = na.omit)
p <- lme(P ~ trt * Nfert, data = subset(bio, loc =="Waseca"),
        random = ~1|Rep/Nfert, na.action = na.omit)

anova(b)
m <- emmeans(b, ~trt, cov.reduce = F)
cld(m)

anova(c)
anova(n)
anova(p)
summary(glht(p, linfct= mcp(trt = "Tukey")))
summary(glht(p1, linfct= mcp(trt = "Tukey")))


totbio <- lme(gm2 ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
```

```
SpecRich <- lme(Total_species_Richness ~ trt * Nfert * loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
PlantRich <- lme(Planted_Species_richness ~ trt * Nfert * loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
unplantRich <- lme(unplanted_species_richness ~ trt * Nfert * loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
funRich <- lme(Functional_richness ~ trt * Nfert * loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
grass <- lme(grassgm2 ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
C3 <- lme(C3gm2 ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
C4 <- lme(C4gm2 ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
leg <- lme(leggm2 ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
mbc <- lme(MBC ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
sgbio <- lme(SGbio ~ trt * Nfert * loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)

plot(totbio)
plot(SpecRich)
plot(PlantRich)
plot(unplantRich)
plot(funRich)
plot(grass)
plot(C3)
plot(C4)
plot(leg)
plot(mbc)
plot(sgbio)

hist(bio$gm2, breaks = 20)
hist(bio$Total_species_Richness, breaks = 20)
hist(bio$Planted_Species_richness, breaks = 20)
hist(bio$unplanted_species_richness, breaks = 20)
hist(bio$Functional_richness, breaks = 20)
hist(bio$grassgm2, breaks = 20)
hist(bio$C3gm2, breaks = 20)
hist(bio$C4gm2, breaks = 20)
hist(bio$leggm2, breaks = 20)
hist(bio$MBC, breaks = 20)
#####

## step 3: refine linear models (remove non-sig intxn) ----
```

```
#####
anova(totbio) #trt:loc
anova(SpecRich)
anova(PlantRich) #trt:loc + trt:Nfert
anova(unplantRich)
anova(funRich) #trt:loc
anova(grass)
anova(C3)
anova(C4)
anova(leg) #trt:loc
anova(mbc)
anova(sgbio) #Nfert:loc

totbio <- lme(gm2 ~ trt + Nfert + loc + trt:loc, data = bio, random = ~1|Rep/Nfert,
na.action = na.omit)
SpecRich <- lme(Total_species_Richness ~ trt + Nfert + loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
PlantRich <- lme(Planted_Species_richness ~ trt + Nfert + loc + trt:loc + trt:Nfert, data =
bio, random = ~1|Rep/Nfert, na.action = na.omit)
unplantRich <- lme(unplanted_species_richness ~ trt + Nfert + loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
funRich <- lme(Functional_richness ~ trt + Nfert + loc + trt:loc, data = bio, random =
~1|Rep/Nfert, na.action = na.omit)
grass <- lme(grassgm2 ~ trt + Nfert + loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
C3 <- lme(C3gm2 ~ trt + Nfert + loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
C4 <- lme(C4gm2 ~ trt + Nfert + loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)
leg <- lme(leggm2 ~ trt + Nfert + loc + trt:loc, data = bio, random = ~1|Rep/Nfert,
na.action = na.omit)
mbc <- lme(MBC ~ trt + Nfert + loc, data = bio, random = ~1|Rep/Nfert, na.action =
na.omit)

#####

## step 4: export ANOVAs including location in model ----
#####
tot <- summary(totbio)
rich <- summary(SpecRich)
plantrich <- summary(PlantRich)
unplantrich <- summary(unplantRich)
funrich <- summary(funRich)
Grass <- summary(grass)
c3 <- summary(C3)
c4 <- summary(C4)
```

```
Leg <- summary(leg)
Mbc <- summary(mbc)

sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_bio_anova.csv')
tot
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_rich_anova.csv')
rich
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_plantrich_anova.csv')
plantrich
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_unplantrich_anova.csv
')
unplantrich
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_runrich_anova.csv')
funrich
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_grass_anova.csv')
Grass
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_c3_anova.csv')
c3
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_c4_anova.csv')
c4
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_leg_anova.csv')
Leg
sink()
sink(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_mbc_anova.csv')
Mbc
sink()

## step 5: write location specific models

## step 5: write location specific models

## step 5: ANOVAs by location ----
#####
byloc <- subset(bio, loc == "Waseca")
totbio <- lme(gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
SpecRich <- lme(Total_species_Richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
```

```
PlantRich <- lme(Planted_Species_richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
funRich <- lme(Functional_richness ~ trt * Nfert, data = byloc, random = ~1|Rep/Nfert,
na.action = na.omit)
grass <- lme(grassgm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
C3 <- lme(C3gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
C4 <- lme(C4gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
leg <- lme(leggm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
mbc <- lme(MBC ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
sgbio <- lme(SGbio ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)

anova(totbio) #trt
anova(SpecRich)
anova(PlantRich)
anova(funRich) #trt:Nfert
anova(grass) #trt
anova(C3)
anova(C4) #trt
anova(leg)
anova(mbc)
anova(sgbio)

summary(glht(totbio, linfct=mcp(trt="Tukey")))
emmeans(grass, ~trt) #1>12

byloc <- subset(bio, loc == "Lamberton")
totbio <- lme(gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
SpecRich <- lme(Total_species_Richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
PlantRich <- lme(Planted_Species_richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
unplantRich <- lme(unplanted_species_richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
funRich <- lme(Functional_richness ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert,
na.action = na.omit)
grass <- lme(grassgm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
C3 <- lme(C3gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
```

```
C4 <- lme(C4gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
leg <- lme(leggm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
mbc <- lme(MBC ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
sgbio <- lme(SGbio ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)


anova(totbio) #trt
anova(SpecRich)
anova(PlantRich) #trt
anova(unplantRich)
anova(funRich)
anova(grass) #trt
anova(C3)
anova(C4) #trt
anova(leg)
anova(mbc)
anova(sgbio) #trt


summary(glht(totbio, linfct=mcp(trt="Tukey")))
summary(glht(PlantRich, linfct=mcp(trt="Tukey")))
emmeans(grass, ~trt) #1>12
summary(glht(sgbio, linfct=mcp(trt="Tukey")))



byloc <- subset(bio, loc == "Becker")
totbio <- lme(gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
SpecRich <- lme(Total_species_Richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
PlantRich <- lme(Planted_Species_richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
unplantRich <- lme(unplanted_species_richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
funRich <- lme(Functional_richness ~ trt, data = byloc, random = ~1|Rep, na.action =
na.omit)
grass <- lme(grassgm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
C4 <- lme(C4gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
leg <- lme(leggm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
```

```
na.omit)
mbc <- lme(MBC ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
sgbio <- lme(SGbio ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)


anova(totbio) #trt + Nfert
anova(SpecRich)
anova(PlantRich) #trt
anova(unplantRich)
anova(funRich)
anova(grass)
anova(C4)
anova(leg)
anova(mbc)
anova(sgbio)

summary(glht(totbio, linfct=mcp(trt="Tukey")))
summary(glht(PlantRich, linfct=mcp(trt="Tukey")))

byloc <- subset(bio, loc == "Roseau")
totbio <- lme(gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
SpecRich <- lme(Total_species_Richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
PlantRich <- lme(Planted_Species_richness ~ trt * Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
unplantRich <- lme(unplanted_species_richness ~ trt + Nfert, data = byloc, random =
~1|Rep/Nfert, na.action = na.omit)
funRich <- lme(Functional_richness ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert,
na.action = na.omit)
grass <- lme(grassgm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
C3 <- lme(C3gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
C4 <- lme(C4gm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
leg <- lme(leggm2 ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
mbc <- lme(MBC ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
sgbio <- lme(SGbio ~ trt + Nfert, data = byloc, random = ~1|Rep/Nfert, na.action =
na.omit)
```

```
anova(totbio)
anova(SpecRich)
anova(PlantRich) #trt + trt:Nfert
anova(unplantRich)
anova(funRich) #trt
anova(grass)
anova(C3)
anova(C4)
anova(leg) #trt
anova(mbc)
anova(sgbio) #trt

#summary(glht(PlantRich, linfct=mcp(trt="Tukey")))
emmeans(PlantRich, ~trt)
summary(glht(funRich, linfct=mcp(trt="Tukey")))
emmeans(leg, ~trt)
summary(glht(sgbio, linfct=mcp(trt="Tukey")))

#####

## step 6: make figures for total biomass, enzymes, final C, delta soil C ----
#####
## MBC----
pdat<-read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly17_jj.csv")
pdat$trt<-factor(pdat$trt)
pdat$Nfert<-factor(pdat$Nfert)

pdat<-pdat[-which(pdat[,"MBC"]>5000),]

SE <- summarySE(pdat, measurevar = "MBC", groupvars = c("trt", "loc"), na.rm=T)
ggplot(SE,
      aes(x=trt, y=MBC)) +
  geom_col() +
  geom_errorbar(aes(ymin = MBC-se, ymax = MBC + se), width = .1) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Soil Microbial Biomass Carbon')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

mbc <- lme(MBC ~ trt * Nfert, random = ~1|Rep/Nfert, subset(pdat, loc =="Roseau"),
na.action=na.omit)
anova(mbc)

## total biomass with N fert----
totbio <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/totbio.csv")
```

```
totbio$gm2 <- totbio$Weight*2; totbio$lbm2 <- totbio$gm2*0.00220462; totbio$lbac <-
totbio$lbm2 * 4046.86; totbio$kgha <- totbio$lbac * 1.121
totbio$Mgha <- totbio$kgha/1000
totbio$trt <- as.factor(totbio$trt)
totbio$Nfert <- as.factor(totbio$Nfert)
levels(totbio$loc)
levels(totbio$loc) <- c("Becker", "Lamberton", "Roseau", "Waseca")

summarySE(totbio, measurevar = "Mgha", groupvars = "loc")
summarySE(totbio, measurevar = "Mgha", groupvars = c("loc", "Nfert"))

SE <- summarySE(subset(totbio, loc == "Waseca"), measurevar = "Mgha", groupvars =
c("trt", "loc", "Nfert"))
pd <- position_dodge(.95)
ggplot(SE,
      aes(fill = Nfert, x=trt, y=Mgha)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Mgha-se, ymax = Mgha + se), position = pd, width = .1) +
  theme_bw() +
  xlab("Species mixture treatment") +
  ylab(bquote('Aboveground biomass (Mg ha'^-1*')')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD"))

## total biomass by N fert----
SE <- summarySE(bio, measurevar = "kgha", groupvars = c("Nfert", "loc"))
pd <- position_dodge(.95)
ggplot(SE,
      aes(x=Nfert, y=kgha)) +
  geom_col() +
  geom_errorbar(aes(ymin = kgha-se, ymax = kgha + se), width = .1) +
  theme_bw() +
  xlab("N fertility") +
  ylab(bquote('Above Ground Biomass (kg ha'^-1*')')) +
  scale_x_discrete(labels=c("1" = "+N", "0" = "-N")) +
  facet_grid(~loc)

## All enzymes----
#SEB <- summarySE(bio, measurevar = "B", groupvars = "loc")
#SEC <- summarySE(bio, measurevar = "C", groupvars = "loc")
#bio$Nagase <- bio$N
#SEN <- summarySE(bio, measurevar = "Nagase", groupvars = "loc")
#SEP <- summarySE(bio, measurevar = "P", groupvars = "loc")
#SEBC <- merge(SEB, SEC, by = "loc")
#SENP <- merge(SEN, SEP, by = "loc")
#enz <- merge(SEBC, SENP, by = "loc")
#writeWorksheetToFile(file='C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_en
```

```
z.xlsx', data=enz, sheet="enz")
#manually converted from wide to long in Excel
enz <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly_enz.csv")
enz$loc <- enz$ï..loc
pd <- position_dodge(.95)
ggplot(enz,
       aes(fill = loc, x=Enzyme, y=Activity)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Activity-SE, ymax = Activity+SE), position=pd, width = .1)
+
  theme_bw() +
  xlab("Enzyme") +
  ylab(expression(paste("Activity (nmol hr"^-1~"g"^-1*")"))) +
  theme(axis.text.x = element_text(angle = 30, hjust = .8)) +
  scale_x_discrete(labels=c
                 ("B" = (expression(paste(beta~"glucosidase"))),
                   "C" = "Cellobiohydrolase",
                   "N" = "N-acetylglucosaminidase",
                   "P" = "Phosphatase")) +
  labs(fill = "Location")


## Enzyme B
SE <- summarySE(bio, measurevar = "B", groupvars = c("trt", "loc"))
wb <- ggplot(subset(SE, loc == "Waseca"),
       aes(x=trt, y=B)) +
  geom_col() +
  geom_errorbar(aes(ymin = B-se, ymax = B + se), width = .1) +
  theme_bw() +
  xlab("Treatment") +
  theme(axis.text.x=element_blank(),axis.title.x=element_blank())+
  ylab(expression(paste(beta~"-glucosidase activity (nmol hr"^-1~"g"^-1~")"))) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD"))

## Enzyme P
SE <- summarySE(bio, measurevar = "P", groupvars = c("trt", "loc"))
rp <- ggplot(subset(SE, loc == "Roseau"),
       aes(x=trt, y=P)) +
  geom_col() +
  geom_errorbar(aes(ymin = P-se, ymax = P + se), width = .1) +
  theme_bw() +
  ylab(expression(paste("Phosphatase activity (nmol hr"^-1~"g"^-1~")"))) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD"))

multiplot(wb, rp)
#### soil C----
```

```
pdat<-read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly17_jj.csv")
pdat$ftrt<-factor(pdat$trt)
pdat$fNfert<-factor(pdat$Nfert)

### Final soil C----
## depth A
SE <- summarySE(pdat, measurevar = "A_C", groupvars = c("ftrt", "loc"))
ggplot(SE,
       aes(x=ftrt, y=A_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = A_C-se, ymax = A_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Soil Carbon at 0-15cm (g kg'^-1*')')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

### Final soil C
## depth B
SE <- summarySE(pdat, measurevar = "B_C", groupvars = c("ftrt", "loc"))
ggplot(SE,
       aes(x=ftrt, y=B_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = B_C-se, ymax = B_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Soil Carbon at 15-30cm (g kg'^-1*')')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD"))

### Final soil C
## depth C
SE <- summarySE(pdat, measurevar = "C_C", groupvars = c("ftrt", "loc"))

ggplot(SE,
       aes(x=ftrt, y=C_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = C_C-se, ymax = C_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Soil Carbon at 30-60cm (g kg'^-1*')')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

### Final soil C
## depth D
SE <- summarySE(subset(pdat, loc!="Roseau"), measurevar = "D_C", groupvars =
```

```
c("ftrt", "loc"))
ggplot(SE,
       aes(x=ftrt, y=D_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = D_C-se, ymax = D_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Soil Carbon at 60-90cm (g kg'^-1*')')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

### Delta soil C----
pdat$difA_C<-pdat$A_C-pdat$A_C13
pdat$difB_C<-pdat$B_C-pdat$B_C13
pdat$difC_C<-pdat$C_C-pdat$C_C13
pdat$difD_C<-pdat$D_C-pdat$D_C13

## Delta soil bar ----
## depth delta A
pdatnoros <- subset(pdat, loc !="Roseau")

SE <- summarySE(pdatnoros, measurevar = "difA_C", groupvars = c("ftrt", "loc"))
ggplot(SE,
       aes(x=ftrt, y=difA_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = difA_C-se, ymax = difA_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Change in Soil Carbon at 0-15cm from 2013 - 2017 (%)')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

## depth delta B
SE <- summarySE(pdatnoros, measurevar = "difB_C", groupvars = c("ftrt", "loc"))
ggplot(SE,
       aes(x=ftrt, y=difB_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = difB_C-se, ymax = difB_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Change in Soil Carbon at 15-30cm from 2013 - 2017 (%)')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

## depth delta C
SE <- summarySE(pdatnoros, measurevar = "difC_C", groupvars = c("ftrt", "loc"))
```

```
SE #Becker 30-60 trt 1 = -0.24
ggplot(SE,
      aes(x=ftrt, y=difC_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = difC_C-se, ymax = difC_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Change in Soil Carbon at 30-60cm from 2013 - 2017 (%)')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

## depth delta D
SE <- summarySE(pdatnoros, measurevar = "difD_C", groupvars = c("ftrt", "loc"))
ggplot(SE,
      aes(x=ftrt, y=difD_C)) +
  geom_col() +
  geom_errorbar(width = 0.1, aes(ymin = difD_C-se, ymax = difD_C + se)) +
  theme_bw() +
  xlab("Treatment") +
  ylab(bquote('Change in Soil Carbon at 60-90cm from 2013 - 2017 (%)')) +
  scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD")) +
  facet_grid(~loc)

