TILLAGE MANAGEMENT INFLUENCE ON MICROBIAL ACTIVITY AND CARBON CYCLING DYNAMICS IN BRAZOS RIVERBOTTOM SOIL

A Thesis

by

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ABSTRACT

Conventional tillage (CT) practices can affect the chemical, physical, and biological health of soil, which alter microbial access to carbon (C) and nutrients from crop residues. Few studies investigate how tillage effects the biological function of soil. This study identifies the effects of CT versus no-till (NT) management in a long-term agricultural system (38 years) by measuring 5 C and nutrient cycling microbial enzymes, as well as C pools. Under NT, soil organic and active C was greater than under CT. Tillage had no significant effect on most extracellular enzyme activities (EEAs). However, there were differences in EEAs across the growing season, where activities were lowest during harvest and highest in early midseason at 0-5 cm depth. Increased C in NT may be due to delayed decomposition due to residue retention. Seasonal effects in enzyme activity may be due to plant-soil interactions associated with nutrient requirements of plants. Switching to NT management can improve the health and function of the soil and increase agricultural C storage.

DEDICATION

I dedicate this thesis to my family.

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1. LITERATURE REVIEW ON THE EFFECTS OF TILLAGE ON SOIL ORGANIC CARBON AND MICROBIAL PROPERTIES

1.1 Introduction

In order to sustain sufficient crop production for the world's increasing population, we must maintain healthy agricultural soils. The soil is Earth's largest terrestrial reservoir of carbon (C) (Ciais *et al.*, 2013; Jackson *et al.*, 2017), and C dynamics in the soil are vital to maintaining the health and function of the soil for crop productivity. Conventional tillage (CT) disrupts soil structure and exposes previously protected soil organic matter (SOM) for microbial decomposition (Six *et al.*, 2000; Blanco-Canqui & Lal, 2004). Long-term CT systems have been reported to increase microbial decomposition and decrease SOM (Roger-Estrade *et al.*, 2010; Thomas *et al.*, 2019; Zhao *et al.*, 2021). Conservation practices, such as no-tillage (NT), have the potential to increase soil C storage in agricultural soils by keeping C protected from microbial degradation in aggregates and within SOM (Paustian *et al.*, 1997; Busari *et al.*, 2015).

Microbes utilize soil organic C (SOC) as their energy source, which also serves to cycle nutrients essential for productive crop growth. They also create secretions that help form organo-mineral associations that help bind soil aggregates together, which can lead to an increase in stabilized soil C (Six *et al.*, 2000; Kogel-Knabner *et al.*, 2008; Blanco-Canqui & Lal, 2004). Eliminating or reducing tillage practices can improve the physical and chemical health of the soil, but it is still unclear how these practices affect biological organisms and their activities in the soil. This review will explore three main areas of interests about tillage and C cycling dynamics: 1) effects of tillage on organic C pools, 2) effect of tillage on microbial activity, and 3) seasonal changes in microbial activity.

1.2 Effects of Tillage on Organic Carbon Pools

Tillage management practices change the dynamics of the C cycle by influencing the distribution of C in the soil profile (Dick, 1983; Karlen *et al.*, 1989; Edwards *et al.*, 1992; Bauer *et al.*, 2002). CT buries and mixes in crop residues, minimizing the amount of residues on the surface. In a CT system, one of the main causes of soil organic carbon (SOC) loss comes from the quick oxidation of the SOC, which occurs when aggregates are broken up exposing previously protected SOC (Six *et al.*, 2000; Blanco-Canqui and Lal, 2004). The destructive nature of CT leads to increased microbial respiration and cycling of nutrients through the soil system (Du *et al.*, 2017). In contrast, conservation tillage practices, such as NT, leave crop residues on the soil surface with no disturbance to soil structure or aggregates.

SOC is complex and made of several pools with different turnover times (Lavallee *et al.*, 2019). Active C is a smaller subset of the total SOC that is readily available for the microbes to decompose (NRCS, 2014). Active C is more sensitive to changes in management practices that affect SOC in agricultural settings, such as tillage management, compared to SOC because active C has a much shorter turnover time (NRCS, 2014).

Many studies have reported less SOC in CT soils when compared to NT (Halpern *et al.*, 2010; Liu *et al.*, 2010; Thomas *et al.*, 2019; Zhao *et al.*, 2021; Shen *et al.*, 2021; He *et al.*, 2021). A chronosequence study comparing three different agricultural study lengths observed that NT had greater SOC compared to CT, and that the difference between the tillage practices became more significant over time (Panettieri *et al.*, 2014). However, NT may not increase SOC compared to CT in all cases. Ye *et al.*, (2020) found that in South Carolina southeastern sandy Coastal Plain soils, a 40-yr study showed no differences in SOC between NT and CT. Another study in sandy soils reported a low proportion of SOC input came from the NT crop residues,

although SOC was greater in NT compared to CT (Halpren *et al.*, 2010). Therefore, sandy soils may be more limited in their capacity to accumulate SOC by NT management compared to fine-textured soils.

There have been mixed reports on tillage effects on active C (Thomas *et al.*, 2019; Ye *et al.*, 2020; Zhao *et al.*, 2021; Shen *et al.*, 2021; He *et al.*, 2021). Some studies noted NT had greater active C compared to CT (Thomas *et al.*, 2019; Ye *et al.*, 2020; He *et al.*, 2021). Conversely, a recent study found that deep ploughing and rotary tillage resulted in greater active C than NT (Shen *et al.*, 2021). In another recent long-term study, no effect of tillage was found on active C (Zaho *et al.*, 2021). The different responses of active C to tillage could be due to the development of significantly more macroaggregates in the top 5 cm in NT compared to CT (Mikha and Rice, 2004). Larger aggregates can better physically protect C within their structure than microaggregates (Nicoloso *et al.*, 2018). Physical protection may help accumulate active C in the NT system (Mikha and Rice, 2004; Aziz *et al.*, 2013). Most studies focus on the chemical and physical effects that tillage management practices have on SOC stabilization, but there is less known about how tillage affects soil biology (Busari *et al.*, 2015).

1.3 Effects of Tillage on Microbial Activity

Microbes secrete extracellular enzymes that break down complex SOM into forms the microbes can easily assimilate, which contributes to nutrient cycling essential for productive plant growth. Extracellular enzyme activities (EEA) are key indicators of microbial SOM decomposition and nutrient cycling (Burns, 1983) and influenced by tillage management (Kladivko, 2001; Helgason *et al.*, 2009; Schmidt *et al.*, 2011). Since EEAs respond rapidly to changes in management (Anon *et al.*, 2001; Mina *et al.*, 2008; Panettieri *et al.*, 2014; Pandey *et*

al., 2014; Mirzavand *et al.*, 2020), measuring EEAs is a recommended practice to detect changes in management (Alvear *et al.*, 2005).

Many studies show an overall increase in EEAs in NT surface soils compared to CT (Mina *et al.*, 2008; Liu *et al.*, 2010; Panettierei *et al.*, 2014; Pandey *et al.*, 2014; Mirzavand *et al.*, 2020; He *et al.*, 2021). In addition, Liu *et al.* (2010) also observed that enzymes, such as alkaline phosphatase, may be influenced by fertilizer applications as well. Some studies in more sandy soils did not observe an effect of tillage on EEAs (Bissett et al., 2013; Ye et al., 2020). This may be due to the limited capacity for sandy soils to increase their SOC in NT management. Without a significant increase in SOC to breakdown, EEAs may not show distinguishable effects of tillage management practices in these sandy soils. Because NT soils rich in clay and silt are typically reported to have greater SOC than CT soils, SOC may be the driving force behind these increased EEAs in NT soils.

