EFFECTS OF LONG-TERM AGRICULTURAL NITROGEN FERTILIZATION ON SOIL ORGANIC CARBON CYCLING

A Thesis

by

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ABSTRACT

The maintenance and improvement of soil organic carbon (SOC) pools is crucial to global food and fiber production as well as the management of carbon (C) emissions. Because SOC and nitrogen (N) cycles are linked, the effects of long-term agricultural N fertilization rates on SOC should be examined to improve soil management practices. Carbon and N were evaluated in soils of a long-term (38 yr) experiment in central Texas under two fertilization rates. A sorghum–wheat–soybean–fallow (SWS) rotation under no tillage was evaluated temporally across two seasons at the surface, and SWS was compared to monoculture wheat with depth to 1 m. No effects of N fertilization rate on SOC were found in the seasonal study, though temporal dynamics were observed. Increased SOC due to a greater rate of N fertilization was reported for the 1-m soil cores.

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NOMENCLATURE

Alpha	α-glucosidase
Beta	β-glucosidase
C	Carbon
CBH	Cellobiohydrolase
CT	Conventional tillage
EEA	Extracellular enzymatic activity
N	Nitrogen
NAG	N-acetyl glucosaminidase
NT	No tillage
SOC	Soil organic carbon

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

1. IMPACT OF AGRICULTURAL FERTILIZATION ON SOIL CARBON CYCLING

1.1 Introduction

Soil organic carbon (SOC) and nitrogen (N) cycles are closely linked and crucial to the understanding of anthropogenic impacts on ecosystems, especially in agricultural settings. As much as 2000 Pg of SOC are held in soils globally, and this pool sustains soil health and long-term food production (Batjes, 2016). Understanding the storage, movement, and interactions of SOC and N is crucial to the responsible management of anthropogenic impacts on this planet. Agricultural N fertilization provides a 30-50% increase in crop yield that much of the human population relies on (Stewart et al., 2005), but there is still uncertainty on how it affects SOC.

The pool of SOC is an integral part of terrestrial ecosystems. It is foundational in soil aggregation and water retention, erosion reduction, soil microbial activity, biodegradation of pollutants, and climate change mitigation (Blanco-Canqui et al., 2013). As SOC is such an important component of these systems, it should be considered when examining anthropogenic influences on the environment. Organic carbon (C) in soil is a dynamic pool, and many factors, including residue input and tillage, are known to impact SOC (Li et al., 2020). Fertilization with N is another common agricultural management practice that could influence SOC through its effect on biomass and organic residues.

Agricultural N fertilization is a significant factor of the human impact on global ecology. From 1980 to 2009, the largest component of global net anthropogenic N inputs was N fertilizer consumption (59-64% of the total) (Han et al., 2020). Such additions of N can have negative effects on local soils and the environment, such as soil acidification when applied in certain

forms. They can also lead to surface water toxicity and downstream eutrophication of water resources (Fenn et al., 1998), which is exacerbated by the poor utilization efficiency of applied N by crops (Robertson and Vitousek, 2009). Management techniques are often used to improve the efficiency of N fertilizer, including timing to plant needs, varying the rate spatially, and focusing N application to the root zone (Robertson and Vitousek, 2009). Since N fertilization is such a ubiquitous agricultural management strategy and foundational to human food production, its effects on SOC must be understood and managed to ensure sustainable crop production.

Soil C and N cycling are primarily driven by soil microbes. Microbes use the decomposition of organic matter to gain energy and to acquire C and N with which to build their biomass. Microscopic organisms are central to decomposition and nutrient cycling in soil (Schimel and Schaeffer, 2012), and these processes of decomposition and respiration are important controls on terrestrial C cycling (Spohn et al., 2016). Most soil N is found in organic matter as complex molecules that are broken down by microbial extracellular enzymes (van der Heijden et al., 2008). As the drivers of both the C and N cycles, microbes are a key element to understanding the effects of N fertilization on the soil C cycle.

The system in soil that ties together C and N inputs, losses, pools, and microbe-driven cycling is complex. Current literature is divided on whether long-term N fertilization increases (Halvorson et al., 1999; Rudrappa et al., 2006; Liu and Greaver, 2010; Ghosh et al., 2018), decreases (Khan et al., 2007; Li et al., 2018; Poffenbarger et al., 2017), or has no net effect on SOC (Wang et al., 2018; Man et al., 2021). If N fertilization increases stored SOC, then this practice provides multiple benefits including increasing yields, improving soil health, and sequestering atmospheric carbon. If N additions decrease SOC, then they would negatively impact long-term soil health and limit the soil's ability to mitigate global C emissions. However,

it is also possible that N additions have no effect on stored SOC. With such a gap in the understanding of the effects of N fertilization on SOC, land managers are left without important knowledge that could influence their decisions. If N fertilization changes SOC, then its effects on SOC and N cycling processes should be explored to better understand and shape future management strategies.

This review provides the context of agricultural N fertilization, the importance of SOC, and discusses the key role of microbes in linking the C and N cycles in soil. In order to understand the influence of N fertilization on SOC, this paper addresses: (i) known effects of agricultural N fertilization on soil; (ii) the influence of N fertilization on SOC; and (iii) microbial activity – the link between the soil N and C cycles. The purpose of this review is to highlight the seemingly contrasting conclusions of current research into the effect of N fertilization on SOC and to identify factors that may be confounding these results.

1.2 Known Effects of Agricultural Nitrogen Fertilization on Soil

Nitrogen is the most common limiting nutrient for both plant and microbial growth and is therefore of great importance in soil. In a native ecosystem, N primarily enters the soil through organic matter deposition, atmospheric deposition, and microbial fixation from the atmosphere. In an agroecosystem, N is commonly added through fertilization, particularly in non-legume crops, to increase crop yield and biomass (Stewart et al., 2005; Lu et al., 2011a) and resupply nutrients removed from harvesting prior crops. However, N addition can also decrease plant symbiosis with N-fixing bacteria (Regus et al., 2017). Losses of soil N include microbial nitrification and denitrification, volatilization, runoff, leaching, and in agricultural settings, crop harvest and removal. Fertilization does not prevent such losses and can even stimulate them (Lu et al., 2011a).

While N fertilization is a ubiquitous practice in agriculture, there are many different types of fertilizers used that have variable effects on soil properties. Nitrate-based fertilizers are prone to significant leaching and require careful application in agriculture (Sebilo et al., 2013; Cao et al., 2018). The chemical effects of the long-term use of ammonium-based fertilizers are centered around ion reactivity and pH. It has been reported that use of ammonium fertilizers decreases soil pH over time, which subsequently reduces cation exchange capacity (CEC) and base saturation (Barak et al., 1997; Khonje et al., 1989; Schroder et al., 2011). The effect is more rapid in neutral to acidic soil. However, the effect can occur in any soil given time. A global review showed that ammonium-based and urea fertilizer additions acidified soil over time across all studies, even those conducted in alkaline soils (Tian and Niu, 2015). Soil acidification can ultimately reduce crop yields and negatively impact overall soil health if left unchecked (Matsuyama et al., 2005; Kibblewhite et al., 2008). Acidification of soil can also affect microbial function (Schroder et al., 2011; Chen et al., 2019a). The management of N fertilization through source, timing, placement, and rate has been used to maximize efficiency and to reduce losses of N to the environment that contribute to eutrophication. Because agricultural N fertilization can impact the chemical characteristics of soil, the N cycle, and ultimately SOC, the optimization of N application to promote plant growth and reduce environmental impacts is a common goal (Singh, 2006).

1.3 Influence of Nitrogen Fertilization on Soil Organic Carbon

While SOC can be stored in soil for decades to centuries, it is not entirely stable. Labile organic C can actively respond rapidly to natural environmental forcing factors, such as changes in soil temperature, moisture, and nutrient availability (Manzoni et al., 2012), as well as agricultural management practices including tillage, cropping rotation, and residue retention (Lal, 2004), which is especially relevant to the more than 15,000,000 km^2 of cropland around the

world (Erb et al., 2007). As N fertilization affects nutrient availability and crop biomass, it also ultimately affects the SOC pool as well.

In many studies, SOC significantly increased under N fertilization in natural and agricultural systems (Halvorson et al., 1999; Rudrappa et al., 2006; Liu and Greaver, 2010; Lu et al., 2011b; Ghosh et al., 2018; Qiu et al., 2020). In a long-term dryland cropping study, Halvorson et al. (1999) examined multiple N rates and found that N fertilization increased plant residue inputs, which resulted in greater SOC at 0-7.5 cm depth compared to no fertilization. A meta-analysis summarizing 257 studies across natural and agricultural systems showed that N fertilization can result in a 20% increase in litter C pools (Lu et al., 2011b). Lu et al. (2011b) also found a small, but significant, increase in SOC storage in agricultural ecosystems following increases in litter C input in their analysis. Ghosh et al. (2018) reported that N fertilization added to the SOC pool by increasing plant biomass. Research on long-term N fertilization by Rudrappa et al. (2006) showed that N-induced increases in root biomass specifically added to stored SOC. Studies that reported increases in SOC due to N fertilization often found a difference in accumulation of SOC by depth, with greater increases near the surface, corresponding with the location of most above- and belowground plant biomass inputs (Ghosh et al., 2018; Halvorson et al., 1999; Liu and Greaver, 2010; Rudrappa et al., 2006). In a meta-analysis of 85 studies in cropland in China, Miao et al. (2019) reported that unbalanced chemical fertilization, balanced chemical fertilization, and manure applications all raised SOC, but that a balanced chemical fertilization regimen with crop residues returned to the surface provided the greatest increase. Halvorson et al. (1999) investigated different rates of N fertilization and found that there was a significant difference in how the soil responded to the individual rates. The potential benefits of balanced N fertilization on SOC have also been discussed at length by Singh (2018). Using N

fertilization that is appropriate in rate and timing for the crop being grown could improve yields while protecting soil health.

The SOC pool has also been found to decrease or remain unchanged by N fertilization. Halvorson et al. (2002) reported no change in SOC after 12 years of N fertilization at multiple rates as compared to a control, even while crop residues increased in an agricultural setting. In a 23-year study of N fertilization, Wang et al. (2018) found no changes to SOC in a rice-barley crop rotation. Both Halvorson et al. (2002) and Wang et al. (2018) suggested that an N-induced elevation of the C turnover rate compensated for the increased crop residue inputs. A loss of SOC due to N fertilization has also been observed and was attributed to crop removal and thus C removal (Khan et al., 2007). In testing multiple N fertilization rates in both continuous maize and maize-soybean rotation, Poffenbarger et al. (2017) found that SOC decreased under some high N rates. Geisseler and Scow (2014) also reported 18 out of 107 studies with reduced SOC due to N fertilization in their meta-analysis. These studies suggest that there are additional variables (e.g. soil texture, climate, crop type, or microbial factors) that affect the influence of N fertilization on SOC storage.

1.4 Microbial Activity: The Link Between Soil Nitrogen and Carbon Cycling

Measures of microbial biomass and community composition can provide insight into how long-term N fertilization affects microbial survival and reproduction. With such a crucial role in nutrient cycling, abundance and diversity of soil microorganisms have been considered a measurement of soil health (Saleem et al., 2019). A review of 107 datasets from 64 long-term trials showed microbial biomass was dependent on soil pH with greater microbial biomass in soils with pH > 7 (Geisseler and Scow, 2014). In an alkaline soil, Bhattacharyya et al. (2012) found increases in microbial biomass under N fertilization and credited them to increased

available C and enhanced metabolic activity. Man et al. (2021) also found an increase in microbial biomass and SOC decomposition due to N fertilization in an acidic soil, suggesting that increased C inputs were regulated by microbial activity.

Zhou et al. (2017) reviewed 454 N addition experiments and found that the initial soil microbiome composition was an important predictor of microbial biomass response to N fertilization with implications for decomposition (i.e., C mineralization). Geisseler and Scow (2014) also proposed that changes in microbial biomass could be attributed to shifts in the microbial community structure. Microbial community composition can determine how it will react to disturbances such as CO_2 fluctuations, temperature changes, C substrate additions, and N fertilization (Allison and Martiny, 2008). The initial composition of microbial communities could be contributing to the varied responses of soil microbes to N fertilization. Wang et al. (2018) and Chen et al. (2019a) found a reduction in microbial diversity, which can affect decomposition, in soil due to N fertilizer addition over time as compared with a no-fertilizer control (Maron et al., 2018). Microbial respiration, a proxy for measuring C mineralization, showed a consistent decrease with increasing fertilization in soils collected across climates in North America (Ramirez et al., 2012). Ramirez et al. (2012) reported that N fertilization shifted the microbial community towards phyla involved in the degradation of easily decomposed C compounds and away from those degrading recalcitrant C. Fog (1988) also found that decomposition was reduced for recalcitrant substrates compared to labile substrates in response to N additions. This would suggest that N additions could decrease soil C in the short term, but that long-term N addition would cause a buildup of recalcitrant soil C. A shift in the microbial community towards fungi was also found by Bardgett et al. (1999) following N fertilization in an

incubation study. Metabolic abilities can also change with microbial community composition, as fungi favor substrates with a higher C:N ratio.

Conventional agricultural tillage has also been shown to negatively impact soil microbial biomass and community diversity, which in turn would affect C and N cycling (Mathew et al., 2012), meaning potential effects of tillage must also be considered. While changes in the population composition can be informative when examining microbial responses to N fertilization, the decomposition activity of the community of a whole must also be understood.