## soil line graphs ----
## Delta C soil line
CN <-
read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/2017_Poly_delta_CN_long.
csv")
CN <- subset(CN, Site != "Roseau")
CN$depth <- as.numeric(as.character(CN$depth))
CN$Nfert <- as.factor(CN$Nfert)
CN$Trt <- as.factor(CN$Trt)
CNSE <- summarySE(CN, measurevar = "delC1317", groupvars = c("Site", "depth"),
na.rm = T)
CNSE$depth <- CNSE$depth*-1
ggplot(CNSE,
      aes(x = delC1317, y = depth, color = Site, shape = Site)) +
#  geom_line(aes(linetype = Site)) +
  geom_point(size = 2) +
  geom_path() +
  labs(color = "Site",
      x = "Change in total soil carbon (%) from 2013-2017",
      y = "Average sampling depth (cm)") +
  scale_y_continuous(breaks = pretty(CNSE$depth), labels = abs(pretty(CNSE$depth)))
```

```
## Final C soil line
brook <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/brook.csv")
brook$carbon <- brook$Carbon......
brook$depth <- as.numeric(as.character(brook$depth))
brook$Trt <- as.factor(brook$trt)
brook$Nfert <- as.factor(brook$Nfert)

brookSE <- summarySE(brook, measurevar = "carbon", groupvars = c("Site", "depth"),
na.rm = T)
brookSE$depth <- brookSE$depth*-1
ggplot(brookSE,
      aes(x = carbon, y = depth, color = Site, shape = Site)) +
  geom_point(size = 2) +
  geom_path() +
  labs(shape = "Site",
      color = "Site",
      x = "Total soil carbon (%) in 2017",
      y = "Average sampling depth (cm)") +
  scale_y_continuous(breaks = pretty(brookSE$depth), labels =
abs(pretty(brookSE$depth)))

#####

## step 7: regressions & ANCOVAs
#####
totbioplant <- lm(gm2 ~ Planted_Species_richness + loc, data = bio)
anova(totbioplant)
summary(totbioplant)
totbiounplant <- lm(gm2 ~ unplanted_species_richness + loc, data = bio)
anova(totbiounplant)
summary(totbiounplant)

SpecRich <-lm(Total_species_Richness ~ unplanted_species_richness + loc, data = bio)
anova(SpecRich)
summary(SpecRich)

PlantRichbio <- lm(Planted_Species_richness ~ gm2 + loc , data = bio)
anova(PlantRichbio)
PlantRichfun <- lm(Planted_Species_richness ~ Functional_richness + loc , data = bio)
anova(PlantRichfun)
PlantRichgrass <- lm(Planted_Species_richness ~ grassgm2 + loc , data = bio)
anova(PlantRichgrass)
summary(PlantRichgrass)
PlantRichleg <- lm(Planted_Species_richness ~ leggm2 + loc , data = bio)
anova(PlantRichleg)
summary(PlantRichleg)
```

```
unplantRichbio <- lm(unplanted_species_richness ~ gm2 + loc, data = bio)
anova(unplantRichbio)
unplantRichspec <- lm(unplanted_species_richness ~ Total_species_Richness + loc, data
= bio)
anova(unplantRichspec)
unplantRichleg <- lm(unplanted_species_richness ~ leggm2 + loc, data = bio)
anova(unplantRichleg)
summary(unplantRichleg)

funRich <- lm(Functional_richness ~ Planted_Species_richness + loc, data = bio)
anova(funRich)
summary(funRich)

mbctotrich <- lm(MBC ~ Total_species_Richness + loc, data = subset(bio [-c(54, 73, 86,
89, 95),]))
anova(mbctotrich)
summary(mbctotrich)

mbcfunrich <- lm(MBC ~ Functional_richness + loc, data = subset(bio [-c(54, 73, 86, 89,
95),]))
anova(mbcfunrich)
summary(mbcfunrich)

mbcfunleg <- lm(MBC ~ leggm2 + loc, data = subset(bio [-c(54, 73, 86, 89, 95),]))
anova(mbcfunleg)
summary(mbcfunleg)

totbiosg <- lm(kgha ~ SGbio + loc, data = bio)
anova(totbiosg)
summary(totbiosg)

richsg <- lm(Total_species_Richness ~ SGbio + loc, data = bio)
anova(richsg)

plantrichsg <- lm(Planted_Species_richness ~ SGbio + loc, data = bio)
anova(plantrichsg)
summary(plantrichsg)

unplantrichsg <- lm(unplanted_species_richness ~ SGbio + loc, data = bio)
anova(unplantrichsg)
summary(unplantrichsg)

mbcsg <- lm(MBC ~ SGbio + loc, data = bio)
anova(mbcsg)
summary(mbcsg)
```

```
plot(totbio)
plot(SpecRich)
plot(PlantRich)
plot(unplantRich)
plot(funRich)
plot(grass)
plot(C3)
plot(C4)
plot(leg)
plot(mbc)

#####

## step 8: regression and ANCOVA figures
#####
ggplot(bio,
     aes(x=Planted_Species_richness, y=gm2, color = loc)) +
  geom_point() +
  geom_abline(slope = -20.16, intercept = 429, size = 1, color = "#F8766D") +
  geom_abline(slope = -20.16, intercept = 946.44, size = 1, color = "#7CAE00") +
  geom_abline(slope = -20.16, intercept = 621.27, size = 1, color = "#00BFC4") +
  geom_abline(slope = -20.16, intercept = 860.61, size = 1, color = "#C77CFF") +
  theme_bw()

ggplot(bio,
     aes(x=unplanted_species_richness, y=gm2, color = loc)) +
  geom_point() +
  geom_abline(slope = 15.3, intercept = 314.54, size = 1, color = "#F8766D") +
  geom_abline(slope = 15.3, intercept = 704.48, size = 1, color = "#7CAE00") +
  geom_abline(slope = 15.3, intercept = 456.41, size = 1, color = "#00BFC4") +
  geom_abline(slope = 15.3, intercept = 675.25, size = 1, color = "#C77CFF") +
  theme_bw()

ggplot(bio,
     aes(x=grassgm2, y=Planted_Species_richness, color = loc)) +
  geom_point() +
  geom_abline(slope = -0.0028, intercept = 4.4, size = 1, color = "#F8766D") +
  geom_abline(slope = -0.0028, intercept = 6.69, size = 1, color = "#7CAE00") +
  geom_abline(slope = -0.0028, intercept = 6.7, size = 1, color = "#00BFC4") +
  geom_abline(slope = -0.0028, intercept = 7.55, size = 1, color = "#C77CFF") +
  theme_bw()

ggplot(bio,
     aes(x=leggm2, y=Planted_Species_richness, color = loc)) +
  geom_point() +
```

```
  geom_abline(slope = 0.0017, intercept = 2.98, size = 1, color = "#F8766D") +
  geom_abline(slope = 0.0017, intercept = 5.83, size = 1, color = "#7CAE00") +
  geom_abline(slope = 0.0017, intercept = 5.66, size = 1, color = "#00BFC4") +
  geom_abline(slope = 0.0017, intercept = 6.77, size = 1, color = "#C77CFF") +
  theme_bw()

ggplot(bio,
       aes(x=Total_species_Richness, y=MBC, color = loc)) +
  geom_point() +
  geom_abline(slope = -2.64, intercept = 378.7, size = 1, color = "#F8766D") +
  geom_abline(slope = -2.64, intercept = 342.0, size = 1, color = "#7CAE00") +
  geom_abline(slope = -2.64, intercept = 850.6, size = 1, color = "#00BFC4") +
  geom_abline(slope = -2.64, intercept = 150.1, size = 1, color = "#C77CFF") +
  ylim(0,1000) +
  theme_bw()

ggplot(bio,
       aes(x=Functional_richness, y=MBC, color = loc)) +
  geom_point() +
  geom_abline(slope = 31.75, intercept = 288, size = 1, color = "#F8766D") +
  geom_abline(slope = 31.75, intercept = 229, size = 1, color = "#7CAE00") +
  geom_abline(slope = 31.75, intercept = 728, size = 1, color = "#00BFC4") +
  geom_abline(slope = 31.75, intercept =  21, size = 1, color = "#C77CFF") +
  ylim(0,1000) +
  theme_bw()

#####

## tables ----

SEP <- summarySE(bio, measurevar = "P", groupvars = c("loc"))
SEB <- summarySE(bio, measurevar = "B", groupvars = c("loc"))
SEC <- summarySE(bio, measurevar = "C", groupvars = c("loc"))
bio$Ni <- bio$N
SEN <- summarySE(bio, measurevar = "Ni", groupvars = c("loc"))
SEP
SEB
SEC
SEN
```

Script two

```
## Created by Jake Jungers
## Edited heavily by Michelle Dobbratz
## Conducting the final polyculture analysis
## Total biomass in need of remediation; use totbio file instead of poly17_jj
## May 21, 2019


##data setup ----
library(lattice)
library(ggplot2)
library(nlme)
library(emmeans)
library(multcompView)
library(multcomp)
library(Rmisc)
data17 <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly17.csv")
data17$Weight <- as.numeric(as.character(data17$Weight))
data17$gm2 <- data17$Weight*2; data17$lbm2 <- data17$gm2*0.00220462;
data17$lbac <- data17$lbm2 * 4046.86; data17$kgha <- data17$lbac * 1.121
data17$B <- as.numeric(as.character(data17$B))
data17$P <- as.numeric(as.character(data17$P))
data17$N <- as.numeric(as.character(data17$N))
data17$C <- as.numeric(as.character(data17$C))

bio <- data17
bio$trt <- as.factor(bio$trt)
bio$Nfert <- as.factor(bio$Nfert)
pdat <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/poly17_jj.csv")
pdat$ftrt<-factor(pdat$trt)
pdat$fNfert<-factor(pdat$Nfert)
soil <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/agvise.csv")
soil$trt <- as.factor(soil$trt)
soil$Nfert <- as.factor(soil$Nfert)

##Soil properties----
ph <- lme(pH~trt*Nfert*loc, random=~1|Rep, data = soil, na.action = na.omit)
anova(ph)
phwas <- lme(pH~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="W", na.action =
na.omit))
anova(phwas)
phl <- lme(pH~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="L", na.action =
na.omit))
anova(phl)
phb <- lme(pH~trt*Nfert, random=~1|Rep,data = soilb, na.action = na.omit)
anova(phb) #Nfert
```

```
phr <- lme(pH~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="R", na.action =
na.omit))
anova(phr)

kb <- lme(K~trt*Nfert, random=~1|Rep,data = soilb, na.action = na.omit)
anova(kb)#Nfert
kl <- lme(K~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="L", na.action =
na.omit))
anova(kl)#Nfert
kr <- lme(K~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="R", na.action =
na.omit))
anova(kr)#Nfert
kw <- lme(K~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="W", na.action =
na.omit))
anova(kw)#Nfert

nb <- lme(NO3~trt*Nfert, random=~1|Rep,data = soilb, na.action = na.omit)
anova(nb)
nl <- lme(NO3~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="L", na.action =
na.omit))
anova(nl) #Nfert
nr <- lme(NO3~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="R", na.action =
na.omit))
anova(nr) #Nfert
nw <- lme(NO3~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="W", na.action =
na.omit))
anova(nw) #Nfert


pb <- lme(P~trt*Nfert, random=~1|Rep,data = soilb, na.action = na.omit)
anova(pb) #Nfert
pl <- lme(P~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="L", na.action =
na.omit))
anova(pl)#Nfert
pr <- lme(P~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="R", na.action =
na.omit))
anova(pr)
pw <- lme(P~trt*Nfert, random=~1|Rep,data = subset(soil, loc =="W", na.action =
na.omit))
anova(pw)#Nfert + intxn


##Total biomass----
totbio <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/totbio.csv")
totbio$ftrt <- as.factor(totbio$trt)
totbio$fNfert <- as.factor(totbio$Nfert)
```

```
bleh <- lme(Weight~ftrt*fNfert, random=~1|Rep, data=subset(totbio, loc=="B"),
          na.action=na.omit)
anova(bleh)
bbt <- emmeans(bleh, ~ftrt*fNfert, cov.reduce = F)
cld(bbt)
con <- contrast(bbt, method = "pairwise")
cld(con)
bbf <- emmeans(bleh, ~fNfert, cov.reduce = F)
cld(bbf)

lam <- lme(Weight~ftrt*fNfert, random=~1|Rep, data=subset(totbio, loc=="L"),
        na.action=na.omit)
anova(lam)

rosbio <- lme(Weight~ftrt*fNfert, random=~1|Rep, data=subset(totbio, loc=="R"),
            na.action=na.omit)
anova(rosbio)


wasbio <- lme(Weight~ftrt*fNfert, random=~1|Rep, data=subset(totbio, loc=="W"),
          na.action=na.omit)
anova(wasbio)
emms3 <- emmeans(wasbio, ~ ftrt*fNfert)
cld(emms3)
con3 <- contrast(emms3, method = "pairwise")
cld(con3)




## EEA ----
eeab <- lm(B~loc, data = bio)
anova(eeab)
cld(emmeans(eeab, ~loc))

eeac <- lm(C~loc, data = bio)
anova(eeac)
cld(emmeans(eeac, ~loc))

eean <- lm(N~loc, data = bio)
anova(eean)
cld(emmeans(eean, ~loc))

eeap <- lm(P~loc, data = bio)
```

```
anova(eeap)
cld(emmeans(eeap, ~loc))

#MBC ----
##outliers; removing for now.
pdat<-pdat[-which(pdat[,"MBC"]>5000),]
#Does MBC vary by treatment? Let's analyze data by location
bwplot(MBC~ftrt|loc*fNfert, data=pdat)
anova(lme(MBC~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(MBC~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(MBC~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(MBC~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))

## final soil ----
#Is there a difference in soil C at shallow depth among treatments?
bwplot(A_C~ftrt|loc*fNfert, data=pdat)
wasAC <- lme(A_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit) # N treatment is significant
anova(wasAC)
wact <- emmeans(wasAC, ~fNfert, cov.reduce = F)
cld(wact)

summary(lme(A_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit)) # Adding N reduced soil C
anova(lme(A_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(A_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(A_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))

#Is there a difference in soil C at 2nd shallowest depth among treatments?
bwplot(B_C~ftrt|loc*fNfert, data=pdat)
anova(lme(B_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(B_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(B_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(B_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))
```

```
#Is there a difference in soil C at 2nd deepest depth among treatments?
bwplot(C_C~ftrt|loc*fNfert, data=pdat)
anova(lme(C_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(C_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
rosCC <- lme(C_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit)
anova(rosCC)
rosct <- emmeans(rosCC, ~ftrt, cov.reduce = F)
cld(rosct)

roscf <- emmeans(rosCC, ~fNfert, cov.reduce = F)
cld(roscf)

anova(lme(C_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))

#Is there a difference in soil C at deepest depth among treatments?
bwplot(D_C~ftrt|loc*fNfert, data=pdat)
anova(lme(D_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit)) # N treatment is significant
anova(lme(D_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(D_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(D_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))

#Look at other depths
bwplot(B_C~ftrt|loc*fNfert, data=pdat)
bwplot(C_C~ftrt|loc*fNfert, data=pdat)
bwplot(D_C~ftrt|loc*fNfert, data=pdat)

## delta C----
#Calculate change in soil C from 2013 to 2017
pdat$difA_C<-pdat$A_C-pdat$A_C13
pdat$difB_C<-pdat$B_C-pdat$B_C13
pdat$difC_C<-pdat$C_C-pdat$C_C13
pdat$difD_C<-pdat$D_C-pdat$D_C13
#Looking at change is soil C at shallow depth (A)
bwplot(difA_C~ftrt|loc*fNfert, data=pdat)
anova(lme(difA_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(difA_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
```

```
        na.action=na.omit))
anova(lme(difA_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(difA_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))
#No differences by treatments

#Looking at change is soil C at 2nd shallowest depth (B)
bwplot(difB_C~ftrt|loc*fNfert, data=pdat)
anova(lme(difB_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(difB_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(difB_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(difB_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))
#No differences by treatments

#Looking at change is soil C at 2nd deepest depth (C)
bwplot(difC_C~ftrt|loc*fNfert, data=pdat)
anova(lme(difC_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(difC_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(difC_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
beckdiffCC <- lme(difC_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat,
loc=="Becker"),
        na.action=na.omit)
anova(beckdiffCC)
beckdcct <- emmeans(beckdiffCC, ~ftrt, cov.reduce = F)
cld(beckdcct)
beckdccn <- emmeans(beckdiffCC, ~fNfert, cov.reduce = F)
cld(beckdccn)

emmeans((lme(difC_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
          na.action=na.omit)), ~ftrt) #1<5; 1<8; 1, 8, and 12 lost carbon.
summary(glht((lme(C_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat,
loc=="Roseau"),
              na.action=na.omit)), linfct=mcp(fNfert="Tukey"))) # Adding N had more C
emmeans((lme(C_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
          na.action=na.omit)), ~fNfert)
#Looking at change is soil C at deepest depth (D)
bwplot(difD_C~ftrt|loc*fNfert, data=pdat)
anova(lme(difD_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
```