1.4 Seasonal Changes in Microbial Activity

Temporal changes in temperature, crop growth, soil moisture, and productivity can alter C availability and microbial activity over a growing season (Joshi *et al.*, 2018). Few studies have investigated the variation of microbial activity over the growing season; however, chemical changes throughout the season, such as root exudates secreted from crops (Franzluebbers *et al.*, 1995; Spedding *et al.*, 2004), pH due to fertilization (Joshi *et al.*, 2018), or changes in soil moisture content (Shi *et al.*, 2013; Singh & Kumar, 2021) can influence SOM and subsequently nutrient cycling. Temporal changes in EEAs have been reported to occur in cropping systems (Singh & Kumar, 2021; Shi *et al.*, 2013; Bissett *et al.*, 2013; He *et al.*, 2021). A recent study in South Dakota took seasonal soil samples, and found increased EEA in spring compared to

summer and autumn which correlated with soil moisture content (Singh & Kumar, 2021). An Australian study, focused on nutrient and crop residue incorporation in wheat, focused on a single season with samples taken before planting, around nutrient/residue incorporation, and around harvest (Bissett *et al.*, 2013). Bisset *et al*. (2013) found significant variation in EEA over time where different EEAs peaked at different times throughout the growing season. A study on maize cropping in South Dakota took samples pre-planting, midseason, and pre-harvest, observing variation in EEAs throughout the growing season that exhibited a strong correlation to soil moisture (Shi *et al.*, 2013). A recent study on winter wheat in China took samples at the sowing, jointing, filling, and harvest stages reported more complex effects (He *et al.*, 2021). They found effects of tillage, soil depth, plant growth stage, and interactive effects between variables on EEAs. He *et al.* (2021) reported cellobiohydrolase (CBH) and beta-glucosidase (β-gluc) activities to increase and decrease throughout the growing season, while N-acetylglucosaminidase (NAG) and phosphatase (Phos) generally had a decreasing trend over the growing season. NT had greater EEA in 0-10 cm compared to the 10-20 cm, while CT had lower EEA in 0-10 cm compared to 10-20 cm.

Seasonal changes in EEA are highly complex and may involve multiple variable interactions. Environmental factors, such as soil moisture, may be a primary driving force behind varied seasonal EEAs (Shi *et al.*, 2013; Singh & Kumar, 2021), but there may be more complex interactions that need to be investigated (He *et al.*, 2021). More studies are needed with multiple collections in a single crop growing season to determine the driving forces behind seasonal changes in microbial activity that affect SOC dynamics in agricultural settings.

1.5 Conclusion

In conclusion, SOC dynamics is a complex area of study involving soil C pools, management practices, and microbes. Soils rich in clay and silts have the potential to store more SOC when switching to NT management, while sandy soils may have limits to their SOC storage capacity with NT alone. The effect of tillage on active C is not definitive and may vary more based on soil type, location, and other environmental factors. NT has reported overall increased EEA compared to NT. However, studies looking at EEAs across a growing season suggest more complex interactions with microbial C cycling that may be overlooked with single point in time sampling. Gaining more knowledge of how tillage affects agricultural soils temporally will help to identify key drivers in soil C dynamics that aid agricultural soils in sequestering more SOC to improve overall soil health and possibly improve crop production.

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2. TILLAGE EFFECTS ON SEASONAL MICROBIAL ACTIVITY AND SOIL ORGANIC CARBON DYNAMICS

2.1 Introduction

Soil contains Earth's largest terrestrial reservoir of carbon (C) (Ciais *et al.*, 2013; Jackson *et al.*, 2017), storing more than 1500 Pg of C or about twice the amount of C in the Earth's atmosphere. In addition, the soil C pool has strong potential to influence the atmospheric C pool and the global climate system (Lal & Stewart, 2019). With the need to increase crop production to sustain an exponentially growing world population (Busari *et al.*, 2015), it is important to identify the human impacts on the C cycle due to long-term use of non-sustainable agricultural management practices. If best management practices, such as no tillage or reduced tillage, are put into place, the world's agricultural soils might store more C than at present (Paustian *et al.*, 1997; Busari *et al.*, 2015), potentially helping to mitigate climate change.

Management practices, such as conservation tillage and crop rotations, have the potential to increase crop productivity and store soil organic C (SOC) (Six *et al.*, 2000; Blanco-Canqui & Lal, 2004; Hobbs *et al.*, 2008; Harden *et al.*, 2018). Conservation tillage, especially no-till (NT), practices are increasingly recognized for enhancing SOC accumulation compared to conventional tillage practices (Six *et al.*, 1999; Halpern *et al.*, 2010; Roger-Estrade *et al.*, 2010; Liu *et al.*, 2015; Zhao *et al.*, 2021) by keeping soil aggregates intact to physically protect SOC (Six *et al.*, 2000; Blanco-Canqui & Lal, 2004; Kogel-Knabner *et al.*, 2008). Microbes help stabilize C by creating secretions that help form organo-mineral associations that bind soil aggregates together to protect SOC (Six *et al.*, 2000; Blanco-Canqui & Lal, 2004; Kogel-Knabner *et al.*, 2008). Increasing SOC, not only helps with regulating climate through C

sequestration but also provides positive co-benefits to the crop through an increase in soil water retention, nutrient retention, crop yield, and resilience to extreme weather events (Busari *et al.*, 2015; Harden *et al.*, 2018).

Active C is a smaller fraction of SOC and is composed of the easily degradable SOC for microbial use (Wander, 2004). Active C is also thought to increase as SOC is increased with improved management (Weil *et al.*, 2003; Hurisso *et al.*, 2016; Awale *et al.*, 2017). CT practices can disrupt the soil surface, break-up soil structure, and expose aggregate-protected C to rapid microbial turnover, resulting in an overall loss of SOC over time (Six *et al.*, 2000; Blanco-Canqui & Lal, 2004; Busari *et al.*, 2015).

Studies show that NT increases SOC pools compared to CT (Halpern *et al.*, 2010; Liu *et al.*, 2010; Thomas *et al.*, 2019; Shen *et al.*, 2021; Zhao *et al.*, 2021; He *et al.*, 2021). A long-term study on silage corn in Canada found 1.1 times greater SOC in NT compared to CT, while also reporting 1.24 times greater active C in NT compared to CT (Thomas *et al.*, 2019). A long-term study in China on a rice-wheat rotation also reported 1.1 times greater SOC in reduced tillage compared to CT systems but saw no effect of tillage on active C (Zhao *et al.*, 2021). Thomas *et al.* (2019) suggests that their NT soils were accumulating active C faster than microbes were consuming it, as they found an effect of tillage on active C but no effect of tillage on soil respiration. A study in China on a wheat-maize rotation, found that NT had 1.09-1.14 times greater SOC than various tillage practices. (Shen *et al.*, 2021). For active C, *Shen et al.* (2021) reported that various tillage treatments increased active C compared to NT. This could be due to the macroaggregates being broken down by tillage (Six *et al.*, 2000; Blanco-Canqui & Lal, 2004; Kogel-Knabner *et al.*, 2008). Shen *et al.* (2021) suggests this could be due to deep plowing that

buries residue deeper in the profile, where there is a relatively high humidity and access to microbes that facilitates decomposition.

Tillage affects not only the total SOC pool, but can also affect SOC pools within different sized aggregates. Shen *et al.* (2021) found that the effect of NT was mainly observed as an increase in large macroaggregates (>5 mm), while deep plowing and subsoiling decreased SOC in microaggregates (<0.25mm). They concluded this was attributable to tillage promoting turnover of macroaggregates along with SOC mineralization in macroaggregates (*Shen et al.*, 2021). However, NT did increase active C in microaggregates rather than macroaggregates, so there is potential for active C to increase long-term under NT treatment in this experiment (Shen *et al.*, 2021). A long-term (49 year) study in Illinois reported greater soil C and active C in NT compared to CT in both large (2-4.75 mm) and small (0.25-2 mm) sized aggregates because less disturbance to the soil can protect and accumulate C with micro and macro-aggregate formation (Wang *et al.*, 2018; Tao *et al.*, 2018; Weidhuner *et al.*, 2021). Most studies focus on chemical and physical effects of tillage and cropping management practices on SOC stabilization, but less is known about how these factors affect the biology of the soil (Busari *et al.*, 2015).