The primary mode of microbial decomposition is the use of extracellular enzymes, and N fertilization has the potential to affect their activity in an environment. Enzymes, such as β-glucosidase (Beta), α-glucosidase (Alpha), and cellobiohydrolase (CBH) break down complex C compounds. Chitin is a source of N in soil and is processed by enzymes such as N-acetyl glucosaminidase (NAG). In a review of 65 N fertilization studies, Jian et al. (2016) found increases in CBH, Alpha, and Beta activities potentially due to improved microbial enzyme resource acquisition leading to greater production of extracellular enzymes. Waldrup and Zak (2006) observed a decrease in oxidative enzyme activity in response to N addition and controlled flux of dissolved organic C. However, in a laboratory incubation with soils from 28 natural systems, Ramirez et al. (2012) reported a 10% reduction in Beta and CBH activities under N fertilization, but they did not account for additional C inputs that result from N fertilization of plants. DeForest et al. (2004) found suppression of cellulose-decomposing enzymes with chronic N additions in a natural system where increases to litter inputs could have been influencing soil C dynamics. In an agricultural setting, Dihman et al. (2019) reported a decrease in dehydrogenase enzyme activity under N fertilization as compared to an unfertilized control after 40 years of management. An earlier study by Bardgett et al. (1999) found no consistent effect of

N fertilization on measures of enzyme activity. It is apparent that, while measures of enzymatic activity can be informative, there is a lack of comparability in the field. The variation in reported enzymatic activity and SOC responses to N fertilization likely reflects that there are other environmental or management factors that are not consistently addressed.

1.5 Conclusion

Nitrogen fertilization can affect SOC, but the reported effects are inconsistent across soils and ecosystems. The complexity of the soil C and N cycles, as linked by microbial activity and modified by field management, has prevented the determination of one universal effect of N fertilization on SOC. Long-term effects on soil pH are one potential indirect driver of SOC shifts under N fertilization. Acidification can influence soil chemical qualities, such as CEC, as well as the soil microbial community, ultimately affecting how SOC is cycled. Plant C inputs can define the availability and accessibility of C for decomposition and incorporation. When considering plant residues, biomass increases due to N fertilization must also be assessed. Residue management, specifically tillage practice, can have a large impact on C availability and must be considered in evaluating SOC dynamics. However, one factor that stood out in this review was the conflicting effects of N fertilization rate on SOC. Tailoring N application rates to plant needs could result in consistent SOC storage across systems.

Future studies should consider N fertilization rate in-depth, and should be controlled for varied environmental factors, such as mean annual temperature and mean annual precipitation, and management factors, such as tillage and cropping rotation. With well-designed studies that control for chemical properties and plant inputs, advances can continue in this important area. Ultimately, N fertilization could be a tool in long-term C storage and climate change mitigation.

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2. EFFECTS OF LONG-TERM AGRICULTURAL NITROGEN FERTILIZATION RATE ON SOIL ORGANIC CARBON AND NITROGEN CYCLES

2.1 Introduction

Agricultural N fertilization is foundational to global food production, but its effect on SOC and microbial nutrient cycling activities is not well understood. Up to 2000 Pg of SOC are stored in soils globally, and this pool is crucial to sustained agricultural production and soil health (Batjes, 2016). As the primary link between the soil SOC and N cycles, soil microbial communities drive nutrient cycling processes, but there is no consensus on how they respond to long-term N fertilization (Lu et al., 2011a; Miao et al., 2019). Availability of N is considered a key limiting factor for plant biomass synthesis and microbial biomass degradation. Changes to N availability affect the quality of residue inputs to the soil as well as the ability of microbes to process organic C compounds that could ultimately impact SOC storage. A more complete understanding of the impact of N fertilization on soil resources would improve management of the soil for sustainable agricultural production. However, literature is inconsistent on how long-term N fertilization impacts SOC storage; studies have reported increases (Ghosh et al., 2018), no changes (Wang et al., 2018), and decreases (Khan et al., 2007) of SOC under N fertilization.

Many studies have documented increases in SOC due to N fertilization and attributed these gains to organic material input from improved crop biomass, both above and below ground (Ghosh et al., 2018; Lu et al., 2011b; Qiu et al., 2020). As SOC increases, soil properties, such as water infiltration, water holding capacity, soil aggregation, microbial activity, and biodegradation of pollutants, subsequently improve (Blanco-Canqui et al., 2013). In conventionally tilled cropping systems, some studies have found that N fertilization resulted in SOC increases compared to an unfertilized control (Ghosh et al., 2018; Qiu et al., 2020). In a

meta-analysis that included both natural and cropping ecosystems by Lu et al. (2011b), small but significant increases in SOC were reported due to N fertilization as compared to a negative control. Ramirez et al. (2012) reported decreases in microbial biomass C and extracellular enzymatic activity (EEA) under N fertilization in an incubation study, proposing that a reduction in SOC mineralization by microbes could contribute to enhanced SOC sequestration. In a study of natural systems, Sinsabaugh et al. (2004) found increased dissolved organic C and decreased EEA activity under N fertilization. In comparison with a no-fertilization control, both attributed suppressed extracellular enzyme activity (EEA) to shifts in bacterial communities, suggesting that the systems were moving towards soil C storage and away from active recycling (Ramirez et al. 2012; Sinsabaugh et al. 2004). This response of EEA to N fertilization, in both an artificial and natural systems, highlights the tight linkage of soil C and N cycling and microbial EEA.

A lack of increase in SOC due to N fertilization has also been observed (Jung and Lal, 2011). Halvorson et al. (2002) showed no change in SOC under N fertilization after 12 years compared to an unfertilized control despite an increase in residue input. Local soil properties and environmental factors could be contributing to the varied response of SOC to N fertilization. Plant residue deposition and microbial biomass addition contribute significantly to SOC (Angst et al., 2018). However, for SOC to remain in the soil long-term, it must be stabilized and stored in the mineral matrix. Storage of SOC is thought to occur through physical protection from EEA in microaggregates and close associations with fine soil particles (Besnard et al., 1996). Soil texture and local climatic factors can dictate the upper limits of SOC storage, and native soil microbiomes can differ in how they respond to changes in organic matter availability (Degens, 1999; Rabbi et al., 2015). Soil physical properties, such as parent material, aggregation, soil type, and topography also affect how a soil responds to fertilization (Angst et al., 2018; Wiesmeier et

al., 2019). Constant SOC, despite N addition, could be due to the soil reaching its physical capacity for SOC storage (Six et al., 2002). Compensation for N-induced increased biomass production with increased microbial decomposition could also explain a lack of effect on SOC. Losses in SOC under long-term N fertilization have been found in comparison to an unfertilized control and attributed to the harvest and removal of biomass in agricultural settings, thus preventing SOC accumulation (Khan et al., 2007). With such mixed results, further research is needed to elucidate the way SOC responds to long-term N fertilization.

Fertilization rate and field management are important factors to consider when determining whether SOC increases, decreases, or remains unchanged under N addition. Comparisons of soil under N fertilization and soils without fertilization are common, but not reflective of standard agricultural practice. Rate of fertilization affects biomass; however, increases in biomass diminish with increasing rate. In a 46-year study of N fertilization, Dhiman et al. (2019) found that SOC increased with fertilization. Increases in SOC from N fertilization at the specific recommended rates have been discussed in a review by Singh (2018), and SOC has been shown to increase up to the recommended fertilization rate then decline (Poffenbarger et al., 2017). Appropriate N fertilization in both rate and timing could improve yields while protecting soil health and increasing SOC. Management factors can also contribute to the wide range of conclusions concerning the effect of N fertilization on SOC. Tillage can increase SOC degradation rates (Halvorson et al., 2002; Alvarez, 2005; Mazzoncini et al., 2011), and until recently, much of the agronomic research measuring changes in SOC due to N fertilization was conducted in conventional tillage (CT) systems where N fertilization is compared to an unfertilized control. The impacts of N fertilization in NT systems have not been well understood, especially with respect to biological parameters such as EEA. Cropping intensity (i.e., number of

crops per year and/or cover cropping practices) and residue management can also affect C inputs to the soil (Mazzoncini et al., 2011; Nyambo et al., 2020). However, few studies evaluate the effect of N fertilization rates on SOC under no tillage (NT).

The goal of the present study was to identify the dynamic effects of long-term N fertilization on soil C and N pools and microbial cycling activities in crop rotation systems under NT production across a growing season. The specific objectives of this project were to:

(1) Quantify the dynamics of soil C and N pools with microbial nutrient cycling activities across a growing season under long-term fertilization practices
(2) Identify correlations between long-term N fertilization rate, shifts in microbial nutrient cycling, and SOC

It is hypothesized that greater N fertilization will increase SOC by removing N limitations to plant growth and thus increasing biomass production. Reduced microbial degradation could also contribute to increased soil C.

2.2 Materials and Methods

2.2.1 Experimental Design

The research site is a long-term management experiment established in 1982 at the Texas A&M Research Farm. It is in south-central Texas (30°32'N 96°26'W) in the Brazos River floodplain on a Belk clay (Fine, mixed, active, thermic, Entic Hapluderts). In this experiment, four N treatments are in a randomized complete block design with four replicates for specific crop rotations produced under CT and NT practices. Only the sorghum (*Sorghum bicolor*) – wheat (*Triticum aestivum*) – soybean (*Glycine max*) – fallow rotation (i.e., SWS) under NT was selected for evaluation. The entire 2-year rotation is replicated such that both phases are present

each year. The different phases are represented by the odd (O) or even (E) year that soybean is grown (i.e., SWSO and SWSE, respectively; Fig. 2.1). The N fertilization rates were applied as no, low, medium, and high N treatments, corresponding to 0, 34, 68, and 102 kg N ha^{-1} applied to wheat and 0, 45, 90, and 135 kg N ha^{-1} applied to sorghum; however, this study focused on two fertilization rates from the experiment (i.e., low and high N treatments).

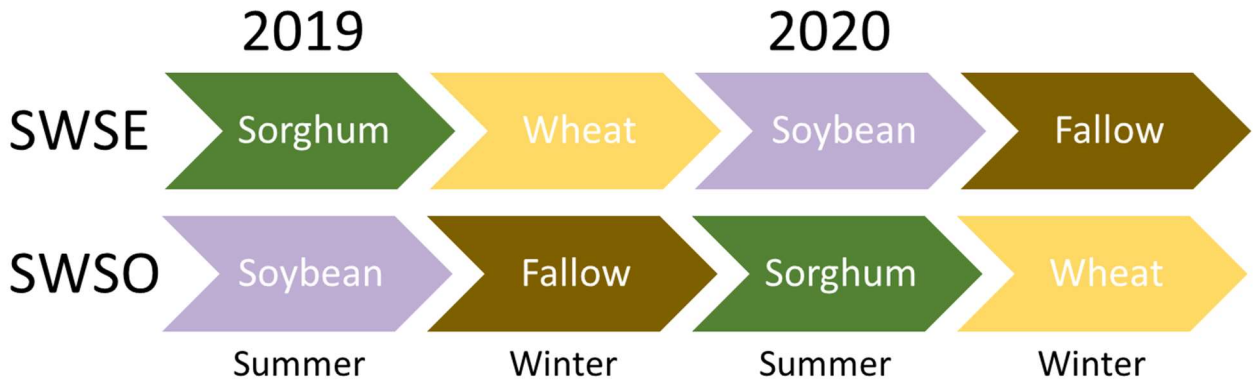

Figure 2.1. Crop rotation sequences used for this study, showing sorghum, wheat, and soybean phases. Years indicate cropping year. Each phase has two fertilization rates, both with four replications (16 plots). Samples were taken during the sorghum and soybean phases (32 samples per sample time).

Wheat (TAM 304) was planted on 20 Nov 2019 at 80,000 seeds per acre and was harvested on 15 May 2020. Glyphosate (Roundup, Bayer) was applied on 1 Jan 2020, and a commercial mix of thiencarbazone-methyl, pyransulfotole and bromoxynil (Huskie Complete, Bayer) was applied 14 Feb 2020. Wheat was fertilized with urea broadcast at the surface of the soil on 17 Feb 2020. Wheat was not sampled during the study period. In 2019, extreme weather events delayed planting of both sorghum and soybean and prohibited the use of commonly grown early maturing varieties. Pioneer sorghum and soybeans were planted on 9 July 2019. In 2020, more typical varieties were used, and crops were planted on schedule. Sorghum was planted on 1 April 2020, and soybean was planted on 3 June 2020. S-metolachlor (Dual Magnum, Syngenta) was applied to the sorghum plots on 2 April 2020, and glyphosate (Roundup, Bayer) was applied 11 May 2020 to both soybean and sorghum to control weeds. Fertilizer N was applied to sorghum as urea broadcast at the surface of the soil on 4 Sep 2019 and 20 May 2020 at the experimental rates. Soybeans were fertilized with TSP on 4 Sep 2019 and 22 July 2020. Due to the delays in planting in 2019, neither soybean nor sorghum was harvested, as the soybean stand was poor, and the sorghum grain was consumed by birds prior to scheduled harvest. In 2020, sorghum and soybean also had poor stands and vegetation was shredded at the scheduled harvest. Thus, yield was not available for evaluation in this study.

Historical data from this site documents increases in SOC over time due to N fertilizer applications. Franzluebbers et al. (1994) reported increased SOC at 5 cm depth after 9 years of N fertilization as compared to the unfertilized control. An increase in grain yield also occurred under N fertilization, as compared with unfertilized plots. The authors suggested that an increase in biomass that likely accompanied the increased yield contributed to the increased SOC (Franzluebbers et al., 1995). Dou et al. (2014) found that after 20 years of consistent

management, particulate and mineral associated organic matter increased under N fertilization and NT management compared to an unfertilized control. At this time, the site has been under the same treatments for over 35 years and was used to further elucidate relationships between long-term N fertilization and SOC dynamics and storage.