```
        na.action=na.omit))
anova(lme(difD_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(difD_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(difD_C~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))

## Jake regressions ----
#Looking at relationships between MBC and plant community variables
xyplot(MBC~gm2|loc*fNfert, data=pdat) #productivity
anova(lme(MBC~gm2, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(MBC~gm2, random=~1|Rep, data=subset(pdat, loc=="Lamberton"),
        na.action=na.omit))
anova(lme(MBC~gm2, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(MBC~gm2, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit))
#Planted species richness
xyplot(MBC~planted_species_richness|loc*fNfert, data=pdat)
anova(lme(MBC~planted_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Waseca"),
        na.action=na.omit))
anova(lme(MBC~planted_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Lamberton"),
        na.action=na.omit))
anova(lme(MBC~planted_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Roseau"),
        na.action=na.omit))
summary(lme(MBC~planted_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Roseau"),
          na.action=na.omit)) #More species results in more MBC
anova(lme(MBC~planted_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Becker"),
        na.action=na.omit))
#total species richness
xyplot(MBC~total_species_richness|loc*fNfert, data=pdat)
anova(lme(MBC~total_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Waseca"),
        na.action=na.omit))
anova(lme(MBC~total_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Lamberton"),
        na.action=na.omit))
anova(lme(MBC~total_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Roseau"),
```

```
        na.action=na.omit))
anova(lme(MBC~total_species_richness, random=~1|Rep, data=subset(pdat,
loc=="Becker"),
        na.action=na.omit)) #If alpha was 0.1, there would be a positive relationship
#between total species richness and MBC here

#Looking at some things
xyplot(gm2~ftrt|loc*fNfert, data=pdat)
xyplot(MBN~leggm2|loc*fNfert, data=pdat)
xyplot(gm2~WeedCover|loc*fNfert, data=pdat)

#Looking at weed cover as a response variable. How does it
#vary by treatment?
bwplot(WeedCover~ftrt|loc*fNfert, data=pdat)
anova(lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Waseca"),
        na.action=na.omit))
anova(lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat,
loc=="Lamberton"),
        na.action=na.omit))
summary(glht((lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat,
loc=="Lamberton"),
                na.action=na.omit)), linfct=mcp(ftrt="Tukey"))) # 12<8; 8>5; 8>1
summary(glht((lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat,
loc=="Lamberton"),
                na.action=na.omit)), linfct=mcp(fNfert="Tukey"))) # Adding N led to more
weeds
anova(lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Roseau"),
        na.action=na.omit))
anova(lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat, loc=="Becker"),
        na.action=na.omit)) #Affected by treatment at Becker
weedmod1<-lme(WeedCover~ftrt*fNfert, random=~1|Rep, data=subset(pdat,
loc=="Becker"),
         na.action=na.omit)
summary(weedmod1)
cld(lsmeans(weedmod1, ~ftrt|fNfert, cov.reduce=F)) #Grass mix has less weed cover
than switchgrass at Becker



## Michelle regressions----
# total biomass and delta C at depth A
anova(lme(delC1317top~kgha*loc, random=~1|Rep, data=bio, na.action=na.omit))

# total biomass and mbc
anova(lme(MBC~kgha*loc, random=~1|Rep, data=bio, na.action=na.omit))
```

```
# total biomass and beta gluc
bio$B <- as.numeric(as.character(bio$B))
anova(lme(B~kgha*loc, random=~1|Rep, data=bio, na.action=na.omit))

## summary tables----
# NO3, pH, P, K
summarySE(soil, measurevar = "NO3", groupvars = c("Site", "Nfert", "trt"), na.rm=T)
#adding N increased NO3
pHSE <- summarySE(soil, measurevar = "pH", groupvars = c("Site", "Nfert"), na.rm=T)
#adding N decreased pH at Becker
KSE <- summarySE(soil, measurevar = "K", groupvars = c("Site", "Nfert"), na.rm=T)
#adding N increased K at Becker and Lamberton; decreased K at Roseau and Waseca
PSE <- summarySE(soil, measurevar = "P", groupvars = c("Site", "Nfert"), na.rm=T)
#adding N increased P at Lamberton; decreased P at Becker and Waseca
write.csv(pHSE,"C:/Users/Michelle/Dropbox/WD/polyculture/CSV/pHSE.csv" )
write.csv(KSE,"C:/Users/Michelle/Dropbox/WD/polyculture/CSV/KSE.csv" )
write.csv(PSE,"C:/Users/Michelle/Dropbox/WD/polyculture/CSV/PSE.csv" )


# N
brook <- read.csv("C:/Users/Michelle/Dropbox/WD/polyculture/CSV/brook.csv")
Ndepthsite <- summarySE(brook, measurevar = "Nitrogen....", groupvars = c("Site",
"Depth"), na.rm=T)
write.csv(Ndepthsite,"C:/Users/Michelle/Dropbox/WD/polyculture/CSV/Ndepthsite.csv"
)

# C
finalC <- summarySE(brook, measurevar = "Carbon......", groupvars = c("Site", "Depth",
"Nfert", "trt"), na.rm=T)
write.csv(finalC, "C:/Users/Michelle/Dropbox/WD/polyculture/CSV/finalC.csv")

#deltaC
pdat$difA_C<-pdat$A_C-pdat$A_C13
pdat$difB_C<-pdat$B_C-pdat$B_C13
pdat$difC_C<-pdat$C_C-pdat$C_C13
pdat$difD_C<-pdat$D_C-pdat$D_C13
deltaA_C <- summarySE(pdat, measurevar = "difA_C", groupvars = c("loc", "Nfert",
"trt"), na.rm=T)
deltaB_C <- summarySE(pdat, measurevar = "difB_C", groupvars = c("loc", "Nfert",
"trt"), na.rm=T)
deltaC_C <- summarySE(pdat, measurevar = "difC_C", groupvars = c("loc", "Nfert",
"trt"), na.rm=T)
deltaD_C <- summarySE(pdat, measurevar = "difD_C", groupvars = c("loc", "Nfert",
"trt"), na.rm=T)
write.csv(deltaA_C, "C:/Users/Michelle/Dropbox/WD/polyculture/CSV/deltaA_C.csv")
write.csv(deltaB_C, "C:/Users/Michelle/Dropbox/WD/polyculture/CSV/deltaB_C.csv")
```

```
write.csv(deltaC_C, "C:/Users/Michelle/Dropbox/WD/polyculture/CSV/deltaC_C.csv")
write.csv(deltaD_C, "C:/Users/Michelle/Dropbox/WD/polyculture/CSV/deltaD_C.csv")
```

CODE USED FOR CHAPTER TWO

```
## Tuesday, August 13
## Michelle Dobbratz
## Analyzing stems, rhizomes, weights, and respective ratios
## as mixed effects
## Y ~ plant type * stand age * phenological stage
## only use dataset t; there's something wrong with dataset tw
## adding in regression analysis

## import and fix up data ----
t <- read.csv("C:/Users/Michelle/Dropbox/WD/tillering/tillering CSV/tillering.csv")
t$blk <- as.factor(t$blk)
t$stand_age <- as.factor(t$stand_age)
t$time_code <- as.factor(t$time_code)
t$height <- as.numeric(as.character(t$height))
t$lodge <- as.numeric(as.character(t$lodge))
t$rhiz_wt <- as.numeric(as.character(t$rhiz_wt))
t$tw <- t$IWG_culm_wt/t$t_tillers
t <- subset(t, trt == "Spring")
t$t_plants <- t$o_plants + t$p_plants + t$s_plants
t$o_plants <- t$o_plants *6.667
t$t_plants <- t$t_plants *6.667
t$p_plants <- t$p_plants *6.667
t$s_plants <- t$s_plants *6.667

t$o_tillers <- t$o_tillers *6.667
t$t_tillers <- t$t_tillers *6.667
t$p_tillers <- t$p_tillers *6.667
t$s_tillers <- t$s_tillers *6.667

t$o_rhiz <- t$o_rhiz *6.667
t$t_rhiz <- t$t_rhiz *6.667
t$p_rhiz <- t$p_rhiz *6.667

t$o_spikes <- t$o_spikes *6.667
t$t_spikes <- t$t_spikes *6.667
t$p_spikes <- t$p_spikes *6.667

t$grain <- as.numeric(as.character(t$grain))
t$weeds <- t$total_wt - t$IWG_wt

levels(tw$stand_age)<-c("1yo", "2yo")
roots <- read.csv("C:/Users/Michelle/Dropbox/WD/tillering/tillering csv/N balance data -
Roots.csv")
roots$blk <- as.factor(roots$blk)
```

```
## quick yield and harvest index ----
t$hi <- t$grain/ (t$IWG_wt*10)
summary(lm(grain ~ stand_age, data = t))
summary(lm(IWG_wt ~ stand_age, data = t))
summary(lm(hi ~ stand_age, data = t))

summarySE(subset(t, time_code == "2"), measurevar = "IWG_wt", groupvars =
"stand_age")
summarySE(subset(t, time_code == "2"), measurevar = "hi", groupvars = "stand_age")

## load packages ----
library(ggplot2)
library(Rmisc)
library(nlme)
library(emmeans)
library(glmm)
library(lme4)
library(ggpubr)

## total, original, propagule plants ----

fit<-glmer(t_plants~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#stand_age + timepoint:stand_age
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(o_plants~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#timepoint + stand_age + intxn
fit <- lm(o_plants ~timepoint*stand_age, data=t)
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(p_plants~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#timepoint + stand_age + intxn
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(s_plants~timepoint*stand_age+(1|blk),data=subset(t, timepoint !=
"Thaw"),family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit) #none
```

```
## total, original, propagule tillers ----
fit<-glmer(t_tillers~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#stand_age + timepoint:stand_age
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(o_tillers~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#timepoint + stand_age + intxn
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(p_tillers~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#timepoint + stand_age + intxn
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(s_tillers~timepoint*stand_age+(1|blk),data=subset(t, timepoint !=
"Thaw"),family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit) #none


##  rhizomes ----
fit<-glmer(t_rhiz~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#stand_age + timepoint
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(o_rhiz~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#timepoint + stand_age
cld(emmeans(fit, ~timepoint*stand_age))

fit<-glmer(p_rhiz~timepoint*stand_age+(1|blk),data=t,family="poisson")
qqnorm(residuals(fit))
plot(residuals(fit, type="deviance"))
summary(fit)#
```

```
## figures ----


## plants, stems, rhizomes,
## plants ----
pd <- position_dodge(.9)

SE <- summarySE(t, measurevar = "o_plants", groupvars = c("time_code", "stand_age"))
op <- ggplot(SE,
      aes(x=stand_age, y=o_plants, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = o_plants-se, ymax = o_plants + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
  ylim(0,900) +
  ylab(bquote('Number of original plants m'^-2*'')) +
  labs(fill="Time code") +
  theme(legend.position="none")


SE <- summarySE(t, measurevar = "p_plants", groupvars = c("time_code", "stand_age"),
na.rm = T)
pp<- ggplot(SE,
      aes(x=stand_age, y=p_plants, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = p_plants-se, ymax = p_plants + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
#  ylim(0,900) +
  ylab(bquote('Number of propagule plants m'^-2*'')) +
  labs(fill="Time code")

ggplot(SE,
      aes(x=stand_age, y=p_plants, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = p_plants-se, ymax = p_plants + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
#  ylim(0,900) +
  ylab(bquote('Number of propagule plants m'^-2*'')) +
  labs(fill="Time code")
```

```
SE <- summarySE(t, measurevar = "s_plants", groupvars = c("time_code", "stand_age"))
sp <- ggplot(SE,
       aes(x=stand_age, y=s_plants, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = s_plants-se, ymax = s_plants + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
  ylim(0,900) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank()) +
  ylab(bquote('Number of seed shatter plants m'^-2*"")) +
  labs(fill="Time code") +
  theme(legend.position="none")


SE <- summarySE(t, measurevar = "t_plants", groupvars = c("time_code", "stand_age"))
tp <- ggplot(SE,
       aes(x=stand_age, y=t_plants, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = t_plants-se, ymax = t_plants + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
  ylim(0,900) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank()) +
  ylab(bquote('Number of total plants m'^-2*"")) +
  labs(fill="Time code") +
  theme(legend.position="none")

multiplot(op, pp, sp, tp, cols = 4)
annotate_figure(plant_plot,
              bottom = text_grob("Age of stand (yrs)"))
plant_plot
pp
## stems ----
pd <- position_dodge(.9)

SE <- summarySE(t, measurevar = "o_tillers", groupvars = c("time_code", "stand_age"))
ot <- ggplot(SE,
            aes(x=stand_age, y=o_tillers, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = o_tillers-se, ymax = o_tillers + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
#  ylim(0,3000) +
```

```
  ylab(bquote('Number of culms from original plants m'^-2*"")) +
  theme(axis.title.x=element_blank())+
  labs(fill="Time code") +
  theme(legend.position="none")


SE <- summarySE(t, measurevar = "p_tillers", groupvars = c("time_code", "stand_age"))
pt<- ggplot(SE,
            aes(x=stand_age, y=p_tillers, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = p_tillers-se, ymax = p_tillers + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
  ylim(0,3000) +
  ylab(bquote('Number of culms from propagule plants m'^-2*"")) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank(),
axis.title.x=element_blank())+
  labs(fill="Time code") +
  theme(legend.position="none")


SE <- summarySE(t, measurevar = "s_tillers", groupvars = c("time_code", "stand_age"))
st <- ggplot(SE,
             aes(x=stand_age, y=s_tillers, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = s_tillers-se, ymax = s_tillers + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
  ylim(0,3000) +
  ylab(bquote('Number of culms from seed shatter plants m'^-2*"")) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank(),
axis.title.x=element_blank())+
  labs(fill="Time code") +
  theme(legend.position="none")


SE <- summarySE(t, measurevar = "t_tillers", groupvars = c("time_code", "stand_age"))
tt <- ggplot(SE,
             aes(x=stand_age, y=t_tillers, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = t_tillers-se, ymax = t_tillers + se), position =
pd) +
  theme_bw() +
  xlab("Age of stand (yrs)") +
```

```
  ylim(0,3000) +
  ylab(bquote('Number of total culms m'^-2*'')) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank(),
axis.title.x=element_blank())+
  labs(fill="Time code") +
  theme(legend.position="none")

multiplot(ot, pt, st, tt, cols = 4)
#####
pd <- position_dodge(.5)
SE <- summarySE(tw, measurevar = "stems", groupvars = c("time_code", "type",
"stand_age"))
ggplot(SE,
       aes(x=time_code, y=stems, shape = type, color = type)) +
  geom_point(position = pd, size = 2) +
  geom_errorbar(width = 0.35, aes(ymin = stems-se, ymax = stems + se), position = pd) +
  theme_bw() +
  xlab("Phenological stage") +
  scale_x_discrete(labels=c("0" = "Thaw", "1" = "Anthesis", "2" = "Harvest", "3" =
"Frost")) +
  ylab(bquote('Number of culms m'^-2*'')) +
  labs(color="Plant type", shape = "Plant type")+
  facet_grid(~stand_age)

SE <- summarySE(tw, measurevar = "stems", groupvars = c("time_code", "type",
"stand_age"))
pd <- position_dodge(.9)
levels(tw$stand_age)<-c("1yo", "2yo")
ggplot(SE,
       aes(x=stand_age, y=stems, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = stems-se, ymax = stems + se), position = pd) +
  theme_bw() +
  xlab("Age of stand") +
  ylab(bquote('Number of culms m'^-2*'')) +
  labs(fill="Time code")+
  facet_grid(~type)
#####
## rhizomes----
pd <- position_dodge(.9)

SE <- summarySE(t, measurevar = "o_rhiz", groupvars = c("time_code", "stand_age"))
or <- ggplot(SE,
             aes(x=stand_age, y=o_rhiz, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = o_rhiz-se, ymax = o_rhiz + se), position = pd)
```

```
+
  theme_bw() +
  xlab("") +
  ylim(0,300) +
  ylab(bquote('Number of rhizomes from original plants m'^-2*"")) +
  labs(fill="Time code") +
  theme(legend.position="none")


SE <- summarySE(t, measurevar = "p_rhiz", groupvars = c("time_code", "stand_age"))
pr<- ggplot(SE,
            aes(x=stand_age, y=p_rhiz, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = p_rhiz-se, ymax = p_rhiz + se), position = pd)
+
  theme_bw() +
  xlab("Age of stand (yrs)") +
  ylim(0,300) +
  ylab(bquote('Number of rhizomes from propagule plants m'^-2*"")) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank())+
  labs(fill="Time code") +
  theme(legend.position="none")

SE <- summarySE(t, measurevar = "t_rhiz", groupvars = c("time_code", "stand_age"))
tr <- ggplot(SE,
             aes(x=stand_age, y=t_rhiz, fill=time_code)) +
  geom_col(position = pd) +
  geom_errorbar(width = 0.35, aes(ymin = t_rhiz-se, ymax = t_rhiz + se), position = pd) +
  theme_bw() +
  xlab("") +
  ylim(0,300) +
  ylab(bquote('Number of total rhizomes m'^-2*"")) +
  theme(axis.text.y=element_blank(),axis.ticks=element_blank())+
  labs(fill="Time code") +
  theme(legend.position="none")

multiplot(or, pr, tr, cols = 3)