Tillage may alter microbial access to C and nutrients from crop residues due to the physical and chemical changes tillage imposes on the soil thereby altering microbial activity (Six *et al.*, 1999; Six *et al.*, 2000; Kladivko, 2001; Helgason *et al.*, 2009; Schmidt *et al.*, 2011). As microbial communities are key drivers of nutrient and C cycling in soils, altering their access to C and other resources may alter the potential of soils to stabilize C (Six *et al.*, 2000; Kogel-Knabner *et al.*, 2008). Extracellular enzyme activities (EEA) are key indicators of SOM decomposition and nutrient cycling by microorganisms (Burns, 1983), which can be influenced by tillage management (Kladivko, 2001; Helgason *et al.*, 2009; Schmidt *et al.*, 2011). Microbes

produce and secrete extracellular enzymes to break down complex organic matter into forms they can easily assimilate. Since EEA responds rapidly to changes in management (Aon *et al.*, 2001; Mina *et al.*, 2008; Panettieri *et al.*, 2014; Pandey *et al.*, 2014; Mirzavand *et al.*, 2020), measuring EEA is recommended for detecting changes in soil health in response to management (Alvear *et al.*, 2005).

Studies have shown that NT increases the majority of EEAs compared to CT (Mina *et al.*, 2008; Pandey *et al.*, 2014; Mirzavand *et al.*, 2020). A study on a wheat-rice rotation in India reported NT to have higher beta-glucosidase (β-gluc), cellobiohydrolase (CBH), and phosphatase (Phos) activities compared to CT due to increased SOC and microbial biomass-C from decreased tillage (Pandey *et al.*, 2014). An increase in the formation and stabilization of macroaggregates may have led to the increase in Phos observed in NT systems compared to CT management (Mirzavand *et al.*, 2020). Similarly, a lentil-finger millet rotation study in India reported greater Phos activity in NT compared to CT; however, there was greater cellulase and β-gluc activity in CT compared to NT (Mina *et al*., 2008).

The overall objective of this research was to determine the effect of long-term tillage on microbial activity and SOC pools. Taking advantage of a long-term agricultural research experiment, EEA, SOC, and active C were measured from soybean-wheat-sorghum-fallow cropping system under CT and NT management across two summer growing seasons. It was hypothesized that compared to NT, CT would lower SOC due to repeated mixing and exposure of SOC and subsequent microbial mineralization of C. Active C and EEA, however, are hypothesized to be greater in CT soils compared to NT due to the increase in microbial access to C from the breakdown of soil structure. Seasonal variation in EEA due to changes in nutrient demand by crops at different growth stages is also expected.

2.2 Methods

2.2.1 Experimental Design

This study was conducted on a 38-year long-term field experiment established in 1982 at the Texas A&M University Research Farm (30.5479572, -96.4349574) near the Brazos River in College Station, TX. The soil is part Ships clay (Very-fine, mixed, active, thermic Chromic Hapluderts) and part Belk clay (Fine, mixed, active, thermic Entic Hapluderts) with the top 10 cm soil containing clay and silty clay texture classes. The soils had about 1-2% inorganic C. The 2-year seasonal study was conducted in the soybean-wheat-sorghum-fallow (*Glycine max*, *Triticum aestivum*, and *Sorghum bicolor*) rotation under CT and NT. The 2-year rotation system is duplicated with one system offset by one year (SWS even and SWS odd, Figure 2.1) so that soybean and sorghum are present each year in different blocks. Within each system, a fertilization rate trial is conducted with a no, low, medium, and high fertilization rate established as a randomized complete block design with four replications. However, only the highest fertilization rate (135 kg N ha^{-1} as urea for sorghum, 102 kg P ha^{-1} to soybean, 135 kg N ha^{-1} as urea for wheat) is used in this study to keep the focus on tillage impacts. The CT consisted of offset disking to flatten rows for wheat and the use of a disk bedder to create rows for soybean and sorghum a few weeks before planting to prep the field. All CT systems were disked after harvest and weeds were controlled using an in-furrow cultivator for the first few weeks (3-6 weeks) after emergence until crops were too tall to operate the tractor in the field. Both CT and NT systems received preplant herbicide applications, CT may have received an in-season herbicide application if needed, and NT typically received 2 in-season herbicide applications. NT fields may have also received 2 herbicide applications in the fallow season if needed. A post-

harvest chemical burn-down was used to kill everything left in the field in all systems; this happened prior to cultivation in the CT systems. Each plot measured 40 m by 4 rows.

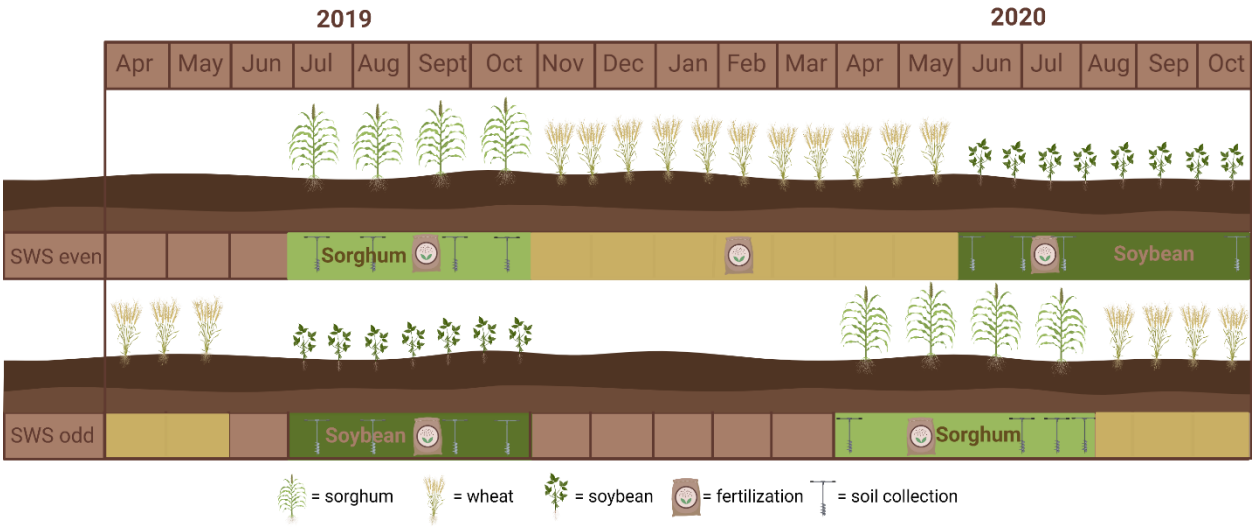

Figure 2.1. Three-crop rotation systems used split by SWS even and SWS odd. Only the growing seasons studied are labeled. Fertilization times and soil sample collection points are shown. Specific dates for sampling time are included in Table 2.1. Created with BioRender.com.

2.2.2 Soil Sampling

Soil samples were taken four times per season (*Planted*, *Midseason1*, *Midseason2*, and *Harvest*) in summer of 2019 and summer of 2020 (Table 2.1). Soil was collected based on crop planting and harvest dates (Figure 2.1; Table 2.1), which was delayed due to inclement weather in 2019. *Planted* was taken within the week after crops were planted. Using a 2.5-cm diameter soil probe, 4 samples were collected in row and 4 were collected between rows at 2 different depths (0-5, 5-10 cm) for each plot. Within and between row samples (8 samples) were

composited for each depth within a plot. The composited and homogenized samples were divided into 3 portions: fresh, freeze-dried, and air dried. Fresh soil was used for gravimetric water content. Freeze-dried soil was used for EEA to "pause" microbial activity at the time of collection (Valaskova & Baldrian, 2006). The rest of the composite sample was air dried at room temperature in the lab and sieved to pass a 2-mm sieve for all other analyses.

Table 2.1. Soil sampling dates for sorghum and soybean during 2019 and 2020.

	Planted	Midseason1	Midseason2	Harvest
Soybean				
2019	10 July	9 Aug	13 Sept	24 Oct
2020	4 June	1 July	13 July	14 Oct
Sorghum				
2019	10 July	9 Aug	13 Sept	24 Oct
2020	2 Apr	1 July	13 July	6 Aug

2.2.3 Physical Analyses

Gravimetric water content was measured on 0-5 and 5-10 cm samples using approximately 5 g of fresh soil on collection day. Fresh soil weights were recorded, dried at 105°C, and reweighed dry. Gravimetric soil water was calculated by subtracting the dry weight from the wet weight and then divided by the dry weight of soil.