2.2.2 Sample Collection

Soil organic C and N cycling was assessed in a series of 0-5 cm and 5-10 cm soil samples taken four times during each growing season of soybean and sorghum in 2019 and 2020. Plots were sampled after planting (planting), twice during the midseason (midseason 1 and midseason 2), and before harvest (harvest) (Table 2.1). Sampling times were selected to be consistent between crop growth stages to avoid variability among phases of the rotation, and they provide an overview of the dynamics of SOC and N pools under the influence of fertilization, crop development, and seasonal changes. There were 8 subsamples collected from each plot with a manual probe (2.54 cm diameter), 4 in crop rows and 4 in furrows, which were composited. All soil samples, except those used for enzyme analysis, were ground to <2 mm, air dried on the lab bench, and stored at room temperature. Soils used for enzyme analysis were freeze-dried the day of sampling. No moisture correction was used.

Table 2.1. Timeline of sampling and fertilization during the study. Fertilization for sorghum occurred as urea and soybean was fertilized with triple superphosphate. Both were broadcast at the surface of the soil.

	Planting	Fertilization	Midseason 1	Midseason 2	Harvest
2019					
Sorghum	10 July	4 Sept	9 Aug	13 Sept	24 Oct
Soybean	10 July	4 Sept	9 Aug	13 Sept	24 Oct
2020					
Sorghum	2 Apr	20 May	1 July	13 July	6 Aug
Soybean	4 June	22 July	1 July	13 July	14 Oct

2.2.3 Analytical Methods

Pools of N measured included total N (TN) and ammonium (NH_4-N) in the soil. Total N was assessed using the Vario El Cube CHNS/Sir Analyzer (Elementar, Ronkonkoma, NY, USA), which combusted 35-40 mg of finely ground soil to gaseous forms and assessed them via gas chromotography. Ammonium-N was extracted from air-dried soils with 2 M potassium chloride in a 1:10 soil:solution ratio. The extracted solutions were analyzed using the Berthelot reaction, in which indophenol blue is formed in the presence of ammonium in solution. The concentration of indophenol blue was assessed via spectrophotometric quantification in comparison with a standard curve ($r^2 \geq 0.95$; Sims et al., 1995).

Pools of C measured included total C, soil inorganic C, SOC, and active C. Total C in the soil was assessed by total combustion analysis as described above. Soil inorganic C was determined by the pressure calcimeter method, in which 6 M hydrochloric acid was used to liberate carbon dioxide from 1 g of soil (Sherrod et al., 2002). Soil organic C was calculated as the difference between total and inorganic C. Active C, i.e., permanganate oxidizable C (POxC), was determined using a colorimetric method in which 2.5 g of soil was reacted with a permanganate solution to assess oxidation (Culman et al., 2012). A spectrophotometer (Synergy H1, Biotek) was used to quantitatively measure color in solutions and compare to a standard curve ($r^2 \geq 0.95$), and absorbance was converted to mg kg^{-1} POxC in soil using the equation reported by Culman et al. (2012).

Enzyme activities were assessed using a modified high-throughput fluorometric method (Sinsabaugh and Klug, 1999; German et al., 2011; Smith et al., 2015). For each sample, 1 g of

soil was suspended in 100 mL of 1 M tris hydroxymethyl aminomethane hydrochloride buffer. A 200 µL aliquot of this slurry was added to the wells in a microplate containing fluorescent-marked substrates, and each plate was incubated at room temperature for 1 (Beta, NAG, Phos) or 3 hr (Alpha, CBH). Each plate also had blank, negative control, and quench wells. At the end of the incubation, 10 µL of 0.5 M NaOH was added to each well to stop the reaction, and fluorescence was measured with a spectrophotometer with an excitation of 360 nm and emission measured at 460 nm (Synergy H1, Biotek). Fluorescence was converted to potential enzymatic activity using the equation reported by Smith et al. (2015).

2.2.4. Data Analysis

Analysis of the factors affecting C and N parameters was performed using a linear mixed effects model with the restricted maximum likelihood (REML) method in JMP Pro version 16 (SAS Institute Inc., Cary, NC). The REML method is a common method for estimating parameters in linear mixed models and is well suited to analysis with small sample size (Harville, 2021). Preliminary analyses revealed a significant effect of depth on most variables. Soil metrics (e.g., TN, SOC, and POxC) had bimodal distributions due to soil depth, except NH_4-N (Table 2.2; Fig. 2.2). The mean of TN, SOC, and POxC were 36, 43, and 63% greater at 0-5 cm than at 5-10 cm depth. The lack of difference with depth in NH_4-N is likely due to high estimated variance associated with year, which is addressed in the results section (Section 2.3.1). Three of five enzyme activity measurements, Beta, Alpha, and CBH, were 209, 174, and 200% greater in the 0-5 cm depth compared to 5-10 cm (Table 2.2; $p < 0.001$ for all). This agrees with known patterns in soil profiles where biologically linked metrics are greater near the soil surface (VeVerka et al., 2019). Due to these differences in C and N pools and enzyme activities between

sample depths, all treatment effects for hypothesis testing were analyzed separately for each depth.

Table 2.2 Mean and standard deviation values for soil C and N properties sampled across two growing seasons at 0-5 and 5-10 cm depth under a sorghum-wheat-soybean-fallow crop rotation. The p-value reflects significant differences by depth ($p \leq 0.05$). Significant p-values are marked in bold.

		0-5 cm		5-10 cm		
	Units	Mean	Std	Mean	Std	P value
Total N	%	0.15	0.02	0.11	0.02	**<0.001**
NH_4-N	mg kg^{-1}	21.3	18.2	19.0	13.0	0.143
SOC	%	1.42	0.23	0.99	0.17	**<0.001**
POxC	mg kg^{-1}	398	79.0	246	73.0	**<0.001**
Beta	mmol g SOC^{-1} hr^{-1}	1230	864	446	296	**<0.001**
NAG	mmol g SOC^{-1} hr^{-1}	111	77.0	75.0	48.0	0.632
Phos	mmol g SOC^{-1} hr^{-1}	737	506	476	299	0.543
Alpha	mmol g SOC^{-1} hr^{-1}	105	79.0	42.0	32.0	**<0.001**
CBH	mmol g SOC^{-1} hr^{-1}	43.0	35.0	16.0	14.0	**<0.001**

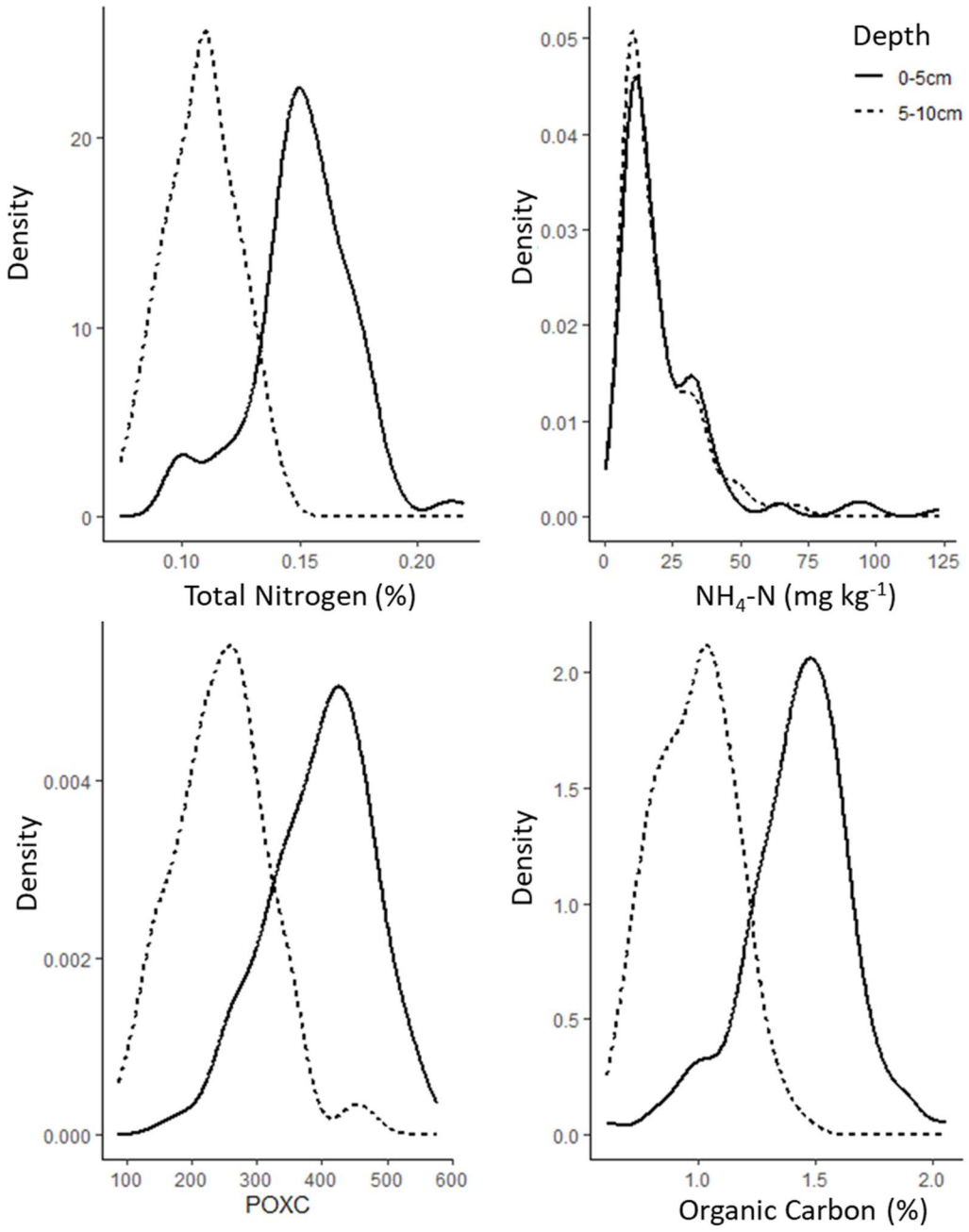

Figure 2.2. Kernel density plots of total nitrogen (TN), ammonium (NH_4-N), soil organic carbon (SOC), and permanganate oxidizable carbon (POxC) in soil collected in 2019 and 2020 under soybean and sorghum. There were taken from a soybean-wheat-sorghum-fallow rotation under no tillage (NT) at 0-5 and 5-10 cm depth increments.

For each depth (i.e., 0-5 cm and 5-10 cm) 128 samples were analyzed (n = 128). N fertilization rate, crop at sampling, and sampling time were considered fixed variables, while plot and year were treated as random variables. Though the experimental design includes blocks, plot was found to account for more variation than block, and thus block was not included in the analysis. Year was considered a random variable as soil metrics showed little variation was due to year (except for NH_4-N as mentioned above). Ultimately, REML models tested 2-way interactions among variables, as Phos and CBH activities were the only response variables that had significant 3-way interactions (Table A.1), and plots revealed inconsistent patterns in the data (Fig. A.1). For simplicity, the 3-way interaction was not included in the models reported in results. Significance was defined as $p \leq 0.05$, and no outliers were removed. For the assessment of EEA, measured activities were log-transformed for normality. Plots were made using JMP version 16 SAS Institute Inc., Cary, NC) and ggplot2 in R version 4.0.5 (R Core Team, Vienna, Austria). All means are reported as mean ± standard deviation.

2.3 Results

2.3.1 Nitrogen

Total N (TN) did not respond to N rate, crop, or sampling time, nor were there any interactions among these effects, at either depth, except that the crop by sampling time interaction was significant at 5-10 cm (Table 2.3). The interaction showed greater TN at midseason 2 sorghum (0.119 ± 0.016 mg kg^{-1}) than soybean (0.105 ± 0.016 mg kg^{-1}; Fig. 2.3).

Table 2.3. F ratios (F) and p values (p) from a generalized linear mixed effects model for soil samples collected across two growing seasons under a sorghum-wheat-soybean-fallow rotation. For each depth (i.e., 0-5 cm and 5-10 cm) 128 samples were analyzed (n = 128). N fertilization rate, crop at sampling, and sampling time were considered fixed variables, where plot and year were treated as random variables. Significance was defined as $p \leq 0.05$ and indicated with bold.