## regression combined----
##grain
t$per_s_tillers <- t$s_tillers/t$t_tillers
summarySE(t, measurevar = "per_s_tillers", groupvars = c("field", "time_code"))

summary(lm(grain ~ IWG_wt, data = t)) #*, +, 0.63
summary(lm(grain ~ t_spikes, data = t))
```

```
summary(lm(grain ~ o_spikes, data = t))
summary(lm(grain ~ p_spikes, data = t))
summary(lm(grain ~ t_plants, data = t))
summary(lm(grain ~ o_plants, data = t))
summary(lm(grain ~ p_plants, data = t)) #., +, R2=0.29
summary(lm(grain ~ t_rhiz, data = t))
summary(lm(grain ~ o_rhiz, data = t))
summary(lm(grain ~ p_rhiz, data = t))
summary(lm(grain ~ t_tillers, data = t))
summary(lm(grain ~ o_tillers, data = t))
summary(lm(grain ~ p_tillers, data = t))
summary(lm(grain ~ s_tillers, data = t))
summary(lm(grain ~ tw, data = t))
summary(lm(grain ~ lodge, data = t))
summary(lm(grain ~ height, data = t))
summary(lm(grain ~ weeds, data = t))

## biomass
summary(lm(IWG_wt ~ t_spikes, data = t)) #*, +, R2=0.11
summary(lm(IWG_wt ~ o_spikes, data = t))
summary(lm(IWG_wt ~ p_spikes, data = t)) #*, +, R2=0.20
summary(lm(IWG_wt ~ t_plants, data = t))
summary(lm(IWG_wt ~ o_plants, data = t)) # **, -, R2= 0.29
summary(lm(IWG_wt ~ p_plants, data = t)) # **, +, R2=0.25
summary(lm(IWG_wt ~ t_rhiz, data = t))
summary(lm(IWG_wt ~ o_rhiz, data = t))
summary(lm(IWG_wt ~ p_rhiz, data = t)) # ., +, R2=0.09
summary(lm(IWG_wt ~ t_tillers, data = t)) #***, +, R2=0.37
summary(lm(IWG_wt ~ o_tillers, data = t)) #**, +, R2=0.32
summary(lm(IWG_wt ~ p_tillers, data = t)) #**, +, R2=0.23
summary(lm(IWG_wt ~ s_tillers, data = t))
summary(lm(IWG_wt ~ tw, data = t)) # *, +, R2=0.16
summary(lm(IWG_wt ~ rhiz_wt, data = t))
summary(lm(IWG_wt ~ lodge, data = t)) # ., +, R2=0.35
summary(lm(IWG_wt ~ height, data = t))
summary(lm(IWG_wt ~ weeds, data = t)) #., +, R2=0.09

summary(lm(p_spikes ~ p_tillers, data = t))
summary(lm(t_spikes ~ p_spikes, data =t)) #***
summary(lm(p_rhiz ~ p_plants, data =t))
summary(lm(p_rhiz ~ p_spikes, data =t)) #
summary(lm(o_rhiz ~ o_spikes, data = t))


## yield interactions
summary(lm(grain ~ veg_wt : p_plants, data = t)) #**, +, R2=0.66
```

```
summary(lm(grain ~ veg_wt * p_plants, data = t)) #*, +, R2=-.89
summary(lm(IWG_wt ~ p_tillers * o_tillers, data = t)) #***, ++-, R2=0.64
summary(lm(IWG_wt ~ p_plants * o_plants, data = t)) #*
summary(lm(IWG_wt ~ t_tillers * tw, data = t))#***, +, R2=0.94

## total spikes
summary(lm(t_spikes ~ IWG_wt, data = t))
summary(lm(t_spikes ~ o_spikes, data = t))
summary(lm(t_spikes ~ p_spikes, data = t))
summary(lm(t_spikes ~ t_plants, data = t))
summary(lm(t_spikes ~ o_plants, data = t))
summary(lm(t_spikes ~ p_plants, data = t))
summary(lm(t_spikes ~ t_rhiz, data = t))
summary(lm(t_spikes ~ o_rhiz, data = t))
summary(lm(t_spikes ~ p_rhiz, data = t))
summary(lm(t_spikes ~ t_tillers, data = t))
summary(lm(t_spikes ~ o_tillers, data = t))
summary(lm(t_spikes ~ p_tillers, data = t))
summary(lm(t_spikes ~ s_tillers, data = t))
summary(lm(t_spikes ~ tw, data = t))
summary(lm(t_spikes ~ lodge, data = t))
summary(lm(t_spikes ~ height, data = t))
summary(lm(t_spikes ~ weeds, data = t))

summary(lm(o_spikes ~ p_spikes, data = t))

## total tillers
summary(lm(t_tillers ~ IWG_wt, data = t))
summary(lm(t_tillers ~ t_spikes, data = t))
summary(lm(t_tillers ~ o_spikes, data = t))
summary(lm(t_tillers ~ p_spikes, data = t))
summary(lm(t_tillers ~ t_plants, data = t))
summary(lm(t_tillers ~ o_plants, data = t))
summary(lm(t_tillers ~ p_plants, data = t))
summary(lm(t_tillers ~ t_rhiz, data = t))
summary(lm(t_tillers ~ o_rhiz, data = t))
summary(lm(t_tillers ~ p_rhiz, data = t))
#summary(lm(t_spikes ~ t_tillers, data = t))
summary(lm(t_tillers ~ o_tillers, data = t))
summary(lm(t_tillers ~ p_tillers, data = t))
summary(lm(t_tillers ~ s_tillers, data = t))
summary(lm(t_tillers ~ tw, data = t))
summary(lm(t_tillers ~ lodge, data = t))
summary(lm(t_tillers ~ height, data = t))
summary(lm(t_tillers ~ weeds, data = t))
```

```
## tw
summary(lm(tw ~ IWG_wt, data = t))
summary(lm(tw ~ t_spikes, data = t))
summary(lm(tw ~ o_spikes, data = t))
summary(lm(tw ~ p_spikes, data = t))
summary(lm(tw ~ t_plants, data = t))
summary(lm(tw ~ o_plants, data = t))
summary(lm(tw ~ p_plants, data = t))
summary(lm(tw ~ t_rhiz, data = t))
summary(lm(tw ~ o_rhiz, data = t))
summary(lm(tw ~ p_rhiz, data = t))
summary(lm(tw ~ t_tillers, data = t))
summary(lm(tw ~ o_tillers, data = t))
summary(lm(tw ~ p_tillers, data = t))
summary(lm(tw ~ s_tillers, data = t))
#summary(lm(t_tillers ~ tw, data = t))
summary(lm(tw ~ lodge, data = t))
summary(lm(tw ~ height, data = t))
summary(lm(tw ~ weeds, data = t))

## lodge
summary(lm(lodge ~ IWG_wt, data = t))
summary(lm(lodge ~ t_spikes, data = t))
summary(lm(lodge ~ o_spikes, data = t))
summary(lm(lodge ~ p_spikes, data = t))
summary(lm(lodge ~ t_plants, data = t))
summary(lm(lodge ~ o_plants, data = t))
summary(lm(lodge ~ p_plants, data = t))
summary(lm(lodge ~ t_rhiz, data = t))
summary(lm(lodge ~ o_rhiz, data = t))
summary(lm(lodge ~ p_rhiz, data = t))
summary(lm(lodge ~ t_tillers, data = t))
summary(lm(lodge ~ o_tillers, data = t))
summary(lm(lodge ~ p_tillers, data = t))
summary(lm(lodge ~ s_tillers, data = t))
summary(lm(lodge ~ tw, data = t))
#summary(lm(tw ~ lodge, data = t))
summary(lm(lodge ~ height, data = t))
summary(lm(lodge ~ weeds, data = t))


## regression 1 yo (ignore comments)----
t<-subset(t, time_code =="2")
##grain
```

```
summary(lm(grain ~ IWG_wt, data = subset(t, field == "R55")))
summary(lm(grain ~ t_spikes, data = subset(t, field == "R55")))
summary(lm(grain ~ o_spikes, data = subset(t, field == "R55")))
summary(lm(grain ~ p_spikes, data = subset(t, field == "R55")))
summary(lm(grain ~ t_plants, data = subset(t, field == "R55")))
summary(lm(grain ~ o_plants, data = subset(t, field == "R55")))
summary(lm(grain ~ p_plants, data = subset(t, field == "R55"))) #., +, R2=0.29
summary(lm(grain ~ t_rhiz, data = subset(t, field == "R55")))
summary(lm(grain ~ t_rhiz, data = subset(t, field == "R55")))
summary(lm(grain ~ p_rhiz, data = subset(t, field == "R55")))
summary(lm(grain ~ t_tillers, data = subset(t, field == "R55")))
summary(lm(grain ~ o_tillers, data = subset(t, field == "R55")))
summary(lm(grain ~ p_tillers, data = subset(t, field == "R55")))
summary(lm(grain ~ s_tillers, data = subset(t, field == "R55")))
summary(lm(grain ~ tw, data = subset(t, field == "R55")))
summary(lm(grain ~ lodge, data = subset(t, field == "R55")))
summary(lm(grain ~ height, data = subset(t, field == "R55")))
summary(lm(grain ~ weeds, data = subset(t, field == "R55")))



## biomass
summary(lm(IWG_wt ~ t_spikes, data = subset(t, field == "R55"))) #*, +, R2=0.11
summary(lm(IWG_wt ~ o_spikes, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ p_spikes, data = subset(t, field == "R55"))) #*, +, R2=0.20
summary(lm(IWG_wt ~ t_plants, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ o_plants, data = subset(t, field == "R55"))) # **, -, R2= 0.29
summary(lm(IWG_wt ~ p_plants, data = subset(t, field == "R55"))) # **, +, R2=0.25
summary(lm(IWG_wt ~ t_rhiz, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ o_rhiz, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ p_rhiz, data = subset(t, field == "R55"))) # ., +, R2=0.09
summary(lm(IWG_wt ~ t_tillers, data = subset(t, field == "R55"))) #***, +, R2=0.37
summary(lm(IWG_wt ~ o_tillers, data = subset(t, field == "R55"))) #**, +, R2=0.32
summary(lm(IWG_wt ~ p_tillers, data = subset(t, field == "R55"))) #**, +, R2=0.23
summary(lm(IWG_wt ~ s_tillers, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ tw, data = subset(t, field == "R55"))) # *, +, R2=0.16
summary(lm(IWG_wt ~ rhiz_wt, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ lodge, data = subset(t, field == "R55"))) # ., +, R2=0.35
summary(lm(IWG_wt ~ height, data = subset(t, field == "R55")))
summary(lm(IWG_wt ~ weeds, data = subset(t, field == "R55"))) #., +, R2=0.09

summary(lm(p_spikes ~ p_tillers, data = subset(t, field == "R55"))) #***
summary(lm(p_spikes ~ p_plants, data = subset(t, field == "R55"))) #
summary(lm(p_rhiz ~ p_plants, data = subset(t, field == "R55"))) #

summary(lm(t_spikes ~ p_spikes, data = subset(t, field == "R55"))) #*
```

```
summary(lm(p_rhiz ~ p_plants, data = subset(t, field == "R55"))) #.
summary(lm(p_rhiz ~ p_spikes, data = subset(t, field == "R55"))) #
summary(lm(o_rhiz ~ o_spikes, data = subset(t, field == "R55"))) #this lack of effect is
interesting
summary(lm(p_rhiz ~ o_plants, data = subset(t, field =="R55")))
summary(lm(lodge ~ t_plants : p_rhiz, data = subset(t, field =="R55")))


## yield interactions
summary(lm(grain ~ veg_wt : p_plants, data = subset(t, field == "R55"))) #
summary(lm(grain ~ veg_wt * p_plants, data = subset(t, field == "R55"))) #NA
summary(lm(IWG_wt ~ p_tillers : o_tillers, data = subset(t, field == "R55"))) #
summary(lm(IWG_wt ~ p_plants * o_plants, data = subset(t, field == "R55"))) #no
benefit
summary(lm(IWG_wt ~ t_tillers * tw, data = subset(t, field == "R55")))#no benefit

## total spikes
summary(lm(t_spikes ~ IWG_wt, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ o_spikes, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ p_spikes, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ t_plants, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ o_plants, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ p_plants, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ t_rhiz, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ o_rhiz, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ p_rhiz, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ t_tillers, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ o_tillers, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ p_tillers, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ s_tillers, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ tw, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ lodge, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ height, data = subset(t, field == "R55")))
summary(lm(t_spikes ~ weeds, data = subset(t, field == "R55")))

summary(lm(o_spikes ~ p_spikes, data = subset(t, field == "R55")))

## total tillers
summary(lm(t_tillers ~ IWG_wt, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ t_spikes, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ o_spikes, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ p_spikes, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ t_plants, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ o_plants, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ p_plants, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ t_rhiz, data = subset(t, field == "R55")))
```

```
summary(lm(t_tillers ~ o_rhiz, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ p_rhiz, data = subset(t, field == "R55")))
#summary(lm(t_spikes ~ t_tillers, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ o_tillers, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ p_tillers, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ s_tillers, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ tw, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ lodge, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ height, data = subset(t, field == "R55")))
summary(lm(t_tillers ~ weeds, data = subset(t, field == "R55")))

summary(lm(t_plants ~ s_tillers, data = subset(t, field == "R55")))


## tw
summary(lm(tw ~ IWG_wt, data = subset(t, field == "R55")))
summary(lm(tw ~ t_spikes, data = subset(t, field == "R55")))
summary(lm(tw ~ o_spikes, data = subset(t, field == "R55")))
summary(lm(tw ~ p_spikes, data = subset(t, field == "R55")))
summary(lm(tw ~ t_plants, data = subset(t, field == "R55")))
summary(lm(tw ~ o_plants, data = subset(t, field == "R55")))
summary(lm(tw ~ p_plants, data = subset(t, field == "R55")))
summary(lm(tw ~ t_rhiz, data = subset(t, field == "R55")))
summary(lm(tw ~ o_rhiz, data = subset(t, field == "R55")))
summary(lm(tw ~ p_rhiz, data = subset(t, field == "R55")))
summary(lm(tw ~ t_tillers, data = subset(t, field == "R55")))
summary(lm(tw ~ o_tillers, data = subset(t, field == "R55")))
summary(lm(tw ~ p_tillers, data = subset(t, field == "R55")))
summary(lm(tw ~ s_tillers, data = subset(t, field == "R55")))
#summary(lm(t_tillers ~ tw, data = subset(t, field == "R55")))
summary(lm(tw ~ lodge, data = subset(t, field == "R55")))
summary(lm(tw ~ height, data = subset(t, field == "R55")))
summary(lm(tw ~ weeds, data = subset(t, field == "R55")))

## lodge
summary(lm(lodge ~ IWG_wt, data = subset(t, field == "R55")))
summary(lm(lodge ~ t_spikes, data = subset(t, field == "R55")))
summary(lm(lodge ~ o_spikes, data = subset(t, field == "R55")))
summary(lm(lodge ~ p_spikes, data = subset(t, field == "R55")))
summary(lm(lodge ~ t_plants, data = subset(t, field == "R55")))
summary(lm(lodge ~ o_plants, data = subset(t, field == "R55")))
summary(lm(lodge ~ p_plants, data = subset(t, field == "R55")))
summary(lm(lodge ~ t_rhiz, data = subset(t, field == "R55")))
summary(lm(lodge ~ o_rhiz, data = subset(t, field == "R55")))
summary(lm(lodge ~ p_rhiz, data = subset(t, field == "R55")))
summary(lm(lodge ~ t_tillers, data = subset(t, field == "R55")))
```

```
summary(lm(lodge ~ o_tillers, data = subset(t, field == "R55")))
summary(lm(lodge ~ p_tillers, data = subset(t, field == "R55")))
summary(lm(lodge ~ s_tillers, data = subset(t, field == "R55")))
summary(lm(lodge ~ tw, data = subset(t, field == "R55")))
#summary(lm(tw ~ lodge, data = subset(t, field == "R55")))
summary(lm(lodge ~ height, data = subset(t, field == "R55")))
summary(lm(lodge ~ weeds, data = subset(t, field == "R55")))