2.2.4 Chemical Analyses

A subsample of the air-dried soil was finely ground to measure total concentrations of C, N, and inorganic C. Total C and N concentrations were measured using the total combustion method with the Vario EL Cube Elementar (Elementar Americas Inc., Ronkonkoma, NY).

Inorganic C concentration was measured using the pressure-calcimeter method developed by Sherrod *et al.* (2002). The percent of SOC was calculated by subtracting the inorganic C from total C. Active C was measured as permanganate oxidizable C (POxC) in mg POxC kg^{-1} soil by the Weil *et al.* (2003) colorimetric method. NH_4^+-N concentration was determined using the Berthelot reaction (Sims *et al.*, 1995). Soil pH was measured using a 2:1 water to soil ratio (Robertson & VanderWulp, 2019) on air-dried soil from two collection dates, one collection before fertilization and one collection after fertilization.

2.2.5 Biological Analyses

A high-throughput fluorometric assay method (German *et al.*, 2011, modified by Smith *et al.*, 2015) was used to measure the potential activity of five extra-cellular enzymes important for C and nutrient cycling: alpha-glucosidase (α-gluc), β-gluc, and CBH for carbon acquisition; N-acetylglucosaminidase (NAG) for nitrogen acquisition; and Phos for phosphorus acquisition. A soil slurry was prepared using 1 g freeze-dried soil in 100 mL tris(hydroxymethyl)aminomethane buffer and incubated at 23°C for 1 h in the presence of β-gluc-, NAG-, and Phos-linked substrates and for 3 h for α-gluc- and CBH fluorescence-linked substrates. Fluorescence values were measured in 96-well plates using a Synergy H1 microplate reader (BioTek; Winooski, VT) at 360/40 nm and 460/40 nm excitation and emission wavelengths, respectively. Potential EEAs were normalized by SOC and calculated using the following equation (Smith *et al*., 2015):

$$Activity\ (\text{mmol}\ g\ SOC^{-1}\ hr^{-1}) = \frac{Net\ Fluorescence\ \times\ Buffer\ Volue\ \times 0.1}{Emission\ Coefficient\ \times\ Homogenate\ Volume\ \times\ Time\ \times\ Mass\ Soil\ Organic\ Carbon}$$

Net Fluorescence was calculated as the difference in fluorescence among assay, slurry and buffer fluorescence and normalized by a quench coefficient. Outliers, data points 1.5 times the interquartile range, among technical replicates (i.e., 16 wells) were removed and samples were

rerun if the standard concentration curves for emission coefficients were not linear (i.e., $R^2 <$ 0.90) using R (version 1.2.1335).

2.2.6 Data Analysis

All data was checked for normality using histograms and qqplots. When necessary, response variables were log transformed for normality prior to statistical analyses. To identify the effect of tillage on SOC and biological properties, a residual maximum likelihood (REML) mixed effects model with standard least squares means were used. The effect of tillage, time group, (*Planted*, *Midseason1*, *Midseason2*, *Harvest*) and crop (sorghum, soybean) were imputed as fixed effects including interactions to the second-degree factorial (i.e., tillage × time group). Year and field block were included as random effects. With the exception of NH_4^+-N (Appendix Figure 1), year did not affect results significantly, so the years were pooled together. Soil depths (0-5, 5-10 cm) were analyzed separately due to a strong bimodal distribution between depths. Tukey's HSD or student t-test were used to show levels of separation in graphs. Multivariate analyses, such as multiple regression correlations, were used to analyze microbial EEA data and Spearman's p-test was used to calculate p-values. Active C to SOC ratios were also analyzed. Significance was set at $p < 0.05$, and all statistical analyses were performed in JMP Pro (Version 16.0.0, SAS Institute Inc.). Means are reported with ± standard error.

2.3 Results

Concentrations of SOC and active C were greater in no-till (NT) soils compared to conventionally tilled (CT) soils (Table 2.2, Figure 2.2). In the top 5 cm, there was approximately 1.5 times greater SOC in NT (14.38 ± 0.27 g SOC kg^{-1} soil) compared to CT (8.88 ± 0.16 g SOC

kg^{-1} soil) (Figure 2.2A). In the 5-10 cm depth, there was 1.2 times greater SOC in NT (9.79 ± 0.21 g SOC kg^{-1} soil) compared to CT (8.13 ± 0.15 g SOC kg^{-1} soil). Active C concentrations showed similar trends as SOC (Figure 2.2B) and were positively correlated with SOC ($r^2 = 0.69$). In the top 5 cm, there was more than 1.7 times greater active C in NT (0.40 ± 0.01 g POxC kg^{-1} soil) compared to CT (0.23 ± 0.007 g POxC kg^{-1} soil, $p = 0.04$). In the 5-10 cm depth, there was almost 1.2 times greater active C in NT (0.24 ± 0.009 g POxC kg^{-1} soil) compared to CT (0.20 ± 0.007 g POxC kg^{-1} soil, $p = 0.002$), albeit a poor model fit (Table 2.1). Both tillage treatments had similar ratios of active C to SOC (0-5 cm 0.027 ± 0.0005 and 5-10 cm 0.025 ± 0.0006, results not shown).

Table 2.2. F statistics and P values for SOC and active C pools (g kg^{-1}) from a residual maximum likelihood (REML) mixed effects model with standard least squares means. Variance component estimates for random variables, year, and block, are included as percent. Whole model fit is shown by the adjusted r^2 value. Significant p values are bolded.

		SOC*		**Active C**	
Effects	df	F ratio	p value	F ratio	p value
0-5 cm					
Tillage	1	42.24	**0.02**	21.29	**0.04**
Time Group	3	0.66	0.58	2.13	0.10
Crop Planted	1	3.44	0.07	0.02	0.89
Time Group×Crop Planted	3	1.04	0.38	2.47	0.07
Time Group×Tillage	3	0.47	0.71	0.95	0.42
Crop Planted×Tillage	1	0.04	0.84	0.42	0.52
Year			1%		6%
Block			18%		22%
Whole Model Fit			r^2= 0.74		r^2= 0.70
5-10 cm					
Tillage	1	29.66	**0.03**	684.21	**0.005**
Time Group	3	1.79	0.15	0.46	0.71
Crop Planted	1	1.48	0.23	0.33	0.56
Time Group×Crop Planted	3	2.37	0.07	2.39	0.07
Time Group×Tillage	3	0.33	0.80	0.23	0.87
Crop Planted×Tillage	1	0.20	0.66	0.15	0.70
Year			5%		20%
Block			1%		0%
Whole Model Fit			r^2= 0.29		r^2= -0.65