		TN		NH_4-N		SOC		POxC	
Effect	df	F	p	F	p	F	p	F	p
0-5 cm									
N Rate	1	0.20	0.658	0.10	0.757	0.11	0.746	0.13	0.720
Crop	1	0.83	0.364	2.45	0.120	8.66	**0.004**	0.12	0.735
Sample Time	3	0.30	0.824	11.5	**<0.001**	2.12	0.103	2.84	**0.042**
Crop × Sample Time	3	0.24	0.869	5.61	**0.001**	0.28	0.838	1.29	0.281
N Rate × Sample Time	3	2.56	0.059	0.01	0.999	2.40	0.073	1.15	0.334
N Rate × Crop	1	0.14	0.713	0.02	0.880	0.59	0.445	0.05	0.817
Est. Variance									
Plot		54.82%		0%		47.48%		41.29%	
Year		0%		38.21%		2.76%		10.15%	
Whole Model Fit		$r^2 = 0.590$		$r^2 = 0.396$		$r^2 = 0.595$		$r^2 = 0.577$	
5-10 cm									
N Rate	1	0.02	0.887	0.37	0.5525	0.09	0.769	0.90	0.360
Crop	1	0.83	0.365	1.29	0.2595	1.98	0.163	0.13	0.716
Sample Time	3	2.24	0.088	12.9	**<0.001**	4.84	**0.004**	0.56	0.644
Crop × Sample Time	3	6.04	**<0.001**	2.01	0.1179	3.12	**0.030**	1.58	0.198
N Rate × Sample Time	3	1.13	0.342	0.02	0.9966	0.63	0.597	0.14	0.936
N Rate × Crop	1	0.37	0.539	0.02	0.9029	0.09	0.771	0.22	0.637
Est. Variance									
Plot		35%		0%		18.15%		0%	
Year		1.59%		52.63%		15.44%		24.01%	
Whole Model Fit		$r^2 = 0.502$		$r^2 = 0.177$		$r^2 = 0.446$		$r^2 = 0.186$	

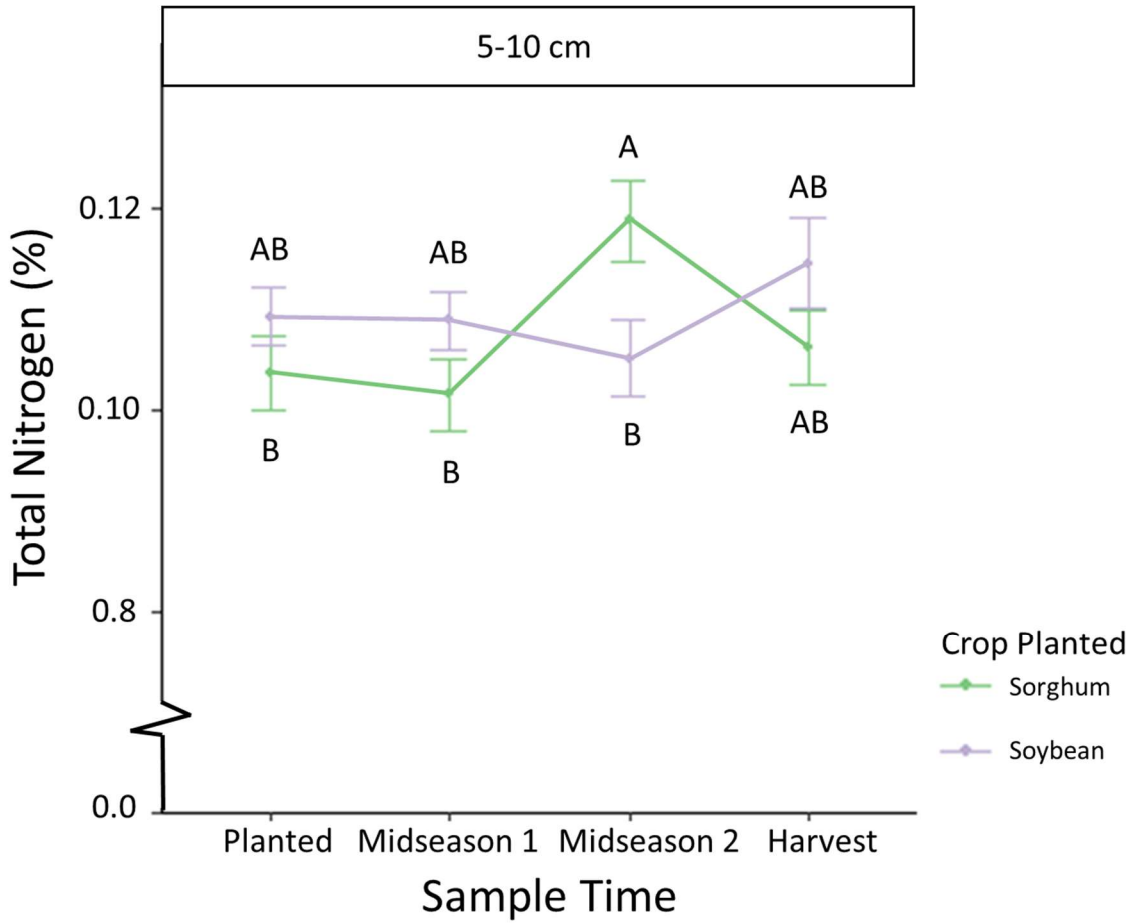

Figure 2.3. Soil total nitrogen (TN) sampled at 5-10 cm in a sorghum-wheat-soybean-fallow crop rotation (SWS) under no tillage (NT) at 4 sample times within two growing seasons under sorghum and soybean. Points represent respective means, with standard error bars indicating data spread. Letters indicate significant differences by Tukey's HSD.

Sampling time affected NH_4–N concentrations at both depths (Table 2.3). While p-values for the effect of sampling time and the interaction of sampling time and crop on NH_4-N were significant at 0-5 cm (Table 2.3), these differences simply reflect a greater concentration at midseason 2 under sorghum compared to soybean, which is expected as no N fertilizer was added to soybean (Fig. 2.4). At 5-10 cm, NH_4-N at midseason 2 (27.7 ± 20.2 mg kg^{-1}) was nearly 70% greater than at all other sampling times. In 2019, the elevated NH_4-N at the midseason 2 sample corresponded to a fertilization event that occurred 9 days earlier. There was no elevation

of NH_4-N concentration in 2020 because sorghum was planted on schedule and fertilization occurred approximately 45 days prior (Fig. A.2). This is also reflected by the large, estimated variance from year that was only observed for NH_4-N with 38 and 53% for 0-5 and 5-10, respectively (Table 2.3).

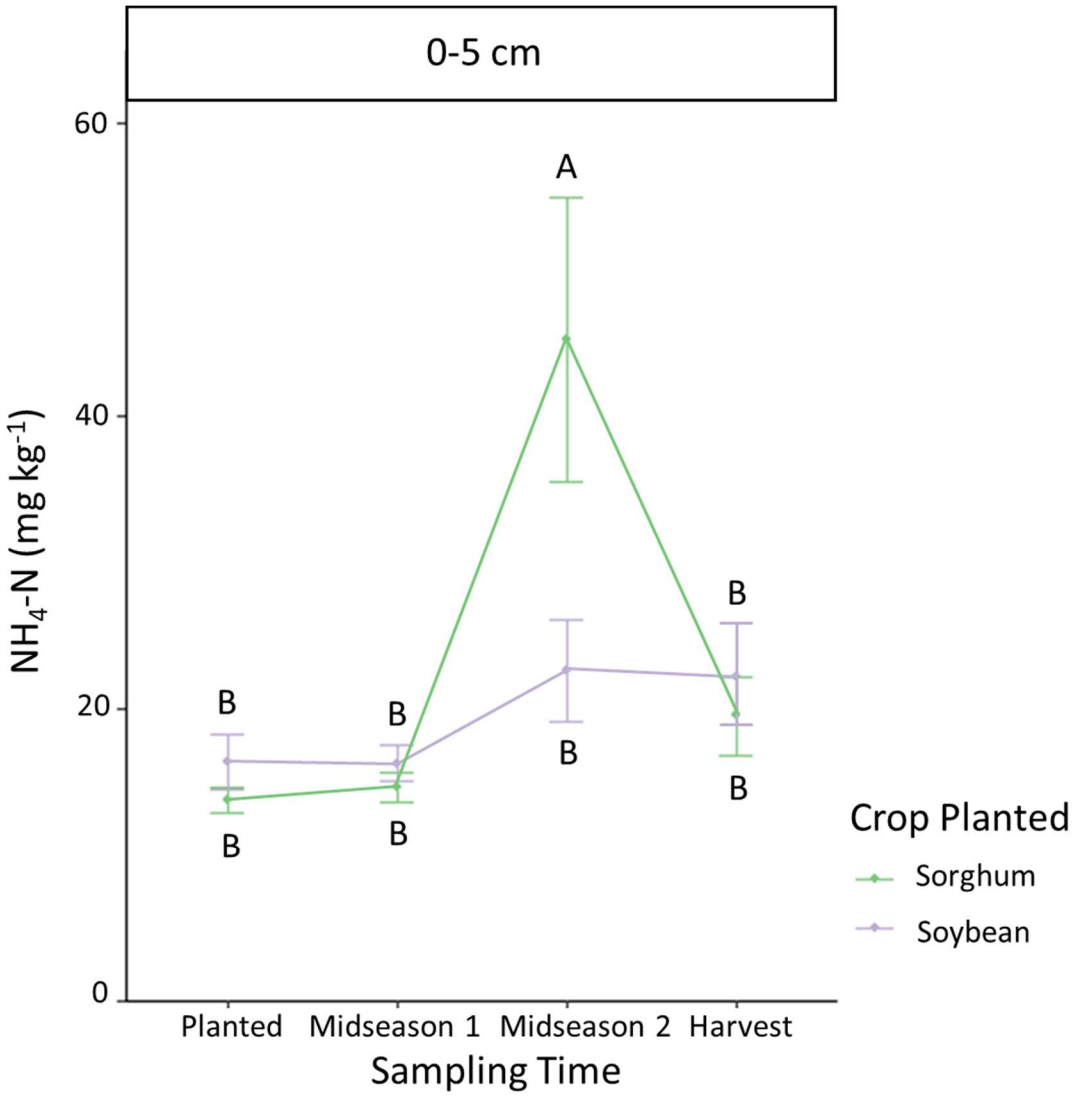

Figure 2.4. Soil ammonium (NH_4-N) concentrations from 0-5 cm depth sampled at across two growing seasons under a sorghum-wheat-soybean-fallow (SWS) crop rotation under no tillage (NT). Points represent respective means, with standard error bars indicating data spread. Letters indicate significant differences by Tukey's HSD.

2.3.2 Carbon

Soil organic C did not differ with N fertilization rate at either depth (Table 2.3), but SOC was affected by crop at the 0-5 cm depth and sampling time at 5-10 cm depth. The crop present in the field during sampling affected SOC at 0-5 cm with greater SOC under soybean (1.47 ± 0.21 %) than under sorghum (1.38 ± 0.24 %; $p = 0.004$; Fig. 2.5). An interactive effect of sampling time and crop present was reported for SOC at 5-10 cm, but a post-hoc mean comparison analysis did not show differences between crops at any individual sampling time. Active C (i.e., POxC) was only influenced by sampling time at 0-5 cm with greater POxC at planting (415 ± 87 mg kg^{-1}) than in midseason 2 (374 ± 77 mg kg^{-1}; Fig. 2.6; Table 2.3).

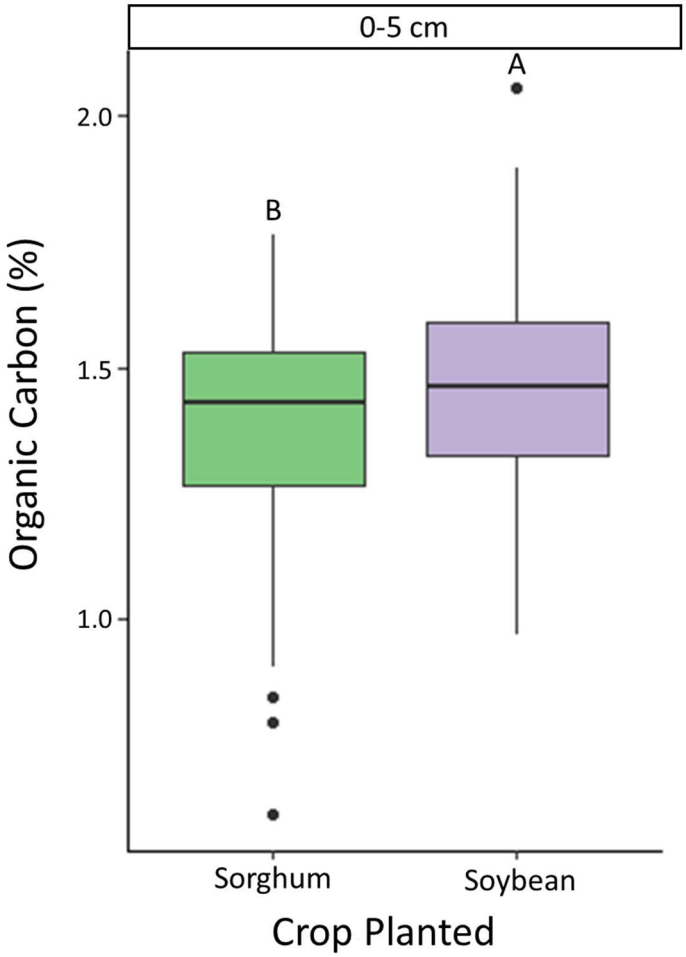

Figure 2.5. Soil organic carbon (SOC) sampled at 0-5 cm under two crops: sorghum and soybean. These were sampled in a sorghum-wheat-soybean-fallow crop rotation under no tillage (NT) across two growing seasons. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Significance difference letters were determined using a Student's t test.