## regression 2 yo (ignore comments) ----
##grain
summary(lm(grain ~ IWG_wt, data = subset(t, field == "R70"))) #*, +, 0.63
summary(lm(grain ~ t_spikes, data = subset(t, field == "R70")))
summary(lm(grain ~ o_spikes, data = subset(t, field == "R70")))
summary(lm(grain ~ p_spikes, data = subset(t, field == "R70")))
summary(lm(grain ~ t_plants, data = subset(t, field == "R70")))
summary(lm(grain ~ o_plants, data = subset(t, field == "R70")))
summary(lm(grain ~ p_plants, data = subset(t, field == "R70"))) #., +, R2=0.29
summary(lm(grain ~ t_rhiz, data = subset(t, field == "R70")))
summary(lm(grain ~ o_rhiz, data = subset(t, field == "R70")))
summary(lm(grain ~ p_rhiz, data = subset(t, field == "R70")))
summary(lm(grain ~ t_tillers, data = subset(t, field == "R70")))
summary(lm(grain ~ o_tillers, data = subset(t, field == "R70")))
summary(lm(grain ~ p_tillers, data = subset(t, field == "R70")))
summary(lm(grain ~ s_tillers, data = subset(t, field == "R70")))
summary(lm(grain ~ tw, data = subset(t, field == "R70")))
summary(lm(grain ~ lodge, data = subset(t, field == "R70")))
summary(lm(grain ~ height, data = subset(t, field == "R70")))
summary(lm(grain ~ weeds, data = subset(t, field == "R70")))

## biomass
summary(lm(IWG_wt ~ t_spikes, data = subset(t, field == "R70"))) #*, +, R2=0.11
summary(lm(IWG_wt ~ o_spikes, data = subset(t, field == "R70")))
summary(lm(IWG_wt ~ p_spikes, data = subset(t, field == "R70"))) #*, +, R2=0.20
summary(lm(IWG_wt ~ t_plants, data = subset(t, field == "R70")))
summary(lm(IWG_wt ~ o_plants, data = subset(t, field == "R70"))) # **, -, R2= 0.29
summary(lm(IWG_wt ~ p_plants, data = subset(t, field == "R70"))) # **, +, R2=0.25
summary(lm(IWG_wt ~ t_rhiz, data = subset(t, field == "R70")))
summary(lm(IWG_wt ~ o_rhiz, data = subset(t, field == "R70")))
summary(lm(IWG_wt ~ p_rhiz, data = subset(t, field == "R70"))) # ., +, R2=0.09
summary(lm(IWG_wt ~ t_tillers, data = subset(t, field == "R70"))) #***, +, R2=0.37
summary(lm(IWG_wt ~ o_tillers, data = subset(t, field == "R70"))) #**, +, R2=0.32
summary(lm(IWG_wt ~ p_tillers, data = subset(t, field == "R70"))) #**, +, R2=0.23
summary(lm(IWG_wt ~ s_tillers, data = subset(t, field == "R70")))
summary(lm(IWG_wt ~ tw, data = subset(t, field == "R70"))) # *, +, R2=0.16
summary(lm(IWG_wt ~ rhiz_wt, data = subset(t, field == "R70")))
```

```
summary(lm(IWG_wt ~ lodge, data = subset(t, field == "R70"))) # ., +, R2=0.35
summary(lm(IWG_wt ~ height, data = subset(t, field == "R70")))
summary(lm(IWG_wt ~ weeds, data = subset(t, field == "R70"))) #., +, R2=0.09

summary(lm(p_spikes ~ p_tillers, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ p_spikes, data =subset(t, field == "R70")))
summary(lm(p_rhiz ~ p_plants, data =subset(t, field == "R70"))) #
summary(lm(p_rhiz ~ p_spikes, data =subset(t, field == "R70"))) #.
summary(lm(o_rhiz ~ o_spikes, data = subset(t, field == "R70")))


## yield interactions
summary(lm(grain ~ veg_wt : p_plants, data = subset(t, field == "R70"))) #.
summary(lm(grain ~ veg_wt * p_plants, data = subset(t, field == "R70"))) #NA
summary(lm(IWG_wt ~ p_tillers * o_tillers, data = subset(t, field == "R70"))) #NA
summary(lm(IWG_wt ~ p_plants * o_plants, data = subset(t, field == "R70"))) #NA
summary(lm(IWG_wt ~ t_tillers * tw, data = subset(t, field == "R70")))#NA

## total spikes
summary(lm(t_spikes ~ IWG_wt, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ o_spikes, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ p_spikes, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ t_plants, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ o_plants, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ p_plants, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ t_rhiz, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ o_rhiz, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ p_rhiz, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ t_tillers, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ o_tillers, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ p_tillers, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ s_tillers, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ tw, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ lodge, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ height, data = subset(t, field == "R70")))
summary(lm(t_spikes ~ weeds, data = subset(t, field == "R70")))

summary(lm(o_spikes ~ p_spikes, data = subset(t, field == "R70")))

## total tillers
summary(lm(t_tillers ~ IWG_wt, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ t_spikes, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ o_spikes, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ p_spikes, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ t_plants, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ o_plants, data = subset(t, field == "R70")))
```

```
summary(lm(t_tillers ~ p_plants, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ t_rhiz, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ o_rhiz, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ p_rhiz, data = subset(t, field == "R70")))
#summary(lm(t_spikes ~ t_tillers, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ o_tillers, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ p_tillers, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ s_tillers, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ tw, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ lodge, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ height, data = subset(t, field == "R70")))
summary(lm(t_tillers ~ weeds, data = subset(t, field == "R70")))

## tw
summary(lm(tw ~ IWG_wt, data = subset(t, field == "R70")))
summary(lm(tw ~ t_spikes, data = subset(t, field == "R70")))
summary(lm(tw ~ o_spikes, data = subset(t, field == "R70")))
summary(lm(tw ~ p_spikes, data = subset(t, field == "R70")))
summary(lm(tw ~ t_plants, data = subset(t, field == "R70")))
summary(lm(tw ~ o_plants, data = subset(t, field == "R70")))
summary(lm(tw ~ p_plants, data = subset(t, field == "R70")))
summary(lm(tw ~ t_rhiz, data = subset(t, field == "R70")))
summary(lm(tw ~ o_rhiz, data = subset(t, field == "R70")))
summary(lm(tw ~ p_rhiz, data = subset(t, field == "R70")))
summary(lm(tw ~ t_tillers, data = subset(t, field == "R70")))
summary(lm(tw ~ o_tillers, data = subset(t, field == "R70")))
summary(lm(tw ~ p_tillers, data = subset(t, field == "R70")))
summary(lm(tw ~ s_tillers, data = subset(t, field == "R70")))
#summary(lm(t_tillers ~ tw, data = subset(t, field == "R70")))
summary(lm(tw ~ lodge, data = subset(t, field == "R70")))
summary(lm(tw ~ height, data = subset(t, field == "R70")))
summary(lm(tw ~ weeds, data = subset(t, field == "R70")))

## lodge
summary(lm(lodge ~ IWG_wt, data = subset(t, field == "R70")))
summary(lm(lodge ~ t_spikes, data = subset(t, field == "R70")))
summary(lm(lodge ~ o_spikes, data = subset(t, field == "R70")))
summary(lm(lodge ~ p_spikes, data = subset(t, field == "R70")))
summary(lm(lodge ~ t_plants, data = subset(t, field == "R70")))
summary(lm(lodge ~ o_plants, data = subset(t, field == "R70")))
summary(lm(lodge ~ p_plants, data = subset(t, field == "R70")))
summary(lm(lodge ~ t_rhiz, data = subset(t, field == "R70")))
summary(lm(lodge ~ o_rhiz, data = subset(t, field == "R70")))
summary(lm(lodge ~ p_rhiz, data = subset(t, field == "R70")))
summary(lm(lodge ~ t_tillers, data = subset(t, field == "R70")))
summary(lm(lodge ~ o_tillers, data = subset(t, field == "R70")))
```

```
summary(lm(lodge ~ p_tillers, data = subset(t, field == "R70")))
summary(lm(lodge ~ s_tillers, data = subset(t, field == "R70")))
summary(lm(lodge ~ tw, data = subset(t, field == "R70")))
#summary(lm(tw ~ lodge, data = subset(t, field == "R70")))
summary(lm(lodge ~ height, data = subset(t, field == "R70")))
summary(lm(lodge ~ weeds, data = subset(t, field == "R70")))




## regression FINAL ----
anthesis <- subset(t, time_code == "1")
harvest <- subset(t, time_code =="2")
anthesis$grain <- harvest$grain
anthesis$IWG_wt <- harvest$IWG_wt
anthesis$lodge <- harvest$lodge

## grain
summary(lm(grain ~ t_rhiz, data = subset(anthesis, field == "R55")))
summary(lm(grain ~ t_plants, data = subset(anthesis, field == "R55")))
summary(lm(grain ~ o_plants, data = subset(anthesis, field == "R55")))
summary(lm(grain ~ t_tillers, data = subset(anthesis, field == "R55")))
summary(lm(grain ~ weeds, data = subset(anthesis, field == "R55")))

summary(lm(grain ~ t_rhiz, data = subset(anthesis, field == "R70")))
summary(lm(grain ~ t_plants, data = subset(anthesis, field == "R70")))
summary(lm(grain ~ o_plants, data = subset(anthesis, field == "R70")))
summary(lm(grain ~ t_tillers, data = subset(anthesis, field == "R70")))
summary(lm(grain ~ p_tillers, data = subset(anthesis, field == "R70")))
summary(lm(grain ~ s_tillers, data = subset(anthesis, field == "R70")))
summary(lm(grain ~ weeds, data = subset(anthesis, field == "R70")))

summary(lm(grain ~ t_spikes, data = subset(harvest, field == "R55")))
summary(lm(grain ~ t_rhiz, data = subset(harvest, field == "R55")))
summary(lm(grain ~ t_plants, data = subset(harvest, field == "R55")))
summary(lm(grain ~ o_plants, data = subset(harvest, field == "R55")))
summary(lm(grain ~ p_tillers, data = subset(harvest, field == "R55")))
summary(lm(grain ~ t_tillers, data = subset(harvest, field == "R55")))
summary(lm(grain ~ weeds, data = subset(harvest, field == "R55")))

summary(lm(grain ~ t_spikes, data = subset(harvest, field == "R70")))
summary(lm(grain ~ t_rhiz, data = subset(harvest, field == "R70")))
summary(lm(grain ~ t_plants, data = subset(harvest, field == "R70")))
summary(lm(grain ~ o_plants, data = subset(harvest, field == "R70")))
summary(lm(grain ~ t_tillers, data = subset(harvest, field == "R70")))
```

```
summary(lm(grain ~ p_tillers, data = subset(harvest, field == "R70")))
summary(lm(grain ~ s_tillers, data = subset(harvest, field == "R70")))
summary(lm(grain ~ weeds, data = subset(harvest, field == "R70")))


## biomass
summary(lm(IWG_wt ~ t_rhiz, data = subset(anthesis, field == "R55")))
summary(lm(IWG_wt ~ t_plants, data = subset(anthesis, field == "R55")))
summary(lm(IWG_wt ~ o_plants, data = subset(anthesis, field == "R55")))
summary(lm(IWG_wt ~ t_tillers, data = subset(anthesis, field == "R55")))
summary(lm(IWG_wt ~ weeds, data = subset(anthesis, field == "R55")))

summary(lm(IWG_wt ~ t_rhiz, data = subset(anthesis, field == "R70")))
summary(lm(IWG_wt ~ t_plants, data = subset(anthesis, field == "R70")))
summary(lm(IWG_wt ~ o_plants, data = subset(anthesis, field == "R70")))
summary(lm(IWG_wt ~ t_tillers, data = subset(anthesis, field == "R70")))
summary(lm(IWG_wt ~ p_tillers, data = subset(anthesis, field == "R70")))
summary(lm(IWG_wt ~ s_tillers, data = subset(anthesis, field == "R70")))
summary(lm(IWG_wt ~ weeds, data = subset(anthesis, field == "R70")))

summary(lm(IWG_wt ~ t_spikes, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ t_rhiz, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ t_plants, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ o_plants, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ p_tillers, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ t_tillers, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ weeds, data = subset(harvest, field == "R55")))

summary(lm(IWG_wt ~ t_spikes, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ t_rhiz, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ t_plants, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ o_plants, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ t_tillers, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ p_tillers, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ s_tillers, data = subset(harvest, field == "R70")))
summary(lm(IWG_wt ~ weeds, data = subset(harvest, field == "R70")))

## spikes
summary(lm(t_spikes ~ t_rhiz, data = subset(anthesis, field == "R55")))
summary(lm(t_spikes ~ t_plants, data = subset(anthesis, field == "R55")))
summary(lm(t_spikes ~ o_plants, data = subset(anthesis, field == "R55")))
summary(lm(t_spikes ~ t_tillers, data = subset(anthesis, field == "R55")))
summary(lm(t_spikes ~ weeds, data = subset(anthesis, field == "R55")))

summary(lm(t_spikes ~ t_rhiz, data = subset(anthesis, field == "R70")))
summary(lm(t_spikes ~ t_plants, data = subset(anthesis, field == "R70")))
```

```
summary(lm(t_spikes ~ o_plants, data = subset(anthesis, field == "R70")))
summary(lm(t_spikes ~ t_tillers, data = subset(anthesis, field == "R70")))
summary(lm(t_spikes ~ p_tillers, data = subset(anthesis, field == "R70")))
summary(lm(t_spikes ~ s_tillers, data = subset(anthesis, field == "R70")))
summary(lm(t_spikes ~ weeds, data = subset(anthesis, field == "R70")))

summary(lm(t_spikes ~ t_spikes, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ t_rhiz, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ t_plants, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ o_plants, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ p_tillers, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ t_tillers, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ weeds, data = subset(harvest, field == "R55")))

summary(lm(t_spikes ~ t_spikes, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ t_rhiz, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ t_plants, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ o_plants, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ t_tillers, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ p_tillers, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ s_tillers, data = subset(harvest, field == "R70")))
summary(lm(t_spikes ~ weeds, data = subset(harvest, field == "R70")))

## lodging score
summary(lm(lodge ~ t_rhiz, data = subset(anthesis, field == "R55")))
summary(lm(lodge ~ t_plants, data = subset(anthesis, field == "R55")))
summary(lm(lodge ~ o_plants, data = subset(anthesis, field == "R55")))
summary(lm(lodge ~ t_tillers, data = subset(anthesis, field == "R55")))
summary(lm(lodge ~ weeds, data = subset(anthesis, field == "R55")))

summary(lm(lodge ~ t_rhiz, data = subset(anthesis, field == "R70")))
summary(lm(lodge ~ t_plants, data = subset(anthesis, field == "R70")))
summary(lm(lodge ~ o_plants, data = subset(anthesis, field == "R70")))
summary(lm(lodge ~ t_tillers, data = subset(anthesis, field == "R70")))
summary(lm(lodge ~ p_tillers, data = subset(anthesis, field == "R70")))
summary(lm(lodge ~ s_tillers, data = subset(anthesis, field == "R70")))
summary(lm(lodge ~ weeds, data = subset(anthesis, field == "R70")))

summary(lm(lodge ~ t_spikes, data = subset(harvest, field == "R55")))
summary(lm(lodge ~ t_rhiz, data = subset(harvest, field == "R55")))
summary(lm(lodge ~ t_plants, data = subset(harvest, field == "R55")))
summary(lm(lodge ~ o_plants, data = subset(harvest, field == "R55")))
summary(lm(lodge ~ p_tillers, data = subset(harvest, field == "R55")))
summary(lm(lodge ~ t_tillers, data = subset(harvest, field == "R55")))
summary(lm(lodge ~ weeds, data = subset(harvest, field == "R55")))
```

```
summary(lm(lodge ~ t_spikes, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ t_rhiz, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ t_plants, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ o_plants, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ t_tillers, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ p_tillers, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ s_tillers, data = subset(harvest, field == "R70")))
summary(lm(lodge ~ weeds, data = subset(harvest, field == "R70")))

summary(lm(grain ~ trt, data = subset(harvest, field == "R55")))
summary(lm(IWG_wt ~ trt, data = subset(harvest, field == "R55")))
summary(lm(t_spikes ~ trt, data = subset(harvest, field == "R55")))
summary(lm(t_plants ~ o_plants, data = subset(harvest, field == "R55")))
summary(lm(t_rhiz ~ p_tillers, data = subset(harvest, field =="R55")))
summary(lm(IWG_wt ~ t_spikes, data = subset(harvest, field == "R70")))
summary(lm(o_plants ~ t_tillers, data = subset(harvest, field == "R55")))
summary(lm(o_plants ~ t_spikes, data = subset(harvest, field == "R55")))
```

CODE USED FOR CHAPTER THREE

```
## CN, mineral N soil for Nbalance
## 6/9/2019
## Modify this code to give better x axis labels
## scale_x_discrete(labels=c("1" = "SG", "5" = "GM", "8" = "LG", "12" = "HD"))


library(alr4)
library(Rmisc)
library(ggplot2)
library(nlme)
library(emmeans)
library(agricolae)

## import ----
CN <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/CN.csv")
CN$perN <- as.numeric(as.character(CN$perN))
CN$blk <- as.factor(CN$blk)