*log transformed for 5-10 cm, but not for 0-5 cm

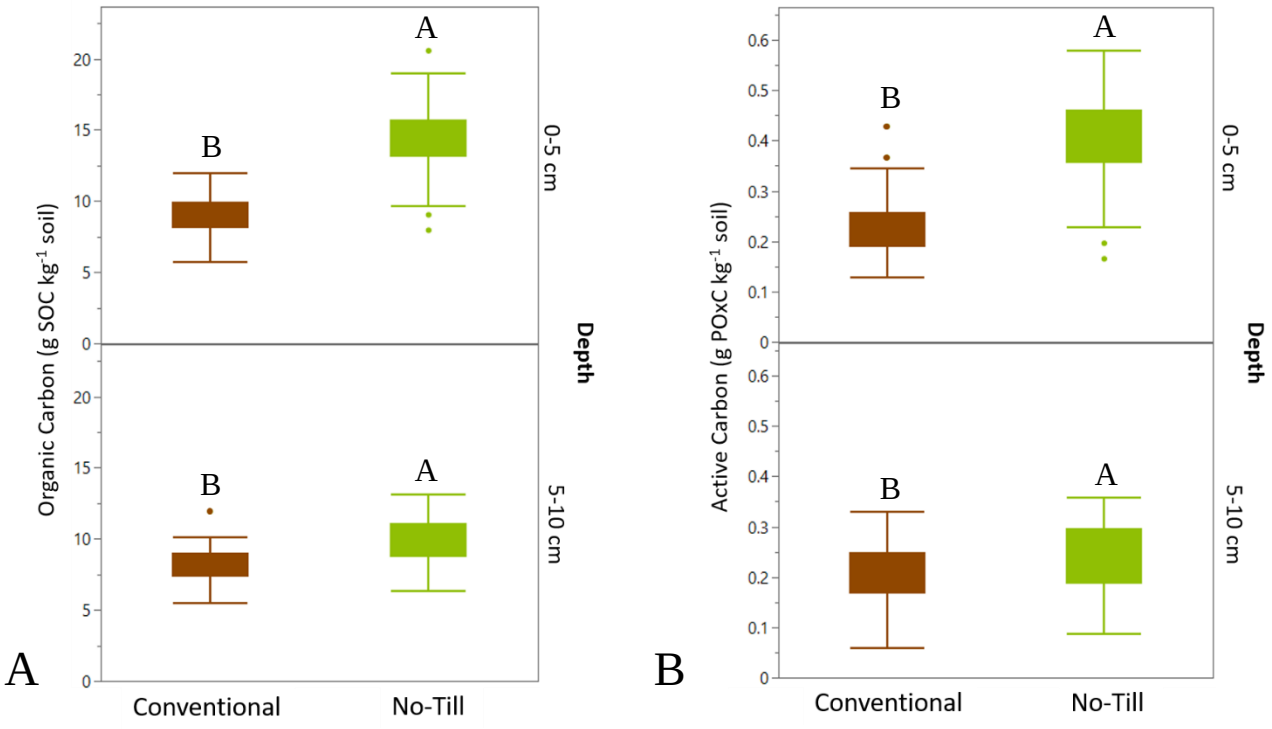

Figure 2.2. A) Mean SOC and B) mean active C at each depth separated by tillage. Both graphs have sorghum and soybean, and time group data pooled together. Significant differences in tillage are shown by different letters from student's t-test.

There was an effect of tillage on total N (TN) in the top 5 cm (Table 2.3). The concentration of TN was 1.5 times greater in NT soils (1.53 ± .03 g N kg^{-1} soil) compared to CT soils (0.99 ± .01 g N kg^{-1} soil) at 0-5 cm (Figure 2.3A). There was an effect of time group on NH_4^+-N concentrations for the 0-5 cm and 5-10 cm (Table 2.3). Both depths saw similar trends with *Midseason2* having the highest concentrations across the growing season. (Figure 2.3B). When estimating the percent variance components of random variables, *Year* represented 50% of variance in NH_4^+-N concentration at both depths suggesting that the effect of Time Group may differ slightly between years (Table 2.3). The increase in NH_4^+-N in *Midseason2* compared to the

rest of the growing season is most likely due to fertilization within two weeks before sampling (Appendix Figure 1). TN had a strong positive correlation with active C ($r^2 = 0.8$, $p < 0.001$) and with SOC ($r^2 = 0.95$, $p < 0.001$). Gravimetric water content had a weak negative correlation with NH_4^+-N ($r^2 = -0.2$, $p = 0.01$).

Table 2.3. F statistics and P-values for soil N pools from a REML mixed effects model with standard least squares means. Variance component estimates for random variables, year and block, are included as percent. Whole model fit is shown by the adjusted r^2 value. Significant p-values are bolded.

		Total N		**NH_4-N***	
Effects	df	F Ratio	p Value	F Ratio	p Value
0-5 cm					
Tillage	1	39.85	**0.02**	1.02	0.42
Time Group	3	1.87	0.14	6.07	**0.0007**
Crop Planted	1	1.20	0.28	0.02	0.89
Time Group×Crop Planted	3	0.68	0.56	1.84	0.14
Time Group×Tillage	3	0.29	0.83	0.38	0.77
Crop Planted×Tillage	1	0.0002	0.96	0.36	0.55
Year			0 %		50%
Block			18%		0%
Whole Model Fit			r^2= 0.73		r^2= 0.37
5-10 cm					
Tillage	1	15.29	0.06	0.62	0.51
Time Group	3	1.36	0.26	3.92	**0.01**
Crop Planted	1	1.38	0.24	0.81	0.37
Time Group×Crop Planted	3	2.60	0.06	0.95	0.42
Time Group×Tillage	3	0.37	0.77	0.42	0.74
Crop Planted×Tillage	1	0.04	0.83	0.64	0.43
Year			0%		50%
Block			6%		0%
Whole Model Fit			r^2=0.28		r^2= 0.36

*log transformed

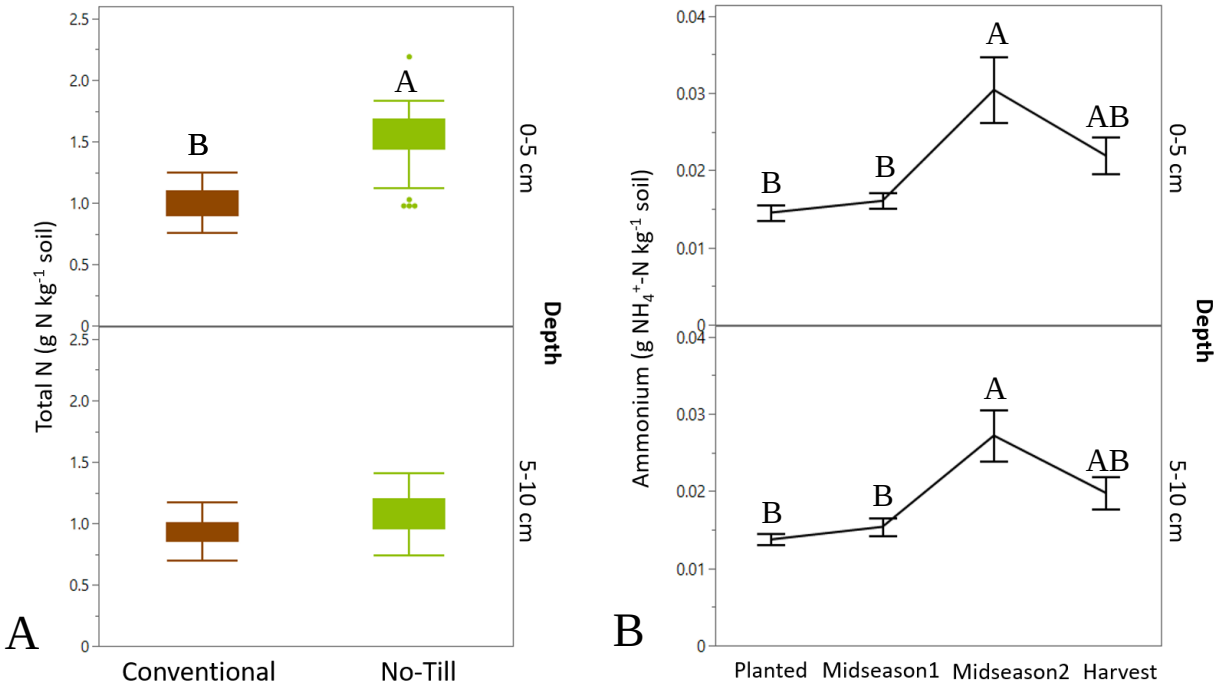

Figure 2.3. A) Mean total N and B) mean NH_4^+-N at each time group separated by tillage with standard error bars. Both graphs have sorghum and soybean data pooled together. Significant differences between tillage in total N graph (A) shown by different letters from student's t-test and time group in NH_4^+-N graph (B) shown by different letters from Tukey's HSD.