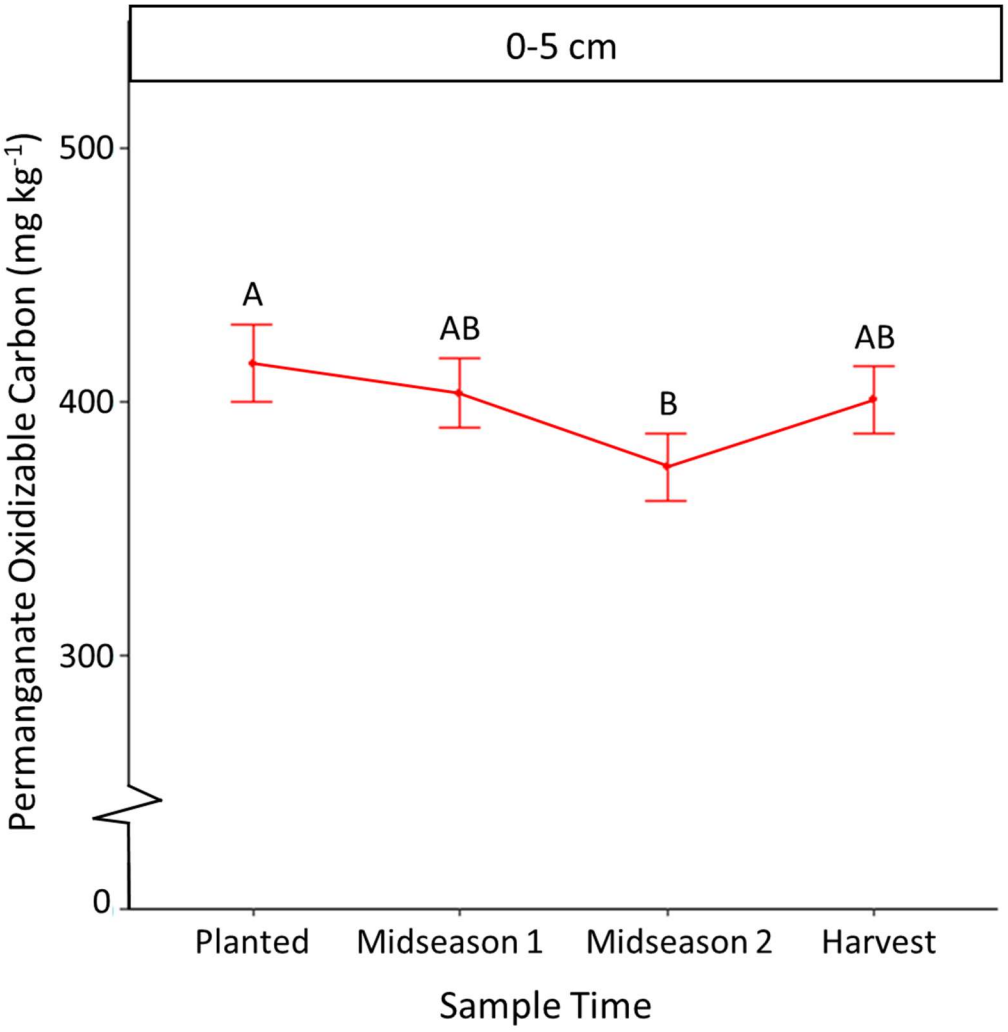

Figure 2.6. Soil permanganate oxidizable carbon (POxC) sampled 8 times across two years. Soils were sampled at 0-5 cm in a sorghum-wheat-soybean-fallow crop rotation under no tillage (NT). Points represent respective means, each derived from 32 data points, with standard error bars indicating data spread. Letters indicate significant differences by Tukey's HSD.

2.3.3 Enzyme Activity

At the surface depth (0-5 cm), soil EEAs were unaffected by N fertilization rate (Table 2.4). There was a significant interaction of sampling time and N fertilization rate on Alpha activity at 0-5 cm where the high rate of fertilization corresponded with less activity at midseason 2 (57.3 ± 58.8 mmol g^{-1} SOC^{-1} hr^{-1}) than the low rate (92.8 ± 48.4 mmol g^{-1} SOC^{-1} hr^{-1}) (Fig. A.3). The crop present at the time of sampling only affected the enzymatic activity of NAG in the 0-5 cm depth with NAG in sorghum having a greater activity (89 ± 55 mmol g^{-1} SOC^{-1} hr^{-1}) than in soybean (68 ± 43 mmol g^{-1} SOC^{-1} hr^{-1})(Fig. 2.7). Sampling time was a significant factor in all EEAs measured at this depth (Table 2.4). For all enzymes, midseason 1 had the greatest activity and harvest had the least activity (Fig. 2.8).

Table 2.4. F ratios (F) and p values (p) from a generalized linear mixed effects model for soil samples collected across two growing seasons under a sorghum-wheat-soybean-fallow (SWS) rotation under no tillage (NT). For each depth (i.e., 0-5 cm and 5-10 cm) 128 samples were analyzed (n = 128). N fertilization rate, crop at sampling, and sampling time were considered fixed variables, where plot and year were treated as random variables. Statistical tests were performed on log-transformed data. Significance was defined as $p \leq 0.05$ and significant p-values are in bold.

		Beta		NAG		Phos		Alpha		CBH	
Effect	df	F	p	F	p	F	p	F	p	F	p
0-5 cm											
N Rate	1	0.25	0.627	0.59	0.457	0.12	0.737	0.45	0.514	<0.001	0.990
Crop	1	2.63	0.108	7.43	**0.008**	2.05	0.156	0.38	0.539	1.17	0.283
Sample Time	3	18.0	**<0.001**	18.2	**<0.001**	18.8	**<0.001**	15.6	**<0.001**	16.2	**<0.001**
Crop × Sample Time	3	0.56	0.642	0.45	0.718	0.16	0.922	0.66	0.577	0.52	0.667
N Rate × Sample Time	3	1.96	0.125	1.94	0.128	0.72	0.540	3.68	**0.015**	2.50	0.064
N Rate × Crop	1	0.83	0.366	0.02	0.879	1.94	0.167	0.71	0.403	2.99	0.087
Est. Variance											
Plot		3.22%		0%		0.00%		0.00%		0.00%	
Year		18.68%		8.22%		20.2%		25.60%		19.27%	
Whole Model		$r^2 = 0.452$		$r^2 = 0.386$		$r^2 = 0.398$		$r^2 = 0.381$		$r^2 = 0.406$	
5-10 cm											
N Rate	1	5.89	**0.029**	0.54	0.474	0.01	0.915	3.28	0.092	2.24	0.157
Crop	1	0.23	0.636	0.88	0.349	1.20	0.277	0.01	0.943	0.96	0.329
Sample Time	3	8.76	**<0.001**	13.7	**<0.001**	9.96	**<0.001**	6.21	**0.007**	5.18	**0.002**
Crop × Sample Time	3	0.37	0.775	0.50	0.687	0.85	0.469	1.06	0.370	0.48	0.700
N Rate × Sample Time	3	3.38	**0.021**	3.30	**0.024**	2.02	0.116	1.45	0.233	0.86	0.465
N Rate × Crop	1	<0.001	0.989	0.09	0.771	0.93	0.337	3.64	0.059	0.51	0.478
Est. Variance											
Plot		2.55%		0.10%		0.00%		0.00%		0.75%	
Year		31%		0.00%		9.56%		30.35%		19.22%	
Whole Model		$r^2 = 0.432$		$r^2 = 0.323$		$r^2 = 0.117$		$r^2 = 0.335$		$r^2 = 0.270$	

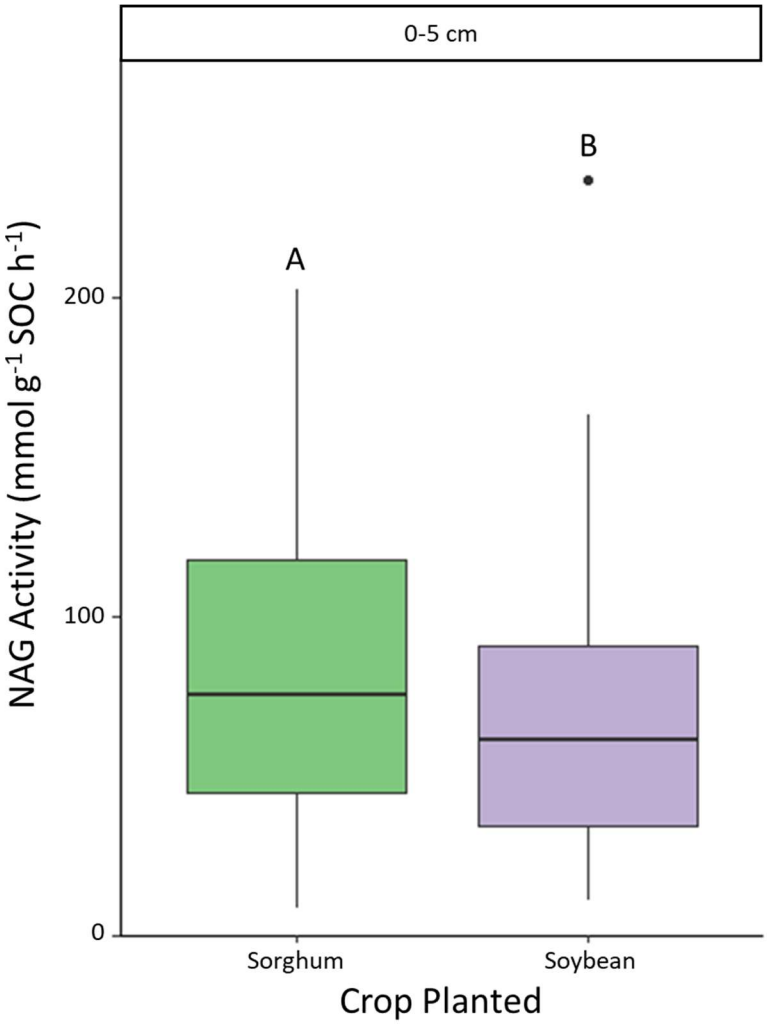

Figure 2.7. Activity of NAG in soil sampled at four time-periods across two years in a sorghum-wheat-soybean-fallow (SWS) crop rotation under no tillage (NT). Data was normalized by SOC. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Letters indicate significant differences as determined by Student's t test.

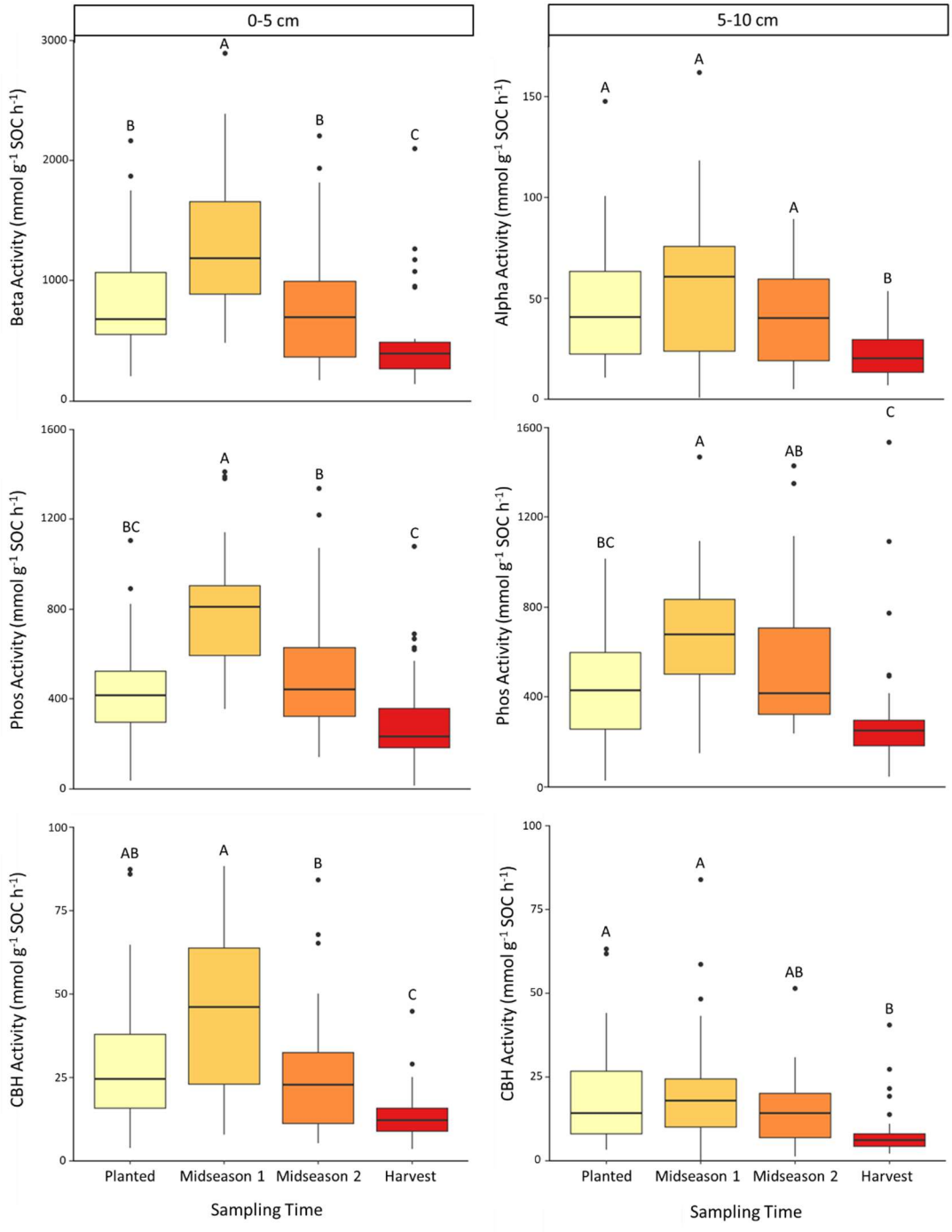

Figure 2.8. Soil extracellular enzyme activities sampled at four time-periods across two years, sampled in a sorghum-wheat-soybean-fallow (SWS) crop rotation under no tillage (NT). Data was normalized by SOC. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Letters indicate significant differences in enzyme activities at sampling times in a given depth as determined by Tukey's HSD.

For soil at 5-10 cm, there was no effect of crop present in the field at the time of sampling on EEAs. Similar to the 0-5 cm depth, all EEAs were affected by sampling time having consistently greater EEA at midseason 1 than at harvest (Fig. 2.8). EEAs at planting and at midseason 2 were comparable to either midseason 1 or harvest or intermediate between the two (Table 2.4, Fig. 2.7). Beta showed an effect of N fertilization rate, and both Beta and NAG had a significant interaction between N fertilization and sampling time (Table 2.4). However, the posthoc mean comparisons did not show direct differences between fertilization rates at any of the sampling times (Fig. A.3). No enzymatic activities were affected by N rate alone.