N <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/tissue_N.csv")
Nitrogen <- N
N <- subset (Nitrogen, field =="R55")
N <- subset(N, plot !="100")
Ni <- subset(Nitrogen, field == "R70")
Ni <- subset(Ni, plot !="avg")
Nit <- subset(Nitrogen, trt =="Control")
Nit <- subset(Nit, plot != "avg")
N$blk <- as.factor(N$blk)
N$time_code <- as.factor(N$time_code)
N$stand_age <- as.factor(N$stand_age)
Nit$blk <- as.factor(Nit$blk)
Nit$time_code <- as.factor(Nit$time_code)
Nit$stand_age <- as.factor(Nit$stand_age)
Nitrogen$stand_age<- as.factor(Nitrogen$stand_age)
Nitrogen$blk <- as.factor(Nitrogen$blk)

t <- read.csv("C:/Users/Michelle/Dropbox/WD/tillering/tillering CSV/tillering.csv")
t$blk <- as.factor(t$blk)
t$stand_age <- as.factor(t$stand_age)
t$time_code <- as.factor(t$time_code)
t$height <- as.numeric(as.character(t$height))
t$lodge <- as.numeric(as.character(t$lodge))
t$rhiz_wt <- as.numeric(as.character(t$rhiz_wt))
t$tw <- t$IWG_culm_wt/t$t_tillers
t$t_plants <- t$o_plants + t$p_plants + t$s_plants
```

```
grain <- read.csv("C:/Users/Michelle/Dropbox/WD/tillering/tillering CSV/grain_wt.csv")
grain$blk <- as.factor(grain$blk)
grain <- subset(grain [-c(20,21),])

dN <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/deltatissueN.csv")
dN$blk <- as.factor(dN$blk)
dN$time_code <- as.factor(dN$time_code)
dN$stand_age <- as.factor(dN$stand_age)

netuptake <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/netuptake.csv")
netuptake$blk <- as.factor(netuptake$blk)
netuptake$time_code <- as.factor(netuptake$time_code)
netuptake$stand_age <- as.factor(netuptake$stand_age)

MineralN <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/MineralN.csv")
MineralN$stand_age <- as.factor(MineralN$stand_age)
MineralN$time_code <- as.factor(MineralN$time_code)
MineralN$blk <- as.factor(MineralN$blk)
MineralN$nit <- MineralN$C.nit..ppm.
MineralN$amm <- MineralN$C.amm..ppm.
mn <- subset(MineralN, type == "Nmin0")

dMinN <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/dMinN.csv")
dMinN$stand_age <- as.factor(dMinN$stand_age)
dMinN$time_code <- as.factor(dMinN$time_code)
dMinN$blk <- as.factor(dMinN$blk)

nmin <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/nmin.csv")
nmin <- subset(nmin, time_code !="3")
nmin$blk <- as.factor(nmin$blk)
nmin$stand_age <-  as.factor(nmin$stand_age)
nmin$time_code <- as.factor(nmin$time_code)

master <- read.csv("C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/master_nbal.csv")
master$stand_age <- as.factor(master$stand_age)
master$time_code <- as.factor(master$time_code)
master$lodge <- as.numeric(as.character(master$lodge))

## Biomass ----
anova(lm(g_m2 ~ trt * stand_age * time_code * type, data = master))
m1 <- aov(g_m2 ~ stand_age + time_code, data = subset(master, type == "IWG"))
m2 <- aov(g_m2 ~ stand_age + time_code, data = subset(master, type == "roots"))
TukeyHSD(m1)
TukeyHSD(m2)
summarySE(subset(master, type == "IWG"), measurevar = "g_m2", groupvars =
```

```
c("time_code", "stand_age"), na.rm = T)
summarySE(subset(master, type == "roots"), measurevar = "g_m2", groupvars =
c("time_code", "stand_age"), na.rm = T)


## Correlations b/t soil and plants ----
iwg <- subset(master, type =="IWG")
grain <- subset(master, type =="grain")
roots<-subset(master, type =="roots")
iwg1 <- subset(master, stand_age =="1")
summary(lm(nit.g_m2 ~ trt, data = master))
summary(lm(delnitg ~ trt, data = master))
summary(lm(delammg ~ trt, data = master))

summary(lm(lodge ~ nit.g_m2, data = subset(iwg, time_code == "2")))
summary(lm(lodge ~ amm.g_m2, data = subset(iwg, time_code == "2"))) #*
summary(lm(amm.g_m2 ~ trt, data = subset(iwg, time_code == "2"))) #*
summary(lm(lodge ~ delnitg, data = subset(iwg, time_code == "2")))
summary(lm(lodge ~ delammg, data = subset(iwg, time_code == "2"))) #.

summary(lm(grain_kgha ~ nit.g_m2, data = subset(grain, time_code == "2")))
summary(lm(grain_kgha ~ amm.g_m2, data = subset(grain, time_code == "2"))) #.
summary(lm(grain_kgha ~ delnitg, data = subset(grain, time_code == "2")))
summary(lm(grain_kgha ~ delammg, data = subset(grain, time_code == "2")))

summary(lm(Corrected..N ~ nit.g_m2 * time_code, data = iwg))
summary(lm(Corrected..N ~ amm.g_m2 * time_code, data = iwg))
summary(lm(Corrected..N ~ delnitg * time_code, data = iwg))
summary(lm(Corrected..N ~ delammg * time_code, data = iwg)) #*

summary(lm(N_g_m2 ~ nit.g_m2 * time_code, data = iwg))
summary(lm(N_g_m2 ~ amm.g_m2 * time_code, data = iwg))
summary(lm(N_g_m2 ~ delnitg * time_code, data = iwg))
summary(lm(N_g_m2 ~ delammg * time_code, data = iwg))

summary(lm(Corrected..N ~ nit.g_m2 * time_code, data = roots)) #**
summary(lm(Corrected..N ~ amm.g_m2 * time_code, data = roots)) #
summary(lm(Corrected..N ~ delnitg* time_code, data = roots))
summary(lm(Corrected..N ~ delammg* time_code, data = roots))

summary(lm(N_g_m2 ~ nit.g_m2 * time_code, data = roots))
summary(lm(N_g_m2 ~ amm.g_m2 * time_code, data = roots))
summary(lm(N_g_m2 ~ delnitg * time_code, data = roots))
summary(lm(N_g_m2 ~ delammg * time_code, data = roots))

summary(lm(Corrected..N ~ nit.g_m2, data = subset(grain, time_code == "2")))
```

```
summary(lm(Corrected..N ~ amm.g_m2, data = subset(grain, time_code == "2"))) #**
summary(lm(Corrected..N ~ delnitg, data = subset(grain, time_code == "2")))
summary(lm(Corrected..N ~ delammg, data = subset(grain, time_code == "2")))

summary(lm(N_g_m2 ~ nit.g_m2, data = subset(grain, time_code == "2")))
summary(lm(N_g_m2 ~ amm.g_m2, data = subset(grain, time_code == "2"))) #.
summary(lm(N_g_m2 ~ delnitg, data = subset(grain, time_code == "2")))
summary(lm(N_g_m2 ~ delammg, data = subset(grain, time_code == "2")))

summary(lm(d_N_g_m2 ~ nit.g_m2, data = subset(iwg, time_code == "2"))) #.
summary(lm(d_N_g_m2 ~ amm.g_m2, data = subset(iwg, time_code == "2")))
summary(lm(d_N_g_m2 ~ delnitg, data = subset(iwg, time_code == "2")))
summary(lm(d_N_g_m2 ~ delammg, data = subset(iwg, time_code == "2")))

summary(lm(g_m2 ~ nit.g_m2 * time_code, data = iwg))
summary(lm(g_m2 ~ amm.g_m2 * time_code, data = iwg))
summary(lm(g_m2 ~ delnitg* time_code, data = iwg))
summary(lm(g_m2 ~ delammg* time_code, data = iwg))

summary(lm(g_m2 ~ nit.g_m2 * time_code, data = roots))
summary(lm(g_m2 ~ amm.g_m2 * time_code, data = roots))
summary(lm(g_m2 ~ delnitg * time_code, data = roots))
summary(lm(g_m2 ~ delammg * time_code, data = roots))

harv <- subset(master, time_code == "2")
summarySE(master, measurevar = "d_N_g_m2", groupvars = "time_code", na.rm = T)
summarySE(master, measurevar = "d_N_g_m2", groupvars = c("trt", "stand_age"), na.rm
= T)

## ANOVA for table ----
anova(lm(N_g_m2 ~ trt * time_code * stand_age, data = tot))
anova(lm(N_g_m2 ~ trt * time_code * stand_age * type, data = Nitrogen))
anova(lm(Corrected..N ~ trt * time_code * stand_age * type, data = Nitrogen))
anova(lm(N_g_m2 ~ trt * time_code * stand_age, data = subset(Nitrogen, type ==
"roots")))
anova(lm(Corrected..N ~ trt * time_code * stand_age, data = subset(Nitrogen, type ==
"roots")))
anova(lm(N_g_m2 ~ trt * time_code * stand_age, data = subset(Nitrogen, type ==
"IWG")))
anova(lm(Corrected..N ~ trt * time_code * stand_age, data = subset(Nitrogen, type ==
"IWG")))
anova(lm(grain_kgha ~ trt * stand_age, data = grain))
anova(lm(N_g_m2 ~ trt * stand_age, data = subset(Nitrogen, type == "grain")))
anova(lm(Corrected..N ~ trt *  stand_age, data = subset(Nitrogen, type == "grain")))
anova(lm(lodge ~ trt * stand_age, data = t))
anova(lm(height ~ trt * stand_age, data = t))
```

```
anova(lm(perN ~ trt * Dkey * Time, data = subset(CN, Field == "R55")))
anova(lm(perN ~ trt * Dkey * Time, data = subset(CN, Field == "R70")))
anova(lm(nit ~ time_code * trt, data = subset(mn, stand_age == "1")))
anova(lm(amm ~ time_code * trt, data = subset(mn, stand_age == "1")))
anova(lm(nit ~ time_code * trt, data = subset(mn, stand_age == "2")))
anova(lm(amm ~ time_code * trt, data = subset(mn, stand_age == "2")))
anova(lme(delnit ~ time_code * trt, random = ~ 1|blk, data = subset(nmin, stand_age
=="1"), na.action=na.omit))
anova(lme(delamm ~ time_code * trt, random = ~ 1|blk, data = subset(nmin, stand_age
=="1"), na.action=na.omit))
anova(lme(delnit ~ time_code * trt, random = ~ 1|blk, data = subset(nmin, stand_age
=="2"), na.action=na.omit))
anova(lme(delamm ~ time_code * trt, random = ~ 1|blk, data = subset(nmin, stand_age
=="2"), na.action=na.omit))
## grain yield
summarySE(grain, measurevar = "grain_kgha", groupvars = c("Field", "trt"))

## X tissue N ----
N <- subset(N, plot != "avg")

tot <- subset(Nitrogen, type == "Total")
all <- lme(N_g_m2 ~ trt + time_code, random = ~1|blk, data = tot )
anova(all) #time_code
cld(emmeans(all, ~time_code)) # 0 = a, 23 = b

tot <- subset(tot, time_code != "0")
all <- lme(N_g_m2 ~ trt + time_code + stand_age, random = ~1|blk, data = tot )
anova(all) #stand_age
cld(emmeans(all, ~trt)) #control = a, split = ab, spring = b

total <- lme(N_g_m2 ~ trt + time_code + type, random = ~1|blk, data = N)
anova(total)
cld(emmeans(total, ~trt + type))

total2 <- lme(Corrected..N ~ trt + time_code + type, random = ~1|blk, data =
subset(Nitrogen, time_code !="0"), na.action = na.omit)
anova(total2)
cld(emmeans(total2, ~trt + time_code + type))
cld(emmeans(total2, ~trt + type))
cld(emmeans(total2, ~type + time_code))


total <- lme(N_g_m2 ~ stand_age * time_code, random = ~1|blk, data =
subset(subset(Nitrogen, trt == "Control"), type =="Total"))
anova(total)
cld(emmeans(total, ~stand_age * time_code))
```

```
all <- lme(Corrected..N ~ type + time_code, random = ~1|blk, data = subset(N, type !=
"Total"))
anova(all)
cld(emmeans(all, ~ type + time_code))
emmeans(all, ~ type + time_code)

all2 <- lme(Corrected..N ~ trt * type, random = ~1|blk, data = subset(N, type != "Total"))
anova(all2)
cld(emmeans(all2, ~type))

## Post-hoc too complicated
all3 <- lme(Corrected..N ~ time_code + type + stand_age, random = ~1|blk, data = Nit,
na.action = na.omit)
anova(all3)
cld(emmeans(all3, ~ type + time_code + stand_age))

all4 <- lme(N_g_m2 ~ time_code + type + stand_age, random = ~1|blk, data = Nit,
na.action = na.omit)
anova(all4)
cld(emmeans(all4, ~ type + time_code + stand_age))

all5 <- lme(N_g_m2 ~ trt * type, random = ~1|blk, data = subset(N, type != "Total"))
anova(all5)
cld(emmeans(all5, ~type))



roots <- lme(Corrected..N ~ trt * time_code, random = ~1|blk, data = subset(N, type ==
"roots"))
anova(roots) #trt, timing, trt:timing
cld(emmeans(roots, ~trt * time_code))

roots <- lme(N_g_m2 ~ trt * time_code, random = ~1|blk, data = subset(N, type ==
"roots"))
anova(roots) #trt, timing, trt:timing
cld(emmeans(roots, ~trt * time_code))

roots <- lme(Corrected..N ~ stand_age * time_code, random = ~1|blk, data = subset(Nit,
type == "roots"))
anova(roots) #trt, timing, trt:timing
cld(emmeans(roots, ~stand_age * time_code))

roots <- lme(N_g_m2 ~ trt * time_code, random = ~1|blk, data = subset(N, type ==
"roots"))
anova(roots) #none
```

```
roots <- lme(N_g_m2 ~ time_code * stand_age, random = ~1|blk, data =
subset(subset(subset(Nitrogen, type == "roots"), trt == "Control"), time_code != "1"))
anova(roots)
cld(emmeans(roots, ~time_code+stand_age))



iwg <- lme(Corrected..N ~ trt * time_code, random = ~1|blk, data = subset(N, type ==
"IWG"))
anova(iwg) #timing, trt
cld(emmeans(iwg, ~time_code + trt))
cld(emmeans(iwg, ~trt))

iwg <- lme(N_g_m2 ~ trt * time_code, random = ~1|blk, data = subset(N, type ==
"IWG"))
anova(iwg) #timing, trt
cld(emmeans(iwg, ~time_code + trt))
cld(emmeans(iwg, ~trt))

iwg <- lme(N_g_m2 ~ stand_age + time_code, random = ~1|blk, data = subset(Nit, type
== "IWG"))
anova(iwg) #stand_age
cld(emmeans(iwg, ~stand_age))

iwg <- lme(Corrected..N ~ stand_age + time_code, random = ~1|blk, data = subset(Nit,
type == "IWG"))
anova(iwg) #stand_age
cld(emmeans(iwg, ~stand_age + time_code))

grain <- lme(Corrected..N ~ trt, random = ~1|blk, data = subset(N, type == "grain"))
anova(grain) #none

grain <- lme(N_g_m2 ~ trt, random = ~1|blk, data = subset(N, type == "grain"))
anova(grain) #none

roots <- lme(N_g_m2 ~ time_code, random = ~1|blk, data = subset(Ni, type == "Roots"))
anova(roots) #time_code
cld(emmeans(roots, ~time_code))

## post-hoc for tables ----
## avg table
nothaw <- subset(subset(Nitrogen, time_code != "0"), plot != "avg")

total <- lm(N_g_m2 ~ trt * stand_age, data = subset(nothaw, type == "Total"))
anova(total) #none
```

```
cld(emmeans(total, ~trt * stand_age))

root <- lm(N_g_m2 ~ trt * stand_age, data = subset(nothaw, type == "roots"))
anova(root)
cld(emmeans(root, ~trt*stand_age))

root <- lm(Corrected..N ~ trt * stand_age, data = subset(nothaw, type == "roots"))
anova(root)
cld(emmeans(root, ~trt*stand_age))

out <- lm(N_g_m2 ~ trt * stand_age, data = subset(nothaw, type == "IWG"))
anova(out)
cld(emmeans(out, ~trt*stand_age))

out <- lme(Corrected..N ~ trt + stand_age, random = ~1|blk, data = subset(nothaw, type
== "IWG"))
anova(out)
cld(emmeans(out, ~trt*stand_age))


out <- lm(N_g_m2 ~ type * trt * stand_age, data = subset(nothaw, type != "Total"))
anova(out)
cld(emmeans(out, ~type*trt*stand_age))

out <- lm(Corrected..N ~ type * trt * stand_age, data = subset(nothaw, type != "Total"))
anova(out)
cld(emmeans(out, ~type*trt*stand_age))

## by sampling time table
Nitrogen$time_code <- as.factor(Nitrogen$time_code)
total <- lm(N_g_m2 ~ trt * stand_age * time_code, data = subset(Nitrogen, type ==
"Total"))
anova(total) #stand_age, time_code, stand_age:time_code
cld(emmeans(total, ~ trt*stand_age*time_code))

out <- lm(N_g_m2 ~ trt * stand_age * time_code, data = subset(Nitrogen, type ==
"roots"))
anova(out)
cld(emmeans(out, ~ trt*stand_age*time_code))

out <- lm(Corrected..N ~ trt * stand_age * time_code, data = subset(Nitrogen, type ==
"roots"))
anova(out)
cld(emmeans(out, ~ trt*stand_age*time_code))

out <- lm(N_g_m2 ~ trt * stand_age * time_code, data = subset(Nitrogen, type ==
```