The effect of tillage on EEA was only observed in the top 5 cm in NAG and at 5-10 cm CBH enzymes, albeit poor model fits (Table 2.4, Figure 2.4). In the top 5 cm, NAG enzyme activity, an enzyme involved in chitin degradation and microbial N uptake, was 1.2 times greater in NT (7.51 ± 0.60 mmol g SOC^{-1} hr^{-1}) compared to CT (6.31 ± 0.53 mmol g SOC^{-1} hr^{-1}) (Figure 2.4A). In the 5-10 cm depth, CBH enzyme activity, an enzyme associated with cellulose

degradation, was 1.3 times lower in NT (1.43 ± 0.17 mmol g SOC^{-1} hr^{-1}) compared to CT (1.89 ± 0.22 mmol g SOC^{-1} hr^{-1}) (Figure 2.4C).

Time group affected all 5 EEAs in the top 5 cm and 4 out of 5 EEAs in the 5-10 cm depth (Table 2.4; Figures 2.4B, 2.4D, 2.5). *Midseason1* had the greatest values for all EEAs measured at 0-5 cm. In the 5-10 cm depth, *Planted* was significantly greater than *Harvest* for all 4 EEAs effected by time group (Table 2.4) In the top 5 cm, β-gluc activity was 1.6, 1.9, and 2.1 times greater at *Midseason1* (118.0 ± 11.10 mmol g SOC^{-1} hr^{-1}) compared to *Planted* (73.89 ± 7.03 mmol g SOC^{-1} hr^{-1}), *Midseason2* (62.80 ± 7.27 mmol g SOC^{-1} hr^{-1}), and *Harvest* (57.25 ± 7.31 mmol g SOC^{-1} hr^{-1}), respectively (Figure 2.5A). In the 5-10 cm depth, β-gluc activity was 1.6 times greater at *Planted* (60.81 ± 7.56 mmol g SOC^{-1} hr^{-1}) compared to *Harvest* (37.52 ± 5.35 mmol g SOC^{-1} hr^{-1}) (Figure 2.5B). In the top 5 cm, NAG activity was 2, 2, and 2.4 times greater in *Midseason1* (11.18 ± 0.82 mmol g SOC^{-1} hr^{-1}) compared to *Planted* (5.92 ± 0.61 mmol g SOC^{-1} hr^{-1}), *Midseason2* (7.36 ± 0.89 mmol g SOC^{-1} hr^{-1}), and *Harvest* (5.66 ± 0.98 mmol g SOC^{-1} hr^{-1}), respectively (Figure 2.4B). In the 5-10 cm depth, NAG activity was 1.3 and 1.6 times lower at *Harvest* (5.66 ± 0.98 mmol g SOC^{-1} hr^{-1}) compared to *Planted* (7.57 ± 0.81 mmol g SOC^{-1} hr^{-1}) and *Midseason1* (9.13 ± 0.55 mmol g SOC^{-1} hr^{-1}), respectively. In the top 5 cm, Phos activity was greatest at *Midseason1* (76.22 ± 7.06 mmol g SOC^{-1} hr^{-1}) and lowest at *Harvest* (31.40 ± 4.28 mmol g SOC^{-1} hr^{-1}) (Figure 2.5C). In the 5-10 cm depth, Phos activity was 1.4, 1.7, and 1.5 times lower at *Harvest* (32.38 ± 5.43 mmol g SOC^{-1} hr^{-1}) compared to *Planted* (46.64 ± 4.50 mmol g SOC^{-1} hr^{-1}), *Midseason1* (54.49 ± 4.25 mmol g SOC^{-1} hr^{-1}), and *Midseason2* (47.66 ± 5.22 mmol g SOC^{-1} hr^{-1}), respectively (Figure 2.5D). In the top 5 cm, α-gluc activity was 3 times greater at *Midseason1* (11.77 ± 1.46 mmol g SOC^{-1} hr^{-1}) compared to *Harvest* (3.75 ± 0.33 mmol

g SOC^{-1} hr^{-1}) (Figure 2.5E). In the 5-10 cm depth, α-gluc activity had no differences among the collection times (Figure 2.5F). In the top 5 cm, CBH activity was 2.9 times greater at *Midseason1* (4.31 ± 0.47 mmol g SOC^{-1} hr^{-1}) compared to *Harvest* (1.49 ± 0.17 mmol g SOC^{-1} hr^{-1}). In the 5-10 cm depth, CBH activity was 2.1 times greater at *Planted* (2.08 ± 0.30 mmol g SOC^{-1} hr^{-1}) compared to *Harvest* (0.97 ± 0.13 mmol g SOC^{-1} hr^{-1}) (Figure 2.4D).

EEA correlated with other measured variables such as gravimetric water content (GWC), active C, and NH_4^+-N (Table 2.5). Gravimetric water content had a weak negative correlation with β-gluc, α-gluc and CBH. The three C cycling enzymes, β-gluc, α-gluc, and CBH, were weakly correlated with active C. β-gluc also had a weak correlation with NH_4^+-N.

Table 2.4. F statistics and P-values for EEAs from a REML mixed effects model with standard least squares means. Variance component estimates for random variables, year, and block, are included as percent. Whole model fit is shown by the adjusted r^2 value. Significant p-values are bolded.

	β-gluc*		**NAG***		**Phos***		**α-gluc***		**CBH***	
Effects	F ratio	p value	F ratio	p value	F ratio	p value	F ratio	p value	F ratio	p value
0-5 cm										
Tillage	1.66	0.33	56.99	**0.03**	2.92	0.23	0.21	0.69	0.03	0.89
Time Group	13.27	**<0.0001**	18.64	**<0.0001**	15.03	**<0.0001**	15.19	**<0.0001**	13.28	**<0.0001**
Crop Planted	0.06	0.81	1.02	0.31	1.71	0.19	0.71	0.40	0.001	0.98
Time Group×Crop Planted	0.70	0.55	0.09	0.97	0.22	0.88	0.66	0.58	1.41	0.24
Time Group×Tillage	0.15	0.92	0.90	0.45	0.83	0.48	0.76	0.52	0.48	0.70
Crop Planted×Tillage	0.29	0.59	1.90	0.17	1.65	0.201	1.16	0.28	0.46	0.50
Year		18%		17%		30%		23%		8%
Block		0%		0%		0%		1%		4%
Whole Model Fit		r^2= 0.28		r^2= 0.28		r^2= 0.36		r^2=0.33		r^2=0.26
5-10 cm										
Tillage	6.18	0.13	0.02	0.24	0.36	0.61	4.46	0.17	20.88	**0.04**
Time Group	2.81	**0.04**	6.10	**<0.0001**	6.50	**<0.0001**	2.15	0.10	3.32	**0.02**
Crop Planted	0.85	0.36	1.40	0.24	1.75	0.19	0.30	0.58	0.58	0.45
Time Group×Crop Planted	0.18	0.91	0.72	0.54	0.33	0.80	0.15	0.93	1.17	0.32
Time Group×Tillage	0.16	0.92	0.11	0.96	0.41	0.74	0.07	0.97	0.10	0.96
Crop Planted×Tillage	0.30	0.58	0.03	0.87	1.04	0.31	1.79	0.18	0.24	0.62
Year		21%		2%		14%		26%		13%
Block		1%		1%		3%		0%		0%
Whole Model Fit		r^2=0.17		r^2=0.09		r^2=0.19		r^2=0.15		r^2=0.07