2.4 Discussion

2.4.1 No Difference in Carbon Pools under Two Rates of Nitrogen Fertilization

Nitrogen fertilization rate did not affect SOC or POxC. Differences in SOC and POxC from fertilization are usually attributed to their effect on biomass (Ghosh et al., 2018; Lu et al., 2011b; Qiu et al., 2020). Although harvest data is not available in this study, increases in yield between these two rates of N fertilization were not observed by Franzluebbers et al. (1995) in the same experimental system. This, in combination with similar soil TN and NH_4-N in both fertilization treatments, suggests that additional fertilizer did not contribute to biomass addition nor soil C and N pools. If crop biomass and C contributions were similar between N rates and soil N levels were unchanged by fertilization rate at the scale measured, then microbial C cycling would remain unchanged. If biomass concentrations did increase due to the higher rate of N fertilization, it is possible the lack of increase in SOC is caused by an upper limit of SOC storage for this soil (Six et al., 2002). If this soil has an upper limit to physical SOC storage, then any excess C inputs due to increased N rate could be lost to microbial degradation and respiration instead of being stored. Thus, even if the low N rate plots were accumulating SOC more slowly

than the high-rate plots, they would eventually reach the same concentration of stored C. These plots have been under the same N fertilization rates for more than 35 years; therefore, if a SOC storage limit exists within this management program, the system may have reached that limit.

While unaffected by N fertilization rate, the crop present during sampling influenced SOC in this study with greater SOC under soybean than sorghum (Fig. 2.5). This may be a reflection of the crop present at the time of sampling, but it also may reflect differences in the winter phase of the rotation. During the winter, wheat consistently preceeded soybean, while fallow preceeded sorghum. The wheat phase of the rotation may contribute additional SOC to the system, and/or the fallow period may result in SOC losses. Since the crops were rotated yearly, this indicates a 0.1% yearly fluctuation in SOC with the phase of the rotation. The crop present at sampling has been reported to affect SOC in these plots previously (Wright et al., 2007) and is likely due to differences in plant physiology including root structure and metabolic tendencies (King and Blesh, 2018), combined with microbial decomposition of differing plant residues. There is a large difference in how much biomass is produced, and thus C deposited, between soybean (5.81 Mg ha^{-1}) and sorghum (15.59 Mg ha^{-1}; Cabelguenne et al., 1999). The C to N ratio of a plant is also a significant factor in how microbes decompose residues (Bending and Turner, 1999), and soybean has a much lower C to N ratio (9.5:1) than sorghum (31:1; Finney et al., 2016; Lynch et al, 2016). Decomposition of residues takes time. Residues from soybean are likely degrading during sorghum production, and sorghum residues degrade during soybean production. However, the most likely explanation is that the wheat residue, which only precedes soybean in the rotation, elevated SOC slightly, which disappears during the fallow that precedes sorghum. Fallow seasons are known to reduce SOC (Burke et al., 2019), particularly in a warm

climate such as Central Texas where winter temperatures are frequently sufficiently warm for microbial activity.

Sampling time also affected C pools with greater POxC at planting than midseason 2 at 0-5 cm (Fig. 2.6). The decrease in POxC from planting to the most active growth period of soybean and sorghum contrasts with other studies. In a NT system with winter cover crops, Burke et al. (2019) found temporal variation in the Texas High Plains and reported the greatest POxC concentrations during the active growth stages of cotton. Culman et al. (2013) also found a pattern of increasing POxC as corn matured. In their NT cover cropped system, residues from the previous crop were retained at the soil surface and were decomposing across the growing season, which is consistent with our study. The decreased POxC over the season may reflect microbial enzymatic activity, breaking down residues and respiring C, removing it from the soil. This explanation is supported by the EEA data: greater EEA in midseason 1 is followed by reduced POxC at midseason 2. There was no effect of crop on POxC, but this may be due to the quick biomass production of sorghum masking the lack of wheat residues that are found in the soybean phase of the rotation.

2.4.2 Sampling Time Drives Extracellular Enzymatic Activities

Microbial EEA can provide a snapshot of potential microbial activity in soil C and N cycling (Burns et al., 2013). β-glucosidase (Beta), α-glucosidase (Alpha), and cellobiohydrolase (CBH) are C-cycling enzymes, breaking down cellulose and starch. N-acetyl glucosaminidase (NAG) activity reflects potential chitin decomposition, and phosphatase (Phos) breaks phosphate groups from organic phosphorus into microbially accessible phosphate. Every EEA measured was significantly affected by sampling time at both depths (Table 2.4). In general, EEAs were either relatively consistent between planting, midseason 1, and midseason 2 (e.g., Beta, Alpha,

and CBH at 5-10 cm) or were maximal at midseason 1 (e.g., Beta, NAG, Phos at 0-5 cm). The peak of potential EEA at midseason 1 sampling time may reflect the point of maximal residue decomposition from the previous phase (i.e., wheat or weeds in fallow), which explains why the pool of active C (i.e., POxC) was lowest at midseason 1. At harvest, POxC may begin to increase as crop residues begin to accumulate. All five EEA measurements were lowest at the harvest sampling time at 5-10 cm, and there was a corresponding increase in SOC. The decrease in EEA may reflect a reduction in the decomposition that leads to SOC loss through respiration (Burns et al., 2013). There is an abundance of data on temporal variations in soil biological metrics, including extracellular enzymatic activity (Debosz et al., 1999; Piotrowska-Dlugosz et al., 2016; Hsiao et al., 2019), POxC (Burke et al., 2019), and NH_4-N (Cain et al., 1999; Riaz et al., 2010). These dynamics are often attributed to seasonal variations in temperature, moisture, and crop growth stage. All of these are likely influencing the dynamics observed in this study.

The crop present at the time of sampling only affected the activity of NAG in the 0-5 cm depth with NAG in sorghum having a greater activity (122 ± 85 mmol g^{-1} SOC^{-1} hr^{-1}) than in soybean (100 ± 67 mmol g^{-1} SOC^{-1} hr^{-1})(Fig. 2.7). This agrees with current research indicating a reduction in NAG activity under soybean following winter wheat harvest (Hsiao et al., 2019) and could be due to a slower decomposition rate of wheat residues under soybean and lack of winter crop preceding sorghum. The N cycle could also be less dynamic under the soybean crop due to a lack of N fertilizer applied and lower crop demand for N from the soil.

2.5 Conclusion

Soil C and N cycling were not affected by long-term (38 years) N fertilization rate, likely due to a lack of difference in plant biomass between the two rates. However, seasonal dynamics and crop effects were observed in C and N pools that provide insight into C and N cycling. The

effect of sampling time on EEAs was especially clear and emphasized the temporal aspect of soil nutrient processes as driven by local environmental factors and plant growth. Future studies should continue to employ repeated samplings across time and, ideally, sample across multiple years to establish a better picture of long-term EEA dynamics. The crop present at sampling had significant effects on SOC and the activity of NAG, a N cycling enzyme. The winter wheat crop that preceded soybean likely contributed to these effects, but physiological differences between soybean and sorghum may also contribute. Future studies could explore the influence of crop type (i.e., legume or grass) on enzymatic activities *in situ*, especially in reference to N cycling. Continued study of soil C and N cycling is crucial to the maintenance of world food and fiber production, as well as soil health, to benefit all those who rely on soil and that which it provides.

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3. AGRICULTURAL SOIL CARBON POOLS: EFFECTS OF NITROGEN FERTILIZATION RATE AT DEPTH

3.1 Introduction

Long-term C storage is at the center of climate change mitigation and agricultural soil health management. Soil organic C (SOC) is a large pool of C with over 1500 Pg stored in the top meter of soil around the world (Jobbagy and Jackson, 2000). Through biological and chemical processes that are driven by soil microbes, more than 15,000,000 km^2 of agricultural land around the world (Erb et al., 2007) has the potential to be a sink for anthropogenic atmospheric C (Lal, 1997). The opportunity for atmospheric C storage is even greater in soils depleted in SOC due to poor soil management practices. Maintenance of SOC is crucial for soil health management because improved crop production is associated with increases in SOC (Gami et al., 2009). While most studies of SOC focus on the top 20-50 cm of soil, recent research indicates that a more complete picture of the soil profile is needed to understand soil C dynamics (Balesdent et al., 2018; Jobbagy and Jackson, 2000; Lal, 2018). Nitrogen fertilization is ubiquitous in agricultural systems, and it is a possible driver of increasing SOC storage at depth (Mujuru et al., 2016). Crop rotation is another possible tool to improve SOC storage through changes in N availability (Osanai et al., 2020). But the literature is inconclusive on how long-term N fertilization in combination with crop rotation affects SOC at depth, particularly under no-tillage (NT) management (Dolan et al., 2006).

Fertilization is used in agriculture to improve crop yield and sustain long-term production. From 1980 to 2009, the largest component of global net anthropogenic N input was N fertilizer consumption, which was up to 743 kg N km^{-2} y^{-1} averaged across the entire terrestrial surface (Han et al., 2020). While it is foundational to maintaining world food production, N

fertilization does have the potential to negatively impact the environment through water contamination and eutrophication (Liu et al., 2015), and by accelerating rates of N trace gas emissions. However, N pollution can be managed by reducing fertilization rates, targeting applications, and careful timing of fertilizers (Diacono et al., 2013). It is possible that N fertilization could increase SOC through improved root biomass and litter deposition or reduced microbial decomposition (Ramirez et al., 2012; Ghosh et al., 2018). This would make N fertilization a possible strategy for reducing atmospheric C through sequestration in soil.

Fertilization increases SOC stored in the soil profile at depth primarily due to contributions to root biomass and eluviation of SOC through the soil profile (Ghosh et al., 2018). The soil benefits from stored SOC through improved water infiltration, aggregation, microbial activity, and biodegradation of pollutants (Blanco-Canqui et al., 2013). Because most biological activity is concentrated at the surface of soil in the aerobic zone, up to 65% of SOC is found in the top 100 cm of soil (Jobbagy and Jackson, 2000). If surface soil is the only pool of SOC considered, profile-wide storage potential and dynamics could easily be missed. In a study by Mujuru et al. (2016), an increase in SOC and total N was observed up to 50 cm depth after 9 years of N fertilization using conventional tillage in both a sandy soil and a clayey soil, potentially due to biomass increases. Sampling up to 120 cm depth, Stewart et al. (2017) found SOC was increased or better retained in the top 30 cm of soil after 6 years of N fertilization under NT, which was attributed to increased residues and root matter from crops. A meta-analysis of 65 studies found consistent increases in SOC in agricultural systems due to N fertilization, as compared to controls without N additions, and attributed these increases to reduced microbial decomposition activity (Jian et al., 2016). Across varied soil types, Jian et al. (2016) attributed reduced decomposition to a reduction in enzymatic breakdown of complex and

recalcitrant lignin compounds that make up a high percentage (≥15%) of wheat and sorghum residues (Tamaki and Mazza, 2011; Wahyuni et al., 2019).

In contrast, Dolan et al. (2006) found no significant differences in SOC due to fertilization after 25 years of N additions, even under NT. While there were differences at individual depths up to 45 cm, Dolan et al. (2006) showed that these were canceled out when examining the entire profile. The importance of sampling a larger portion of the profile was emphasized by this study, noting that differences would have been reported if the entire profile had not been considered (Dolan et al., 2006). Another long-term study found no changes in SOC due to N fertilization after 22 years of cropping and tillage as compared to a control attributing the stability of SOC to a compensatory increase in microbial activity (Lou et al., 2011).

It is also possible that N fertilization ultimately decreases stored SOC under agricultural production. Zhong et al. (2015) reported decreases in SOC up to 120 cm depth under their highest rate of N fertilization (360 kg urea ha^{-1}) following 9 years of N fertilization in an experiment under a tilled wheat cropping system. The effect of N fertilization on SOC may not be linear, instead increasing up to a threshold and then decreasing based on contribution of N to additional biomass or to microbial degradation. This trend would suggest that an optimal N fertilization rate, based on plant N needs, is critical to evaluating SOC storage potential (Zhong et al., 2015; Singh, 2018). In a comprehensive meta-analysis by Alvarez (2005) including 137 studies, an increase in SOC was reported under N fertilization only when crop residues were returned to the soil surface. Alvarez et al. (2005) also noted that sites with a higher mean annual temperature and finer soil texture had sequestered less SOC, emphasizing the effect of individual soil properties on SOC storage potential.

Management practices, such as tillage and crop rotation, can also influence SOC dynamics. Tillage can affect SOC storage and can have different effects at different depths (Deen and Kataki, 2003). Olson and Al-Kaisi (2015) found significant differences between SOC storage under NT as compared to moldboard plow tillage, and different patterns of storage at depth. Under tillage, SOC was lost from 0-20 cm but held constant from 20-75 cm, where the NT plots stored SOC at 0-5 cm and lost SOC from 5-35 cm (Olson and Al-Kaisi, 2015). With such large potential differences between C storage under CT and NT systems, SOC studies must consider tillage management. However, studies of the effects of N fertilization on SOC storage under NT are limited. Crop rotation can also affect SOC, with increased crop intensity resulting in increased SOC storage due to greater biomass inputs into soil (Mazzoncini et al., 2011; Nyambo et al., 2020). McDaniel et al. (2014) reported that SOC increased with increasing N fertilization rate and with increasing number of rotated crops. In a long-term rotation and fertilization trial in Syria, researchers found increased SOC under a tilled crop rotation as compared to no rotation and with N fertilization as compared to no N additions (Ryan et al., 2008). Ryan et al. (2008) reported SOC data for the top 20 cm of soil but found no interactions between N fertilization and crop rotation. Similarly, Jagadamma et al. (2007) showed increases in SOC up to 30 cm depth due to N fertilization as compared to a control without fertilization. In contrast to Ryan et al. (2008), Jagadamma et al. (2007) also reported that continuous corn stored more SOC than a rotation of corn and soybean. While generally crop rotations are understood to improve soil health and SOC through year-round residue inputs (McDaniel et al., 2014), results from the study by Jagadamma et al. (2007) suggest that further investigation is needed.

The overall aim of this study was to quantify effects of long-term (>35 years) N fertilization on soil C and N pools and SOC storage down to 1 m depth under rotated and

monoculture wheat production under NT management. It is hypothesized that increased N fertilization consistently improved biomass production over the long-term study resulting in an increase in SOC pools at all depths up to 1 m. Crop rotation is hypothesized to increase stored SOC at depth as compared to the wheat monoculture due to consistent, rather than seasonal, biomass additions.