```
"IWG"))
anova(out)
cld(emmeans(out, ~ trt*stand_age*time_code))

out <- lm(Corrected..N ~ trt * stand_age * time_code, data = subset(Nitrogen, type ==
"IWG"))
anova(out)
cld(emmeans(out, ~ trt*stand_age*time_code))

out <- lm(d_N_g_m2 ~ trt * stand_age, data = subset(dN, time_code =="0-2"))
anova(out)
cld(emmeans(out, ~trt*stand_age))

## grain table
total <- lm(N_g_m2 ~ trt * stand_age, data = subset(Nitrogen, type == "grain"), na.action
= na.omit)
anova(total)
cld(emmeans(total, ~ trt*stand_age))

total <- lm(Corrected..N ~ trt * stand_age, data = subset(Nitrogen, type == "grain"))
anova(total)
cld(emmeans(total, ~ stand_age))

lod <- lme(lodge ~ stand_age * trt, random = ~1|blk, data = t, na.action = na.omit)
anova(lod)
cld(emmeans(lod, ~stand_age * trt))

ht <- lme(height ~ stand_age * trt, random = ~1|blk, data = t, na.action = na.omit)
anova(ht)
cld(emmeans(ht, ~stand_age * trt))

grain$stand_age <- as.factor(grain$stand_age)
gr <- lme(grain_kgha ~ stand_age * trt, random = ~1|blk, data = grain, na.action =
na.omit)
anova(gr)
cld(emmeans(gr, ~stand_age * trt))

## table 5
total2 <- lme(N_g_m2 ~ trt + time_code + type, random = ~1|blk, data =
subset(subset(Nitrogen, time_code !="0"), type != "Total"), na.action = na.omit)
anova(total2)
cld(emmeans(total2, ~trt + type))
cld(emmeans(total2, ~type + time_code))

total2 <- lme(Corrected..N ~ trt + time_code + type, random = ~1|blk, data =
subset(subset(Nitrogen, time_code !="0"), type != "Total"), na.action = na.omit)
```

```
anova(total2)

cld(emmeans(total2, ~trt + type))
cld(emmeans(total2, ~type + time_code))

## tables for results section
summarySE(subset(Nitrogen, type !="Total"), measurevar = "N_g_m2", groupvars =
c("type"))
summarySE(subset(Nitrogen, type !="Total"), measurevar = "Corrected..N", groupvars =
c("type"), na.rm = T)
summarySE(subset(dN, time_code =="0-2"), measurevar = "d_N_g_m2", groupvars =
c("trt", "stand_age"))
summarySE(grain, measurevar = "grain_gm2", groupvars = c("stand_age", "trt"))
out <- lm(grain_gm2 ~ trt * stand_age, data = grain)
anova(out)
cld(emmeans(out, ~ trt*stand_age))
grain$stand_age <- as.factor(grain$stand_age)

## tables ----
summarySE(subset(Nitrogen, type == "Total"), measurevar = "N_g_m2", groupvars =
c("trt", "stand_age"))
summarySE(subset(subset(Nitrogen, type == "Total"), time_code == "0"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"))
summarySE(subset(subset(Nitrogen, type == "Total"), time_code == "2"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "Total"), time_code == "3"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)

summarySE(subset(Nitrogen, type == "roots"), measurevar = "N_g_m2", groupvars =
c("trt", "stand_age"))summarySE(subset(subset(Nitrogen, type == "roots"), time_code ==
"0"), measurevar = "N_g_m2", groupvars = c("trt", "stand_age"))
summarySE(subset(subset(Nitrogen, type == "roots"), time_code == "2"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "roots"), time_code == "3"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)

summarySE(subset(Nitrogen, type == "roots"), measurevar = "Corrected..N", groupvars
= c("trt", "stand_age"))
summarySE(subset(subset(Nitrogen, type == "roots"), time_code == "0"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"))
summarySE(subset(subset(Nitrogen, type == "roots"), time_code == "2"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "roots"), time_code == "3"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)

summarySE(subset(Nitrogen, type == "IWG"), measurevar = "N_g_m2", groupvars =
```

```
c("trt", "stand_age"))
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "0"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm=T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "1"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm=T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "2"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "3"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)

summarySE(subset(Nitrogen, type == "IWG"), measurevar = "Corrected..N", groupvars
= c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "0"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "1"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "2"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "IWG"), time_code == "3"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)

summarySE(subset(subset(Nitrogen, type == "grain"), time_code == "2"), measurevar =
"N_g_m2", groupvars = c("trt", "stand_age"), na.rm = T)
summarySE(subset(subset(Nitrogen, type == "grain"), time_code == "2"), measurevar =
"Corrected..N", groupvars = c("trt", "stand_age"), na.rm = T)


summarySE(subset(Nitrogen, type != "Total"), measurevar = "N_g_m2", groupvars =
c("type", "stand_age"))

## figures ----
## Type by stand age table
SE <- summarySE(subset(Nitrogen, type != "Total"), measurevar = "N_g_m2",
groupvars = c("type", "stand_age"))

###### Total tissue ----
## X Total nitrogen by type and treatment; R55
SE <- summarySE(subset(N, type != "Total"), measurevar = "N_g_m2", groupvars =
c("type", "trt"))
ggplot(SE,
      aes(fill = type, x=trt, y=N_g_m2)) +
  geom_bar(stat="identity") +
  theme_bw() +
  xlab("Treatment") +
  ylab(expression(paste("Total plant nitrogen (g N ",m^-2,")")))
```

```
## X Total N by type, stacked
SE <- summarySE(subset(subset(N, type != "Total"), time_code !="1"), measurevar =
"N_g_m2", groupvars = c("type", "trt", "time_code"), na.rm =T)
pd <- position_dodge(0)
ggplot(SE,
      aes(fill = type, x=trt, y=N_g_m2)) +
  geom_bar(stat="identity") +
  theme_bw() +
  xlab("Treatment") +
  ylab(expression(paste("Total plant nitrogen (g N ",m^-2,")"))) +
  facet_grid(~time_code)

## X Total nitrogen by type and treatment; controls for 1yo and 2yo
SE <- summarySE(subset(subset(subset(Nitrogen, type != "Total"), trt == "Control"),
time_code != "1"), measurevar = "N_g_m2", groupvars = c("type", "time_code",
"stand_age"), na.rm = T)
ggplot(SE,
      aes(fill = type, x=time_code, y=N_g_m2)) +
  geom_bar(stat="identity") +
  theme_bw() +
  xlab("Timing") +
  ylab(expression(paste("Total plant nitrogen (g N ",m^-2,")"))) +
  facet_grid(~stand_age)

## X Percent N by type
SE <- summarySE(subset(N, type != "Total"), measurevar = "Corrected..N", groupvars =
c("type", "time_code"), na.rm = T)
pd <- position_dodge(.95)
ggplot(SE,
      aes(fill = type, x=time_code, y=Corrected..N)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N + se), position = pd,
width = .1) +
  theme_bw() +
  xlab("Sampling time") +
  ylab("Total nitrogen (%)")

ggplot(SE,
      aes(fill = time_code, x=type, y=Corrected..N)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N + se), position = pd,
width = .1) +
  theme_bw() +
  xlab("Tissue type") +
  ylab("Nitrogen concentration (%)")
```

```
## X % N by type (adjacent) and treatment (consolidated by sampling time) in 1yo
SE <- summarySE(subset(N, type != "Total"), measurevar = "Corrected..N", groupvars =
c("type", "trt"), na.rm = T)
pd <- position_dodge(.95)
ggplot(SE,
       aes(fill = trt, x=type, y=Corrected..N)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N + se), position = pd,
width = .1) +
  theme_bw() +
  xlab("Type") +
  ylab("Nitrogen concentration (%)")

## X % N by stand age and sampling time in control plots
SE <- summarySE(subset(subset(Nitrogen, type != "Total"), trt == "Control"),
measurevar = "Corrected..N", groupvars = c("type", "time_code", "stand_age"), na.rm =
T)
ggplot(SE,
       aes(fill = time_code, x=type, y=Corrected..N)) +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N + se), position = pd,
width = .1) +
  geom_bar(position=pd, stat="identity") +
  theme_bw() +
  xlab("Tissue type") +
  ylab("Total nitrogen (%)") +
  facet_grid(~stand_age)

## X Total N by stand age and sampling time in control plots'
SE <- summarySE(subset(subset(subset(Nitrogen, type != "Total"), trt == "Control"), plot
!= "avg"), measurevar = "N_g_m2", groupvars = c("type", "time_code", "stand_age"),
na.rm = T)
ggplot(SE,
       aes(fill = time_code, x=type, y=N_g_m2)) +
  geom_errorbar(aes(ymin = N_g_m2-se, ymax = N_g_m2 + se), position = pd, width =
.1) +
  geom_bar(position=pd, stat="identity") +
  theme_bw() +
  xlab("Type") +
  ylab(expression(paste("Total plant nitrogen (g N ",m^-2,")"))) +
  facet_grid(~stand_age)

## Total N by type (adjacent) and treatment (consolidated by sampling time) in 1yo
SE <- summarySE(subset(N, type != "Total"), measurevar = "N_g_m2", groupvars =
c("type", "trt"), na.rm = T)
pd <- position_dodge(.95)
ggplot(SE,
```

```
      aes(fill = trt, x=type, y=N_g_m2)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = N_g_m2-se, ymax = N_g_m2 + se), position = pd, width =
.1) +
  theme_bw() +
  xlab("Type") +
  ylab(expression(paste("Total plant nitrogen (g N ",m^-2,")")))

###### Roots ----
roots <- subset(N, type =="roots")

## X Percent nitrogen in roots of 1yo by timing and treatment
SE <- summarySE(subset(roots, time_code != "1"), measurevar = "Corrected..N",
groupvars = c("trt", "time_code"))
pd <- position_dodge(.95)
ggplot(SE,
      aes(fill = trt, x=time_code, y=Corrected..N)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N + se), position = pd,
width = .1) +
  theme_bw() +
  xlab("Timing") +
  ylab("Total nitrogen in root tissue (%)")

## X Roots total N by treatment and time in 1yo
SE <- summarySE(subset(roots, time_code != "1"), measurevar = "N_g_m2", groupvars
= c("trt", "time_code"))
pd <- position_dodge(.95)
ggplot(SE,
      aes(fill = trt, x=time_code, y=N_g_m2)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = N_g_m2-se, ymax = N_g_m2 + se), position = pd, width =
.1) +
  theme_bw() +
  xlab("Timing") +
  ylab(expression(paste("Total root nitrogen (g N ",m^-2,")")))

## X Roots total N in control
SE <- summarySE(subset(subset(subset(Nitrogen, type == "roots"), trt == "Control"),
time_code != "1"), measurevar = "N_g_m2", groupvars = c("time_code", "stand_age"))
ggplot(SE,
      aes(x=time_code, y=N_g_m2)) +
  geom_bar(stat="identity") +
  geom_errorbar(aes(ymin = N_g_m2-se, ymax = N_g_m2+se), width = .1) +
  theme_bw() +
  xlab("Sampling time") +
```

```
  ylab(expression(paste("Total root nitrogen (g N ",m^-2,")"))) +
  facet_grid(~stand_age)

## Roots %N in control
SE <- summarySE(subset(subset(subset(Nitrogen, type == "roots"), trt == "Control"),
time_code != "1"), measurevar = "Corrected..N", groupvars = c("time_code",
"stand_age"))
ggplot(SE,
       aes(x=time_code, y=Corrected..N)) +
  geom_bar(stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N+se), width = .1) +
  theme_bw() +
  xlab("Sampling time") +
  ylab("Total nitrogen in root tissue (%)") +
  facet_grid(~stand_age)

###### IWG ----
iwg <- subset(Nitrogen, type =="IWG")

## X IWG total nitrogen by trt and time 1yo
SE <- summarySE(subset(iwg, stand_age == "1"), measurevar = "N_g_m2", groupvars =
c("trt", "time_code"))
pd <- position_dodge(.95)
ggplot(SE,
       aes(fill = trt, x=time_code, y=N_g_m2)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = N_g_m2-se, ymax = N_g_m2 + se), position = pd, width =
.1) +
  theme_bw() +
  xlab("Timing") +
  ylab(expression(paste("Total shoot nitrogen (g N ",m^-2,")")))

## IWG %N by trt and time 1yo
SE <- summarySE(subset(iwg, stand_age == "1"), measurevar = "Corrected..N",
groupvars = c("trt", "time_code"))
pd <- position_dodge(.95)
ggplot(SE,
       aes(fill = trt, x=time_code, y=Corrected..N)) +
  geom_bar(position=pd, stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N + se), position = pd,
width = .1) +
  theme_bw() +
  xlab("Timing") +
  ylab("Nitrogen in shoot tissue (%)")

## IWG total N by stand age and time control
```

```
SE <- summarySE(subset(subset(Nitrogen, type == "IWG"), trt == "Control"),
measurevar = "N_g_m2", groupvars = c("time_code", "stand_age"))
ggplot(SE,
       aes(x=time_code, y=N_g_m2)) +
  geom_bar(stat="identity") +
  geom_errorbar(aes(ymin = N_g_m2-se, ymax = N_g_m2+se), width = .1) +
  theme_bw() +
  xlab("Sampling time") +
  ylab(expression(paste("Total shoot nitrogen (g N ",m^-2,")"))) +
  facet_grid(~stand_age)

## IWG %N by stand age and time control
SE <- summarySE(subset(subset(Nitrogen, type == "IWG"), trt == "Control"),
measurevar = "Corrected..N", groupvars = c("time_code", "stand_age"), na.rm=T)
ggplot(SE,
       aes(x=time_code, y=Corrected..N)) +
  geom_bar(stat="identity") +
  geom_errorbar(aes(ymin = Corrected..N-se, ymax = Corrected..N+se), width = .1) +
  theme_bw() +
  xlab("Sampling time") +
  ylab("Nitrogen in shoot tissue (%)")+
  facet_grid(~stand_age)

## X Nmin ----
r55nout <- lme(delnit ~ time_code * trt, random = ~ 1|blk, data = subset(nmin, stand_age
=="1"), na.action=na.omit)
r55aout <- lme(delamm ~ time_code * trt, random = ~ 1|blk, data = subset(nmin,
stand_age =="1"), na.action=na.omit)
anova(r55nout)#time_code; time_code*trt no intercept
anova(r55aout)#none no intercept
cld(emmeans(r55nout, ~trt*time_code))

r70nout <- lme(delnit ~ time_code * trt, random = ~ 1|blk, data = subset(nmin, stand_age
=="2"), na.action=na.omit)
r70aout <- lme(delamm ~ time_code * trt, random = ~ 1|blk, data = subset(nmin,
stand_age =="2"), na.action=na.omit)
anova(r70nout)#time_code; time_code*trt no intercept
cld(emmeans(r70nout, ~trt*time_code))
anova(r70aout)#none but intercept

nmin$delnitg <- nmin$delnit*0.195
SE <- summarySE(nmin, measurevar = "delnitg", groupvars = c("stand_age",
"time_code", "trt"), na.rm = TRUE)
pd<- position_dodge(.5)
addline_format <- function(x,...){
  gsub('\\s','\n',x)
```

```
}
ggplot(SE,
      aes(x = time_code, group = trt, y = delnitg, color = trt, shape = trt, linetype = trt)) +
  geom_point(size = 2, position = pd) +
  geom_errorbar(aes(ymin = delnitg-se, ymax = delnitg + se), width = .1, position = pd) +
  labs(color = "Treatment",
      shape = "Treatment",
      x = "Sampling time") +
  geom_line(position = pd) +
  geom_hline(yintercept = 0) +
  scale_x_discrete(labels=addline_format(c("0" = "Green-up to anthesis", "1" = "Anthesis
to harvest", "2" = "Harvest to dormancy"))) +
  theme(legend.position = "none") +
  ylab(expression(paste("Net mineralized nitrite/nitrate (g N ",m^-2,")"))) +
  facet_grid(~stand_age)

nmin$delammg <- nmin$delamm*0.195
SE <- summarySE(nmin, measurevar = "delammg", groupvars = c("stand_age",
"time_code", "trt"), na.rm = T)
pd<- position_dodge(.5)
ggplot(SE,
      aes(x = time_code, group = trt, linetype = trt, y = delammg, color = trt, shape = trt))
+
  geom_point(size = 2, position = pd) +
  geom_errorbar(aes(ymin = delammg-se, ymax = delammg + se), width = .1, position =
pd) +
  geom_hline(yintercept = 0) +
  geom_line(position = pd) +
  theme(legend.position = "none") +
  scale_x_discrete(labels=addline_format(c("0" = "Green-up to anthesis", "1" = "Anthesis
to harvest", "2" = "Harvest to dormancy"))) +
  labs(color = "Treatment",
      shape = "Treatment",
      x = "Sampling time") +
  ylab(expression(paste("Net mineralized ammonia/ammonium (g N ",m^-2,")"))) +
  #  scale_y_continuous(breaks = pretty(SE$Dkey), labels = abs(pretty(SE$Dkey))) +
  facet_grid(~stand_age)