*log transformed

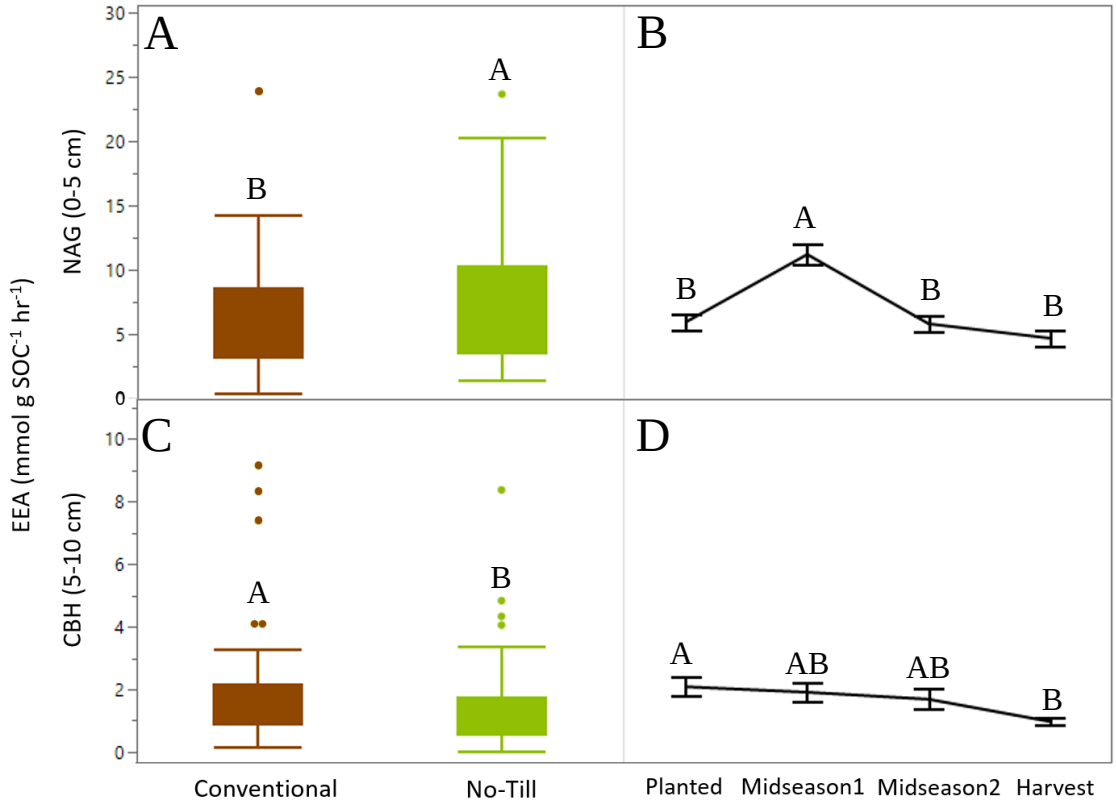

Figure 2.4. Tillage and time group effects on NAG and CBH EEAs. A) Mean enzyme activity for NAG (0-5 cm) by tillage. B) Mean enzyme activity for NAG (0-5 cm) by time group with standard error bars. C) Mean enzyme activity for CBH (5-10 cm) by tillage. D) Mean enzyme activity for CBH (5-10 cm) by time group with standard error bars. Graphs split by tillage (A and C) have time group and crops pooled together with significant differences shown by different letters from student's t-test. Graphs split by time group (B and D) have tillage and crops pooled together with significant differences shown by different letters from Tukey's HSD. Statistics performed on log-transformed data.

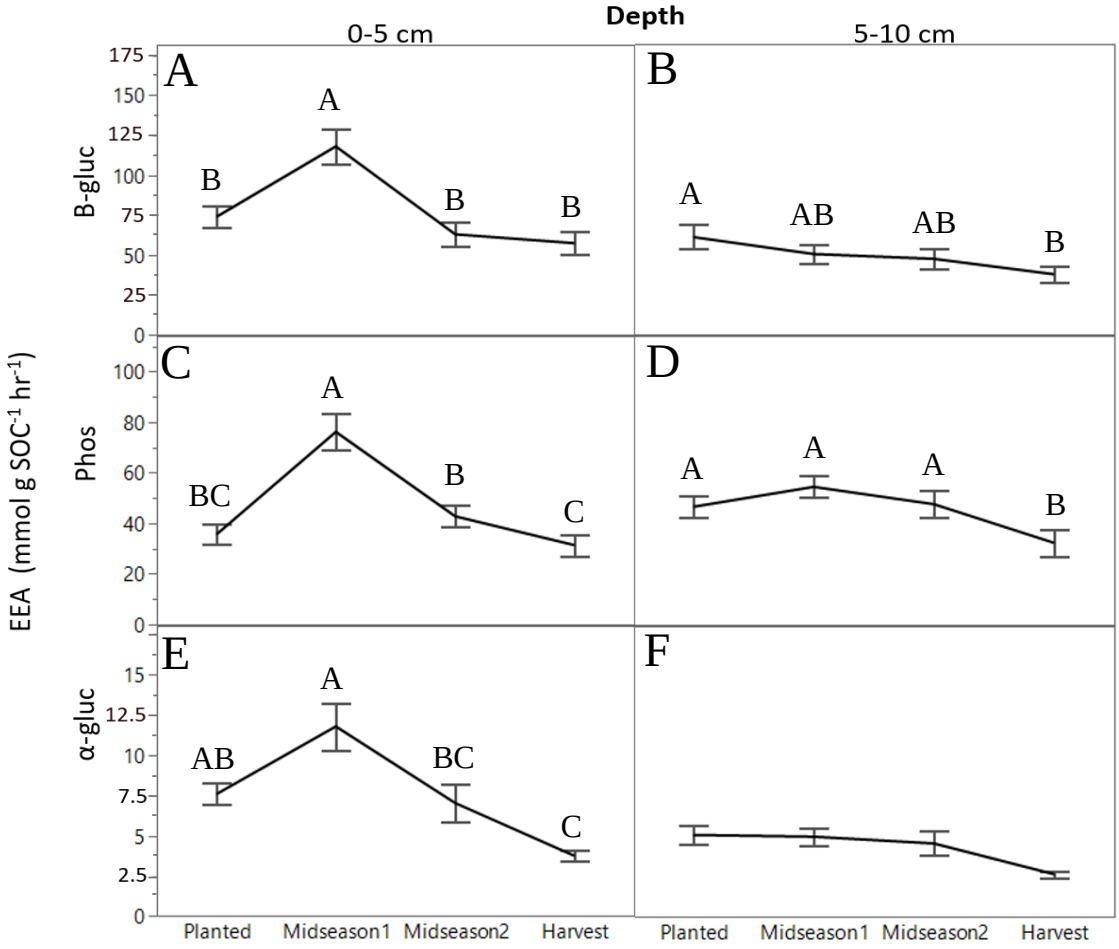

Figure 2.5. EEAs split by time group for A) β-gluc 0-5 cm, B) β-gluc 5-10 cm, C) Phos 0-5 cm, D) Phos 5-10 cm, E) α-gluc 0-5 cm, and F) α-gluc 5-10 cm with standard error bars. Tillage and crop data are pooled together. Significant differences shown with different letters from Tukey's HSD.

Table 2.5. Multivariate correlation table using Spearman's for EEAs with other measured variables. Both crops, both depths, and all 4-time groups included. Significance levels from Spearman's p test indicated as following: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significant p-values are bolded. GWC is the gravimetric water content.

	SOC	**Active C**	**Total N**	**NH_4^+-N**	**GWC**
B-gluc	0.11	**0.26** ***	0.06	**0.18** **	**-0.13***
NAG	-0.06	-0.03	-0.08	-0.12	0.03
Phos	-0.03	0.03	-0.08	-0.05	-0.06
α-gluc	0.08	**0.20** **	0.05	0.11	**-0.23** ***
CBH	0.11	**0.19** **	0.09	0.02	**-0.20 *****

2.4 Discussion

This study assessed the effect of tillage on C cycling dynamics and EEAs in a replicated field study that has been maintained under the same cropping sequence, tillage, and fertilization regime since 1982, allowing the opportunity to study the long-term impacts of tillage on soil C pools and enzyme activity. The results of this study show that NT increased both active and SOC, but did not increase EEA when activities were normalized by SOC. However, EEAs differed over the growing season with all EEAs peaking at *Midseason1* in the top 5 cm, while *Planted* was significantly greater than *Harvest* for EEAs in 5-10 cm.

Consistent with current literature, both SOC and active C concentrations were greater in NT compared to CT in the top 10 cm of the soil (Halpern *et al.*, 2010; Liu *et*

al., 2010; Thomas *et al.*, 2019; Zhao *et al.*, 2021; Shen *et al.*, 2021; He *et al.*, 2021). NT management can conserve soil structure and protect aggregate-stored C compared to CT managed soils (Du *et al.*, 2017; Zhao *et al.*, 2021). Crop residues left in place by NT, rather than incorporated deeper in the soil by tillage (i.e., CT), can lower the degradation rate of organic matter (Six *et al.*, 1999; Yadvinder-Singh, 2010). Several studies looking at the effects of residue placement (incorporated vs left on surface) showed that incorporating residues significantly increased the decomposition rate of the residues (Ghiaey & Alberts 1993; Beare *et al.*, 2002; Gupta & Ladha, 2010; Wang & Saninju, 2014; Zhang *et al.*, 2018). In my study, the only difference among treatments was whether the residues were incorporated or not. Zhang *et al.* (2018) also found that NT increased SOC and macroaggregation along with a significant positive correlation between SOC and macroaggregation, further emphasizing the importance of physical protection of SOC by the macroaggregates maintained in NT management.