3.2 Materials and Methods

3.2.1 Experimental Plots

This experiment was conducted in south-central Texas on a Belk clay (fine, mixed, active, thermic, Entic Hapluderts). The site is in the Brazos River floodplain at a long-term management site established in 1982 at the Texas A&M Research Farm (30°32'N 96°26'W). Four replicates of four N treatments are in a randomized complete block design, and this is replicated in its entirety by crop system and by tillage treatment. Two crop systems were assessed: a rotation of sorghum (*Sorghum bicolor*) – wheat (*Triticum aestivum*) – soybean (*Glycine max*) – fallow (SWS) and a monoculture of wheat (MONO). Both systems were produced under no tillage (NT) or conventional tillage (CT); however, only NT systems were evaluated. The systems were fertilized at four different N rates during the sorghum and wheat phases. Fertilization occurred on 17 Feb 2020 as urea broadcast at the surface of the soil. The fertilization rates were classified as no, low, medium, and high and were 0, 45, 90, and 135 kg N ha^{-1} for sorghum and 0, 34, 68, and 102 kg N ha^{-1} for wheat, respectively. Soybean did not receive N fertilizer. High N (i.e., 102 and 135 kg N ha^{-1}) and low N (i.e., 34 and 45 kg N ha^{-1}) rates of fertilization with NT wheat established in either a rotated or monoculture system were chosen for this study.

3.2.2 Sampling

Two types of soil cores were taken on 20 May 2020 after the winter wheat harvest in both cropping systems. These were collected with a truck–mounted Giddings hydraulic probe (Giddings Machine Company, CO). For soil chemical analysis, 1-m cores were taken to assess soil C and N storage at depth. For each plot, three 5-cm diameter cores were collected, separated into sections by depth (0-5, 5-10, 10-15, 15-30, 30-60, 60-100 cm), and composited by depth in the field. Soil samples were air-dried and ground to pass a 2-mm sieve.

To measure soil bulk density (BD), one 7.6-cm diameter core was collected in each plot to 1-m depth. These cores were preserved in plastic tubes and taken to the lab to be divided into sections by depth (0-5, 5-10, 10-15, 15-30, 30-60, 60-100 cm). Bulk density was calculated by dividing the weight of the soil (oven-dried at 105 ˚C) by the volume of each depth section. Soil C stocks were calculated by converting SOC concentration (%) to SOC mass per hectare using soil BD and sample depth.

3.2.3 Analytical Methods

Soil N and C pools were analyzed to assess the impacts of long-term N fertilization on SOC at depth. Nitrogen pools measured included total N (TN) and ammonium (NH_4-N) in the soil. Total N was assessed using combustion elemental analysis with the Vario El Cube CHNS Analyzer (Elementar, Ronkonkoma, NY, USA). For each sample, 35-40 mg soil were fine ground and completely combusted to gaseous forms, which were quantified by gas chromotography. To measure NH_4-N concentration, air-dried soils were extracted with 2 M potassium chloride in a 1:10 ratio. The extractants were analyzed using the Berthelot reaction, in which indophenol blue is formed in the presence of ammonium in solution. The concentration of indophenol blue was assessed using spectrophotometry and compared to a standard curve ($r^2 \geq 0.95$; Sims et al., 1995). To quantify soil C pools, total C (TC), soil inorganic C (IC), SOC, and

active C (POxC) were measured. Soil TC was assessed by total combustion analysis as described above. The pressure calcimeter method was used to determine IC, where 1 g soil was reacted with hydrochloric acid and carbon dioxide evolution was measured (Sherrod et al., 2002). Soil organic C was calculated as the difference between TC and IC. Permanganate oxidizable C (i.e., active C) was measured using a colorimetric method (Culman et al., 2012). In this method, 2.5 g soil were reacted with a permanganate solution to assess oxidation. The color was quantified spectrophotometrically (Synergy H1, Biotek) and compared to a standard curve ($r^2 \geq 0.95$). The equation described by Culman et al. (2012) was used to convert the absorbance values to mg kg^{-1} of POxC in soil.

3.2.4 Data Analysis

The analysis of factors influencing soil C and N was performed with a linear regression mixed effects model with the restricted maximum likelihood (REML) method in JMP version 15 (SAS Institute Inc.). This method was chosen as it is well suited to analysis with a small sample size and is a common method for estimating parameters in linear mixed models (Harville, 2021). Crop system, N fertilization rate, and depth were considered fixed effects, while plot number was assessed as a random effect. Tukey's honest significant differences was used for posthoc mean comparisons. While the original experimental design includes blocks, these did not have a significant effect on metrics assessed and thus were not considered in analysis.

3.3 Results and Discussion

3.3.1 Bulk Density Affected by Depth and Nitrogen Fertilization Rate

Soil BD was affected by N fertilization rate ($p = 0.023$) and by depth ($p < 0.001$) (Table 3.1; Fig. 3.1); however, there was no interaction with any of the main effects evaluated. Soil BD

was less under high N fertilization (1.35 ± 0.16 g cm^{-1}) than the low N rate (1.41 ± 0.19 g cm^{-1}; Fig. 3.1 A). Decreases in BD with mineral fertilization have been reported before and are typically attributed to improved root growth and biomass additions to the soil that lead to increased SOC (Li et al., 2018; Lin et al., 2019; Lan et al., 2020). Halvorson et al. (2002) also reported decreasing BD under increasing N addition in the topsoil of a NT cropping system, attributing the changes to increased surface SOC.

Table 3.1 F ratios (F) and p-values (p) for carbon metrics from a generalized linear mixed effects model for soil samples collected under a sorghum-wheat-soybean-fallow rotation (SWS) and a monoculture of wheat (MONO). Samples were collected to 1 m depth in 2020 and analyzed in increments (i.e., 0-5, 5-10, 10-15, 15-30, 30-60, 60-100 cm). Nitrogen fertilization rate (N rate), cropping system (Crop), and depth were considered fixed variables. Significance was defined as $p \leq 0.05$.

		Bulk Density		**Active C**		**SOC**		**SOC Stocks**	
Effect	**df**	F	p	F	p	F	p	F	p
N Rate	1	7.01	**0.02**	12.8	**0.004**	5.38	**0.04**	0.97	0.35
Crop	1	2.46	0.14	10.4	**0.01**	7.84	**0.02**	0.01	0.92
Depth	5	36.5	**<0.0001**	112	**<0.0001**	295	**<0.0001**	10.9	**<0.0001**
N Rate × Crop	1	0.05	0.82	3.04	0.11	2.25	0.16	2.66	0.13
Depth × N Rate	5	0.78	0.57	2.42	**0.05**	1.96	0.10	0.34	0.89
Crop × Depth	5	0.79	0.56	5.00	**0.001**	14.4	**<0.0001**	2.93	**0.02**
Depth × Crop × Depth	5	1.24	0.30	1.60	0.17	1.80	0.13	1.39	0.24
Model Fit (r^2)		0.73		0.90		0.96		0.65	

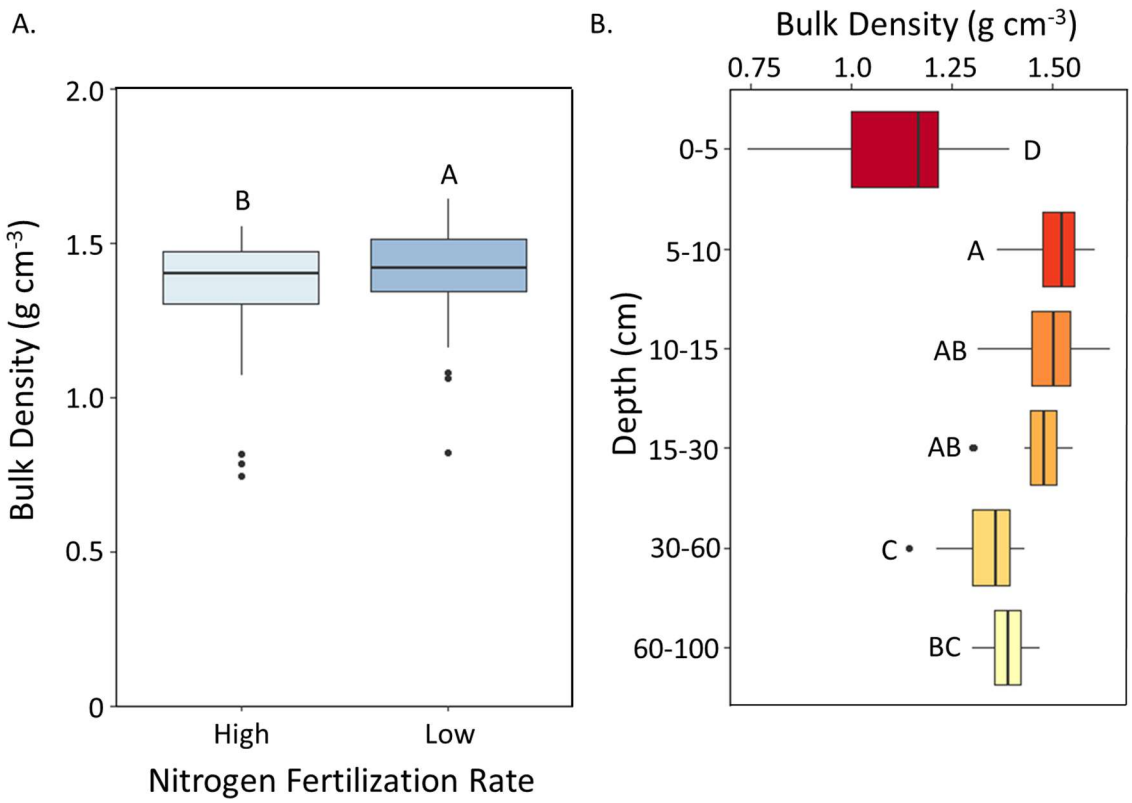

Figure 3.1. Bulk density for soil sampled following harvest of wheat in both a rotated (SWS) and monoculture (MONO) cropping system evaluated in 2020 following more than 35 years of consistent management. A. High and low nitrogen fertilization rates are indicated by color, and letters indicate significant differences as determined by the Student's t test. Error bars indicate +/- standard deviation. B. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Letters indicate significant differences as determined by Tukey's HSD.

Soil BD was lowest at 0-5 cm and greatest in the 5-30 cm depths (Fig. 3.1 B). The greater SOC at the surface (Section 3.3.2) is likely responsible for the much lower soil BD at the surface (Rawls, 1982), as there was not a significant texture difference between 0-5 and 10-15 cm. Select samples were analyzed for soil texture, and the average clay content at 0-5 cm was 47.5 ± 2.1%, where the average at 10-15 cm was 52.7 ± 4.3 % (data not shown). Variability in BD was high at 0-5 cm compared to all other depths, as surface soils are particularly subject to spatial environmental factors (i.e., extremely local compaction from equipment or workers in the field).

3.3.2 Additional Nitrogen Increases Soil Organic Carbon in Surface Soil

Soil organic C was influenced by N fertilization rate and an interaction between cropping system and depth (Table 3.1). Overall, SOC was greater under the high N fertilization rate (0.61 ± 0.45 %) compared to the low fertilization rate (0.52 ± 0.42 %, Fig. 3.2 A). This supports the hypothesis that SOC increases with additional N. Increases in SOC due to greater N fertilization could be attributed to improved crop residue inputs (Stewart et al., 2017). In the SWS system yields were historically greater under high N fertilization rate compared to no N but not statistically different from the low N rate (Franzluebbers et al., 1995). Trends have continued in recent years, although no yield data was available for evaluation in the years of this study. Residues may have increased or reduced microbial degradation of lignin compounds could be contributing to increased SOC, as described by Jian et al. (2016).

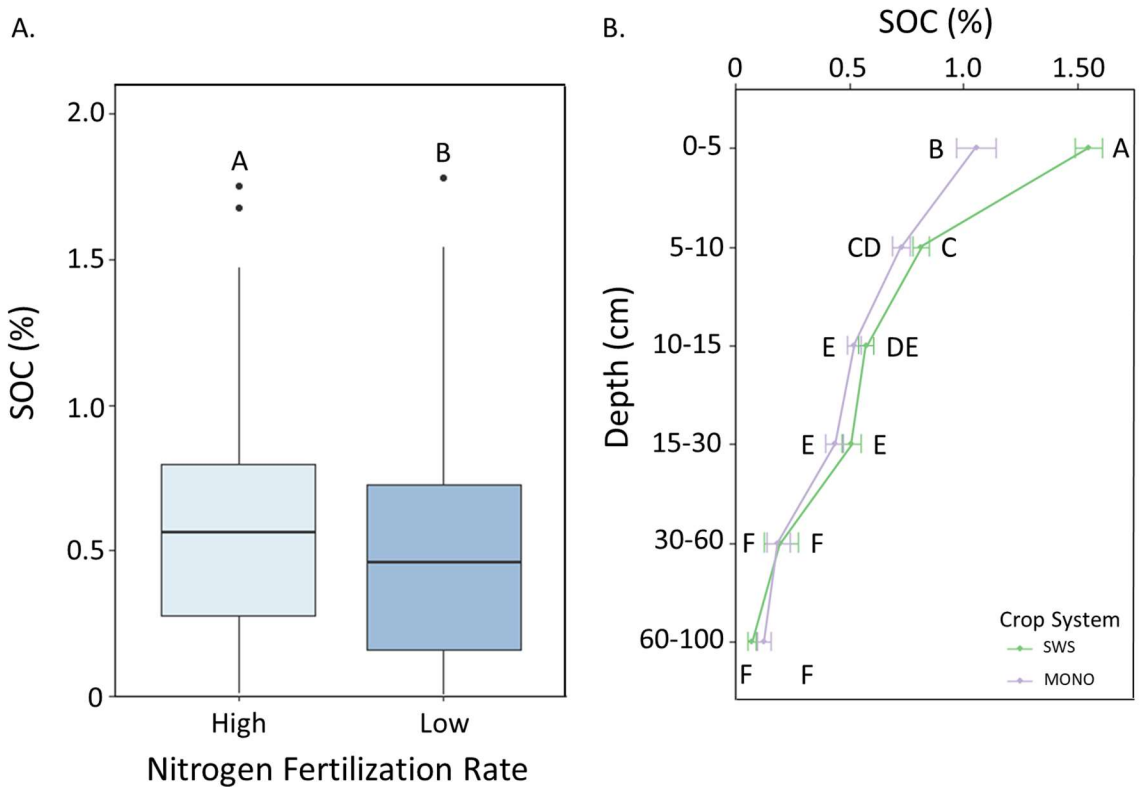

Figure 3.2. Soil organic carbon concentration (SOC) for soil sampled following harvest of wheat from a rotation (SWS) or monoculture (MONO) system under two nitrogen fertilization rates. These were evaluated in 2020 following more than 35 years of consistent management. A. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Differing letters indicate significant differences as determined by Student's t test. B. Error bars indicate +/- standard error and differing letters indicate a significant difference as determined by Tukey's HSD.