## X Mineral N ----
nitout <- lm(nit ~ stand_age * time_code * trt, data = mn)
outlierTest(nitout)
ammout <- lm(amm ~ stand_age * time_code * trt, data = mn)
outlierTest(ammout)
aout <- mn[-c(22, 47),]
ammout <- lm(amm ~ stand_age * time_code * trt, data = aout)
nout <- mn[-c(32,35),]
```

```
nitout <- lm(nit ~ stand_age * time_code * trt, data = nout)

nitout <- lme(nit ~ stand_age * time_code * trt, random = ~ 1|blk, data = nout)
ammout <- lm(amm ~ stand_age * time_code * trt, data = aout)
anova(ammout) #all regressors and interactions
anova(nitout) #stand_age, time_code, trt, stand_age:time_code, stand_age:trt,
time_code:trt, 3way

r55nitout <- lme(nit ~ time_code * trt, random = ~ 1|blk, data = subset(nout, stand_age
=="1"))
r55ammout <- lm(amm ~ time_code * trt, data = subset(aout, stand_age =="1"))
anova(r55ammout) #all regressors and interactions
anova(r55nitout) #all regressors and interactions
cld(emmeans(r55ammout, ~trt*time_code))
cld(emmeans(r55nitout, ~trt*time_code))
cld(emmeans(r55nitout, ~trt))

r70nitout <- lme(nit ~ time_code * trt, random = ~ 1|blk, data = subset(nout, stand_age
=="2"))
r70ammout <- lm(amm ~ time_code * trt, data = subset(aout, stand_age =="2"))
anova(r70ammout) #all regressors and interactions
anova(r70nitout) #time_code and time_code:trt
cld(emmeans(r70ammout, ~trt))
cld(emmeans(r70ammout, ~time_code))
cld(emmeans(r70nitout, ~time_code))
cld(emmeans(r70nitout, ~trt*time_code))

nout$nitg <- nout$nit*0.1935
SE <- summarySE(nout, measurevar = "nitg", groupvars = c("stand_age", "time_code",
"trt"), na.rm = T)
pd<- position_dodge(.5)
ggplot(SE,
      aes(x = time_code, group = trt, linetype = trt, y = nitg, color = trt, shape = trt)) +
  geom_point(size = 2, position = pd) +
  geom_errorbar(aes(ymin = nitg-se, ymax = nitg + se), width = .1, position = pd) +
  labs(color = "Treatment",
      shape = "Treatment",
      x = "Sampling time") +
  ylab(expression(paste("Soil nitrite/nitrate (g N ",m^-2,")"))) +
  geom_line(position = pd) +
  theme(legend.position = "none") +
  scale_x_discrete(labels=c("0" = "Green up", "1" = "Anthesis", "2" = "Harvest", "3" =
"Dormancy")) +
  facet_grid(~stand_age)

aout$ammg <- aout$amm*0.1935
```

```
SE <- summarySE(aout, measurevar = "ammg", groupvars = c("stand_age", "time_code",
"trt"), na.rm = T)
pd<- position_dodge(.5)
ggplot(SE,
      aes(x = time_code, group = trt, linetype = trt, y = ammg, color = trt, shape = trt)) +
  geom_point(size = 2, position = pd) +
  geom_errorbar(aes(ymin = ammg-se, ymax = ammg + se), width = .1, position = pd) +
  labs(color = "Treatment",
      shape = "Treatment",
      x = "Sampling time") +
  ylab(expression(paste("Soil ammonia/ammonium (g N ",m^-2,")"))) +
  #  scale_y_continuous(breaks = pretty(SE$Dkey), labels = abs(pretty(SE$Dkey))) +
  geom_line(position = pd) +
  theme(legend.position = "none") +
  scale_x_discrete(labels=c("0" = "Green up", "1" = "Anthesis", "2" = "Harvest", "3" =
"Dormancy")) +
  facet_grid(~stand_age)

## change in mineral N ----
dmin <- lme(d_nit_g_m2 ~ stand_age * time_code * trt, random = ~ 1|blk, data =
dMinN)
anova(dmin) #none

dmin <- lme(d_amm_g_m2 ~ stand_age * time_code * trt, random = ~ 1|blk, data =
dMinN)
anova(dmin) #none

## X total soil N ----
out <- lm(perN ~ Field + trt + Dkey + Time + Field:Dkey, data = CN)
outlierTest(out)
NoOut<- CN[-c(454, 455, 457, 462, 464, 465, 466, 467, 469, 470, 471, 472, 473, 474,
468),]
anova(out)

out <- lme(perN ~ Field + trt + Dkey + Time, random = ~ 1|blk, data = NoOut)
summary(out) #Field, Depth, Time
cld(emmeans(out, ~ Field + Dkey + Time))
cld(emmeans(out, ~ Field))
cld(emmeans(out, ~ Time))

r55out <- lme(perN ~ trt + Dkey + Time, random = ~ 1|blk, data = subset(NoOut, Field
== "R55"))
summary(r55out) #Depth, Time; no intxns
cld(emmeans(r55out, ~ Time))

r70out <- lme(perN ~ trt + Dkey + Time, random = ~ 1|blk, data = subset(NoOut, Field
```

```
== "R70"))
summary(r70out) #Depth, Time; no intxns
cld(emmeans(r70out, ~ Time))
brookSE <- summarySE(brook, measurevar = "carbon", groupvars = c("Site", "depth"),
na.rm = T)

NoOut$Dkey <- NoOut$Dkey*-1
SE <- summarySE(NoOut, measurevar = "perN", groupvars = c("Field", "Time",
"Dkey"))
ggplot(SE,
       aes(x = perN, y = Dkey, color = Time, shape = Time)) +
  #  geom_line(aes(linetype = Site)) +
  geom_point(size = 2) +
  geom_path() +
  labs(color = "Time",
       x = "Total Soil N (%)",
       y = "Average sampling depth (cm)") +
  scale_y_continuous(breaks = pretty(SE$Dkey), labels = abs(pretty(SE$Dkey))) +
  facet_grid(~Field)




## delta tissue N ----
total <- lme(d_N_g_m2 ~ trt + time_code, random = ~1|blk, data = subset(dN, stand_age
== "1"))
anova(total) #time_code
cld(emmeans(total, ~time_code))

total <- lme(d_N_g_m2 ~ stand_age * time_code, random = ~1|blk, data = subset(dN, trt
== "Control"))
anova(total) #time_code
cld(emmeans(total, ~stand_age + time_code))

SE <- summarySE(subset(dN, trt == "Control"), measurevar = "d_N_g_m2", groupvars =
c("time_code", "stand_age"))
ggplot(SE,
       aes(x=time_code, y=d_N_g_m2)) +
  geom_bar(stat="identity") +
  geom_errorbar(aes(ymin = d_N_g_m2-se, ymax = d_N_g_m2+se), width = .1) +
  theme_bw() +
  xlab("Sampling time") +
  ylab("Change in total nitrogen (gN/m2)") +
  facet_grid(~stand_age)

SE <- summarySE(subset(dN, stand_age == "1"), measurevar = "d_N_g_m2", groupvars
```

```
= c("time_code", "trt"))
ggplot(SE,
       aes(x=trt, y=d_N_g_m2)) +
  geom_bar(stat="identity") +
  geom_errorbar(aes(ymin = d_N_g_m2-se, ymax = d_N_g_m2+se), width = .1) +
  theme_bw() +
  xlab("Sampling time") +
  ylab("Change in total nitrogen (gN/m2)") +
  facet_grid(~time_code)

## net uptake ----
total <- lme(d_N_g_m2 ~ trt, random = ~1|blk, data = subset(netuptake, stand_age ==
"1"))
anova(total) #time_code
cld(emmeans(total, ~trt))

total <- lme(d_N_g_m2 ~ stand_age, random = ~1|blk, data = subset(netuptake, trt ==
"Control"))
anova(total) #time_code
cld(emmeans(total, ~stand_age))

SE <- summarySE(subset(netuptake, trt == "Control"), measurevar = "d_N_g_m2",
groupvars = c("stand_age"))


SE <- summarySE(subset(netuptake, stand_age == "1"), measurevar = "d_N_g_m2",
groupvars = c("trt"))



## tables ----
#SEx <- summarySE(N, measurevar = "Corrected..N", groupvars = c("type", "timing",
"trt"), na.rm = T)
#write.csv(SEx, file = "C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/N_summary.csv")

#SEiwg <- summarySE(subset(N, type =="IWG"), measurevar =
#                  "Corrected..N", groupvars = c("timing", "trt"), na.rm = T)
#write.csv(SEiwg, file = "C:/Users/Michelle/Dropbox/WD/nbal/nbal
csv/N_iwg_summary.csv")

#SEroots <- summarySE(subset(N, type =="roots"), measurevar =
#                  "Corrected..N", groupvars = c("timing", "trt"), na.rm = T)
#write.csv(SEroots, file = "C:/Users/Michelle/Dropbox/WD/nbal/nbal
csv/N_roots_summary.csv")

#SEgrain <- summarySE(subset(N, type =="grain"), measurevar =
```

```
#                     "Corrected..N", groupvars = c("timing", "trt"), na.rm = T)
#write.csv(SEgrain, file = "C:/Users/Michelle/Dropbox/WD/nbal/nbal
csv/N_grain_summary.csv")

#SEx <- summarySE(N, measurevar = "Corrected..C", groupvars = c("type", "timing",
"trt"), na.rm = T)
#write.csv(SEx, file = "C:/Users/Michelle/Dropbox/WD/nbal/nbal csv/C_summary.csv")
```

APPENDIX NUMBER TWO- EXTENDED MATERIALS AND METHODS SECTION FOR CHAPTER TWO

Location and Site Preparation

Research was conducted on a Tallula silt loam (Coarse-silty, mixed, superactive, mesic Typic Hapludoll) at the Rosemount Research and Outreach Center (44°41'05"N 93°04'13"W) 24 miles south of St. Paul, MN. An improved population of intermediate wheatgrass from the fifth cycle of breeding for increased grain yield by The Land Institute (Salina, KS) was used for this study. The previous crop of soybean (*Glycine max*) was terminated prior to seedbed preparation, after which IWG was planted with a cone seeder in 15 cm rows in September of 2016 and 2017. Data collection occurred in 2018 on one year old (1 yo) and two year old (2 yo) intermediate wheatgrass fields. 80 kg N ha^{-1} in the form of urea was applied by hand to both fields in early May. Early May was chosen for the earliest fertilization because abundant late snow cover precluded fertilization during April.

Vegetative Tissue Sampling

Sampling for rhizomes and plants took place in early May (as soon as the ground thawed), mid-June (anthesis onset), mid-August (harvest), and late October (after frost). These sampling times were chosen to correspond with the physiological events of spring tillering, elongation, seed fill, and post-harvest fall regrowth. Rhizomes were obtained as part of another experiment in the same fields- see Dobbratz et al. 2019 for full details. In brief, a Giddings probe was used to collect soil down to 90 cm, which was divided into 15 cm increments. Each plot was sub sampled twice, and the sub samples were combined. Samples were frozen until they could be washed. During root washing, rhizomes were removed, dried at 35° C for five days, and weighed. Due to labor shortage, the roots and rhizomes from the mid-June timepoint were not processed.

Plant sampling was conducted by marking three 15 cm by 33 cm quadrats at predetermined sampling locations in each plot. Each marked area was excavated to a depth of 20 cm. Excess soil was gently removed from the belowground biomass, taking care to keep plants in-tact. All plant matter was stored at 4° C until sorted.

Plant Sorting, Categorization, and Tiller Counting

In the lab, biomass was cleaned and grouped by originating individual. First, belowground biomass from a given plot was cleaned of all excess soil, taking care not to damage roots, rhizomes, or crowns. Then, an individual plant was identified. A group of tillers was considered an individual plant if each tiller connected directly to the same crown. A tiller or group of tillers connecting to a crown via a rhizome longer than 1 cm was considered a separate individual. When a rhizome or rhizomes would connect off a rhizome, or one rhizome would bear more than one crown, each crown was considered a separate individual.

Plants were designated as either "original" (originating from seed planted by a researcher), "vegetative propagule" (originating from a rhizome), or "seed propagule" (originating from seed shatter) based on the above- and belowground morphology. Vegetative propagules were identified by the presence of a rhizome growing upward through the center of the crown of the plant. Rhizome direction can be determined not only by position in crown (rhizomes originate from the side of a crown of a parent plant and become the meristem of a vegetative propagule) but also the orientation of the sheath. Like tillers, rhizomes have sheaths that open acropetally. Therefore, it can be determined which plant is a vegetative propagule by observing rhizome morphology. Original plants were designated based on 1) the absence of a basipetal rhizome and 2) the presence of more advanced development than would be expected for a seedling that

originated from seed shatter. Seed propagules were nearly always identifiable based on the presence of a lemma and palea, however any plant that was missing an inward rhizome and was too small to be an original plant was considered a seed propagule. Based on this very liberal assessment, we found no evidence that seed propagules live more than a few months. No seed propagules had more than two tillers, and few had more than one tiller.

After designation, the tillers, rhizomes, and spikes of a plant were counted. Because grain was removed at harvest, spikes were not counted at this time. At anthesis, spikes were counted, and roughly 10% were found to still be in elongation. It is thus possible that due to delayed reproductive development, some spikes were not counted. However, given that the vast majority of the field was in anthesis at sampling, if a tiller was so delayed in its development that there was no spike in elongation at the time, it is unlikely such a tiller would contribute to grain yield. Rhizomes were counted by cleaning belowground material and identifying any structure with sheaths (rhizomes are also typically thicker, more translucent, and less branching than roots). A rhizome was counted as belonging to a plant if it originated from the crown of said plant. Any tiller connecting to the crown of a plant was categorized as belonging to that plant. At some times, smaller structures (usually less than 2 cm) were difficult to differentiate between tiller and rhizome. In these cases, the direction of growth, transparency, color, and location on the crown would be considered. For example, an upward growing, opaque, darker green structure near the top of the crown was considered to be a tiller.

Harvest Sampling and Observations

At harvest in mid-August, additional sampling and observations took place. Grain was removed, threshed, dried at 35° C until constant weight, and weighed to determine grain yield. Heights were taken by measuring from the soil surface to the tallest part of five plants per plot. Lodging was assessed on a 1-10 scale, where 1 = all plants in the plot were fully upright, 5 = most plants were at a 45° angle, and 10 = all plants lying down.

Statistical Analysis

Statistical analysis was conducted in R (R Core Team, 2019). A generalized linear mixed model (glmer) with a Poisson distribution was used for the count variables, such as number of plants, number of tillers, and number of rhizomes. All other response variables were analyzed with the nlme package (Pinhero et al., 2019). Treatment and stand age were considered fixed effects and block was considered a random effect. For post hoc analysis, the emmeans package (Lenth, 2019) was used. Means were separated using Tukey's HSD.

APPENDIX THREE- IMAGES OF ABOVE- AND BELOWGROUND MORPHOLOGY IN INTERMEDIATE WHEATGRASS

Above are one year old intermediate wheatgrass plants sampled in early May 2018. Left- a young individual exhibits first order tillering and rhizome production. Center- two small vegetative propagules originate from the same rhizome. Right- several meristems emerge from a damaged crown.

Above are two year old intermediate wheatgrass plants sampled in early May 2018. Left- a mature rhizome sends up multiple vegetative propagules. Right- a rhizome grows in excess of 70 cm (without sending up any vegetative propagules) before turning upward.

Above, shattered seed germinates in a 2 year old intermediate wheatgrass stand, sampled in early May 2018. Seed hulls, highlighted with yellow boxes, encompass the base of seed propagules. Left- two seed propagules originate from the same spike fragment. Right- a seed propagule emerges near an original plant, provided for potential confusion with original plant tillers.

Above, two new individuals emerge from within a mass of original plant stems, sampled from a 2 year old stand in early May 2018. Highlighted in yellow, the base of the seed propagule is framed by a hull. Nearly all seed propagules sampled throughout the growing season had this feature. Highlighted in red, the base of the vegetative propagule is thick, red, and encircled by acropetally opening sheaths. These sheaths open toward meristematic growth, even below the soil surface, allowing vegetative propagules to be distinguished with certainty from original plants by the presence of a rhizome growing *into and through* the crown, rather than away from via an axillary branch. Aside from the aforementioned differences, seed propagules and vegetative propagules have qualitative differences. Vegetative propagules have thick, reddish bases, which seed propagules have a thin, yellowish base. Seed propagules became increasingly etiolated throughout the growing season, while vegetative propagules exhibited growth patterns more similar to first year plants.