Results from this study supported the hypothesis that CT would have lower SOC, but they did not support the hypothesis that active C would be greater in CT because both pools of C were greater with NT. The physical protection of soil C in aggregates and soil structure maintained in NT may help to accumulate active C as well (Mikha & Rice, 2004; Aziz *et al.*, 2013). Although NT increased C pools in this experiment, the ratio of active C to SOC did not change between tillage treatments. In this study, about 3% of the SOC was readily available for microbes to use regardless of tillage management.

For the majority of enzymes measured, there was no effect of tillage on EEAs. This research did not agree with the hypothesis that increased tillage leads to increased soil microbial EEA. Tillage did affect NAG at 0-5 cm and CBH at 5-10 cm. When normalizing EEAs by soil mass and not SOC, there was a significant decrease in EEAs in CT soils compared to NT soils, highlighting the importance of SOC availability in driving microbial activity (He et al., 2021) (data not shown). The effect of tillage on EEAs observed in other studies may be due to differences in how EEAs were calculated (i.e., with or without normalizing by SOC) and may reflect differences in substrate availability (Mina *et al.*, 2008; Pandey *et al.*, 2014; Mirzavand *et al.*, 2020). The effect of tillage was observed, however, on SOC-normalized CBH, which is consistent with the decrease observed in another cellulose-degrading enzyme (Mina *et al.,* 2008). Mina *et al.* (2008) did not address why they may have seen contrasting cellulase activity compared to other studies, but this could be due to tillage distributing more plant debris with cellulose into the profile for improved enzyme access. Since most studies report that NT has greater SOC than CT (Halpern *et al.*, 2010; Liu *et al.*, 2010; Thomas *et al.*, 2019; Zhao *et al.*, 2021; Shen *et al.*, 2021; He *et al.*, 2021), it may be important to normalize EEAs by SOC to determine if there is a direct effect of tillage on EEA or if increased EEA is an indirect effect of increased C pools. Normalizing EEAs by SOC may reveal that EEAs are more affected by access to C pools than tillage.

Consistent with the hypotheses and current literature, EEAs varied across the growing season (Shi *et al.*, 2013; Reardon *et al.*, 2019; Singh & Kumar, 2021). All of the enzymes in the top 5 cm showed similar trends throughout the season with peak activity

in *Midseason1*. For the 5-10 cm depth, *Planted* was significantly different than *Harvest*. Shifting edaphic soil properties throughout the growing season could be influencing enzyme activity. Some of these edaphic properties that have been shown to influence EEAs are soil moisture (Shi *et al.*, 2013; Singh & Kumar, 2021), nutrient concentration (Reardon *et al.*, 2019), and fertilization application (Liu *et al.*, 2010). The activity of three enzymes, β-gluc, α-gluc, and CBH, negatively correlated with soil moisture, but the relationship was weak. The three C cycling enzymes (β-gluc, α-gluc, and CBH) also correlated with active C, although a weak relationship. However, the C cycling enzymes did not correlate with SOC which could mean that active C is a reliable measurement of the actively cycled C in the soil as the EEAs increased with increasing active C. Multiple collections over a growing season is not common, as most seasonal studies are based on one sample collection per season (Mina *et al.*, 2008; Liu *et al.*, 2010; Panettierei *et al.*, 2014; Pandey *et al.*, 2014; Mirzavand *et al.*, 2020; Singh & Kumar, 2021). Collecting multiple soil samples in a single season has provided insights into a much more biologically variable and complex relationship with other edaphic soil properties (Bissett *et al.*, 2013; Shi *et al.*, 2013; He *et al.*, 2021).

2.5 Conclusion

This research concludes that CT reduces C pools compared to NT, but microbial EEAs are more sensitive to sampling time within the season in this long-term tillage study. The results of this study suggest that substrate availability (i.e., SOC or active C) and plant-soil interactions associated with plant nutrient requirements drive soil

microbial EEAs over a growing season regardless of tillage management. This study also highlights the complexity of EEAs throughout the growing season and suggests more than one sampling time throughout a growing season. Residues left in place by NT may help build SOC and active C pools by slowing down SOM degradation and protecting C pools with macroaggregation in the soil. Slowing down SOM degradation could help farmers keep their land healthy and productive for longer in the future. If agricultural soils are able to store more SOC, this could also help mitigate global climate change. Switching to NT is likely to improve the health and function of the soil and, in turn, help agricultural systems store more C.

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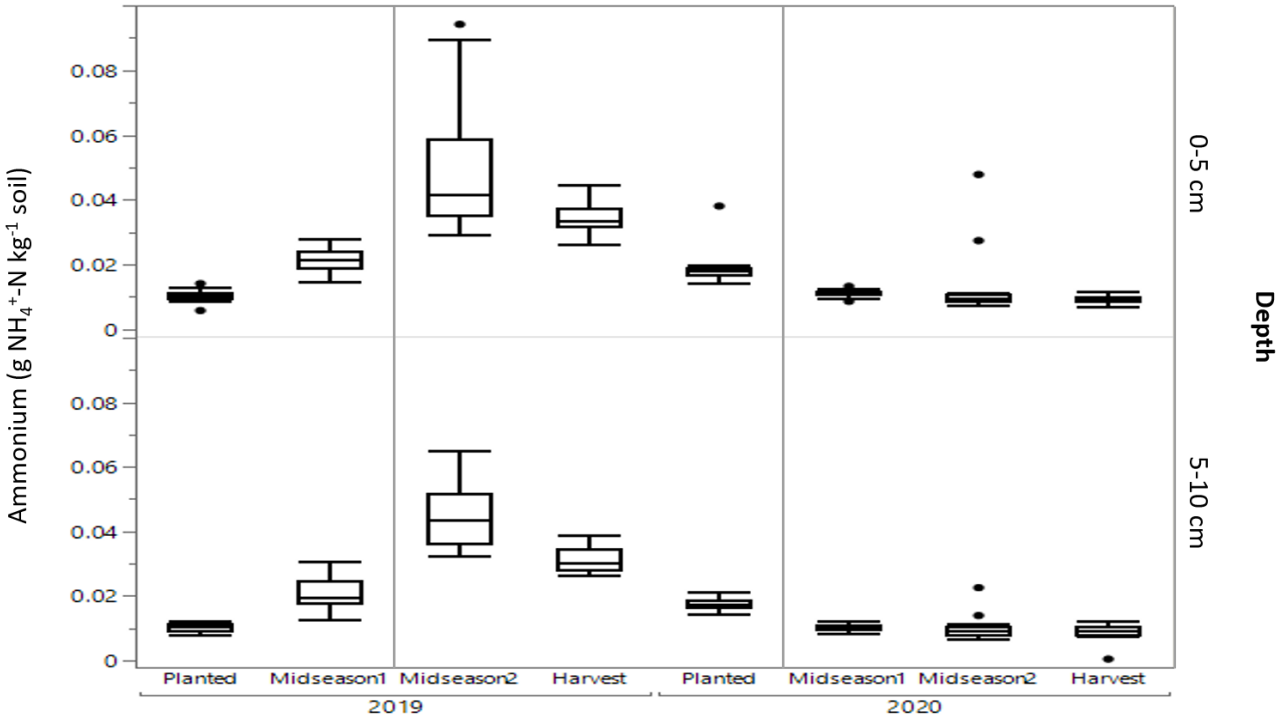
Appendix 1



Appendix Figure 1. NH_4^+-N over the growing season, separated by year and depth. Lines going down across figure represent fertilization.