An interactive effect of crop system and depth on SOC was identified. At 0-5 cm, greater SOC was found under SWS (1.50 ± 0.17 %) than under MONO (1.06 ± 0.24 %), and SOC decreased with increasing soil depth (Fig. 3.2 B). Previous studies in this same experimental system also found SOC was up to 22% greater in the SWS rotation than in MONO (Franzluebbers et al., 1994). The consistent presence of crops, and thus biomass inputs, in the SWS may be responsible for this additional SOC as the fallow period compared to the MONO system. Many studies have reported increased SOC under crop rotation as compared to

monocropping with a fallow between growing seasons (McDaniel et al. 2014; Nyambo et al., 2020; Ryan et al., 2008), and biological metrics are commonly found to decrease with depth. Since this experiment is under NT, residue inputs remain concentrated at the soil surface (0-5 cm) and may be less likely to affect the subsurface, resulting in consistent SOC concentrations at depth (Stewart et al., 2017). There was notable variability in SOC under SWS as compared to MONO at all depths, with standard deviation of 0.51 and 0.35%, respectively, that could be attributed to root morphology, contribution of N from soybean, and profile mixing in the rotation (Kulmatiski and Kardol, 2008). A limitation of this dataset is that there are only 4 data points that represent one combination of cropping system and N fertilization rate, and while each data point was a composite of 3 samples, this may have influenced some results.

Calculated SOC stocks to 1 m depth were variable with a significant effect of depth, and an interaction between depth and cropping system (Table 3.1; Fig. 3.3). At 0-5 cm, SWS had a greater stock of SOC (86,144 ± 21,707 kg C ha^{-1}) than MONO (56,370 ± 15,393 kg C ha^{-1}). Many studies have found greater SOC under crop rotations (McDaniel et al. 2014; Nyambo et al., 2020; Ryan et al., 2008), but these only sampled to 30 cm or less. Since soil BD is used to calculate SOC stocks, variability in that metric can also affect these results. Greater BD causes larger calculated SOC stocks, and BD at 0-5 was highly variable (Fig. 3.1 B). However, the differences observed here could also be linked to texture changes that were observed in the soil profiles at depth. Zinn et al. (2007) and Needelman et al. (1999) found that soil texture and minerology are significant factors in how SOC is stored. This experiment also highlights the importance of considering the soil profile as a whole – the C stocks from 30-100 cm accounted for more than 30% of the total C stocks measured in the study.

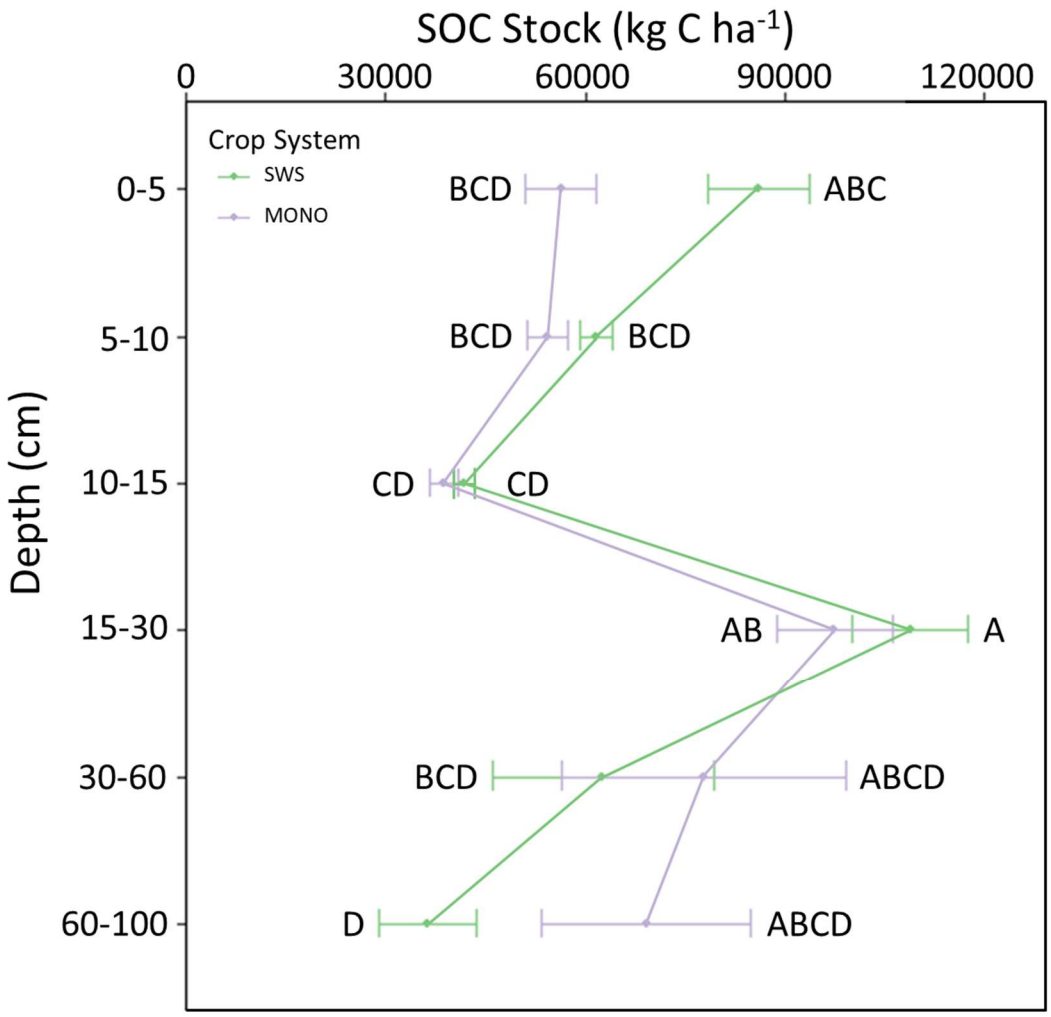

Figure 3.3. Soil organic carbon (SOC) stock for soil sampled following harvest of wheat from a rotation (SWS) or monoculture (MONO) system following more than 35 years of consistent management. These were evaluated in 2020 under two nitrogen fertilization rates. Error bars indicate +/- standard error and letters indicate significant differences in SOC stocks as determined by Tukey's HSD.

Active C, as measured by POxC, responded to N fertilization rate, crop rotation, and depth, but 2-way interactions with depth were observed (Table 3.1). Concentrations of POxC decreased with depth, which was expected and consistent with current literature (Jagadamma et al., 2019). The concentration of POxC was greater under the high rate of N fertilization (373 ± 61 mg kg^{-1}) than under the low rate (278 ± 103 mg kg^{-1}) but only at 0-5 cm (Fig. 3.4 A). This agrees with the hypothesized increases in active C due to increased N fertilization rate and follows the pattern of SOC being affected only in the surface soil, particularly in a NT system. Similarly, the effect of crop rotation was only observed 0-5 cm, with greater POxC under SWS (389 ± 51 mg kg^{-1}) than under MONO (261 ± 87 mg kg^{-1}; Fig. 3.4 B). It is not surprising that the SWS rotation would have greater C as crops are continually in the field, except in alternate winters, while the MONO is fallow every summer (~5-6 mo y^{-1}). This agrees with current literature, as Sprunger et al. (2020) reported that even in systems with the same cropping intensity, diversifying the cropping system increased active C. Liu et al. (2006) also found that crop rotation systems have greater SOC stocks than monocultures. Increases in POxC and SOC have been reported under N fertilization and attributed to improved plant growth and production (Mi et al., 2016). Known to be sensitive to management, POxC can provide a picture of the labile pool of C in soil (Culman et al., 2012).

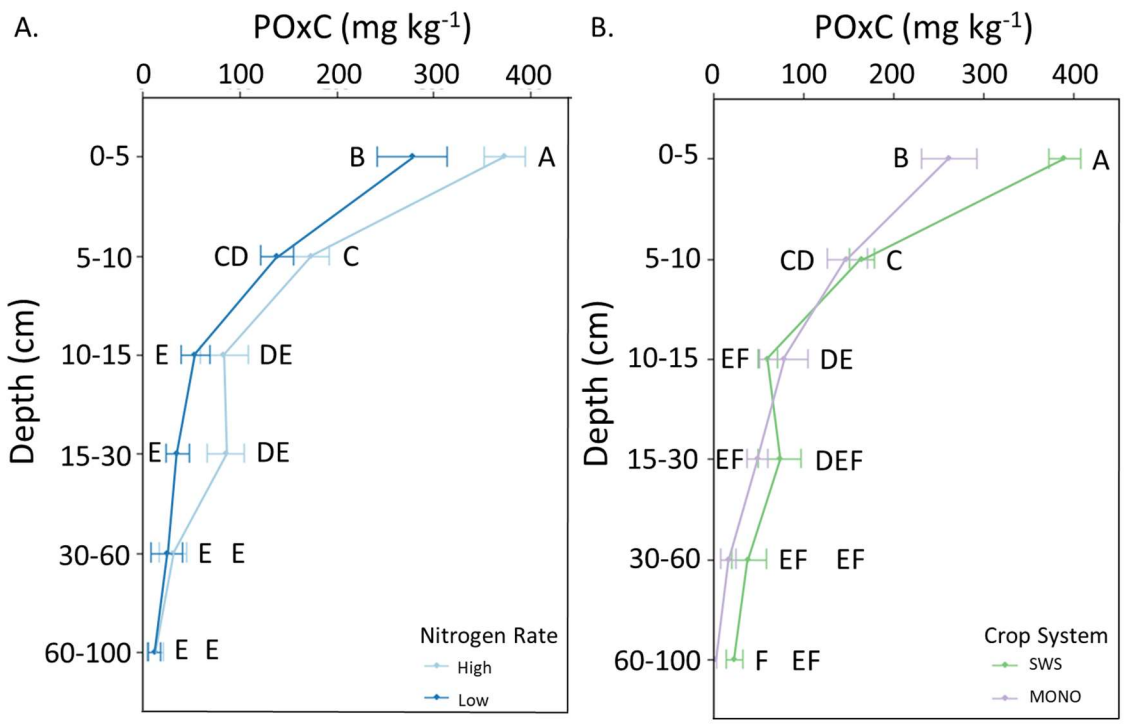

Figure 3.4 Permanganate oxidizable carbon (POxC) concentration for soil sampled following harvest of wheat from a rotation (SWS) or monoculture (MONO) system under two nitrogen fertilization rates. These were evaluated in 2020 following more than 35 years of consistent management. Error bars indicate +/- standard error. A. POxC concentrations by depth and N rate. B. POxC by depth and crop rotation. Colors indicate treatments and differing letters indicate a significant difference as determined by Tukey's HSD.

3.3.3 Soil Nitrogen Pools Unaffected by Fertilization Rate

There were no main effects of N fertilization rate on soil TN or NH_4-N, but both showed an interactive effect of depth by cropping system (Table 3.2; Fig. 3.5). Total N was greater in SWS (0.165 ± 0.012 %) than in MONO (0.119 ± 0.024 %) at 0-5 cm. The TN for all samples ranged from 0.015 to 0.186% and was comparable to ranges reported by Gami et al. (2009) and Halvorson et al. (1995), which were 0.015 to 0.2 % and 0.05 to 0.146%, respectively. The decrease of soil TN with depth observed here appears to be linear and is like that observed by Zhang et al. (2016) in their exploration of soil N in northern China.

Table 3.2. F ratios (F) and p-values (p) for nitrogen metrics from a generalized linear mixed effects model for soil samples collected under a sorghum-wheat-soybean-fallow rotation and a monoculture of wheat. Samples were collected to 1 m depth in 2020 and analyzed in increments (i.e., 0-5, 5-10, 10-15, 15-30, 30-60, 60-100 cm). N fertilization rate, crop at sampling, and depth were considered fixed variables. Significance was defined as $p \leq 0.05$.

		TN		**NH_4-N**	
Effect	df	F	p	F	p
N Rate	1	3.10	0.10	1.76	0.21
Crop	1	4.46	0.06	0.80	0.39
Depth	5	226	**<0.0001**	27.3	**<0.0001**
N Rate × Crop	1	2.03	0.18	2.12	0.17
Depth × N Rate	5	2.19	0.07	0.90	0.49
Crop × Depth	5	11.7	**<0.0001**	3.25	**0.01**
Depth × Crop × Depth	5	1.34	0.26	0.91	0.48
Model Fit (r^2)		0.96		0.79	

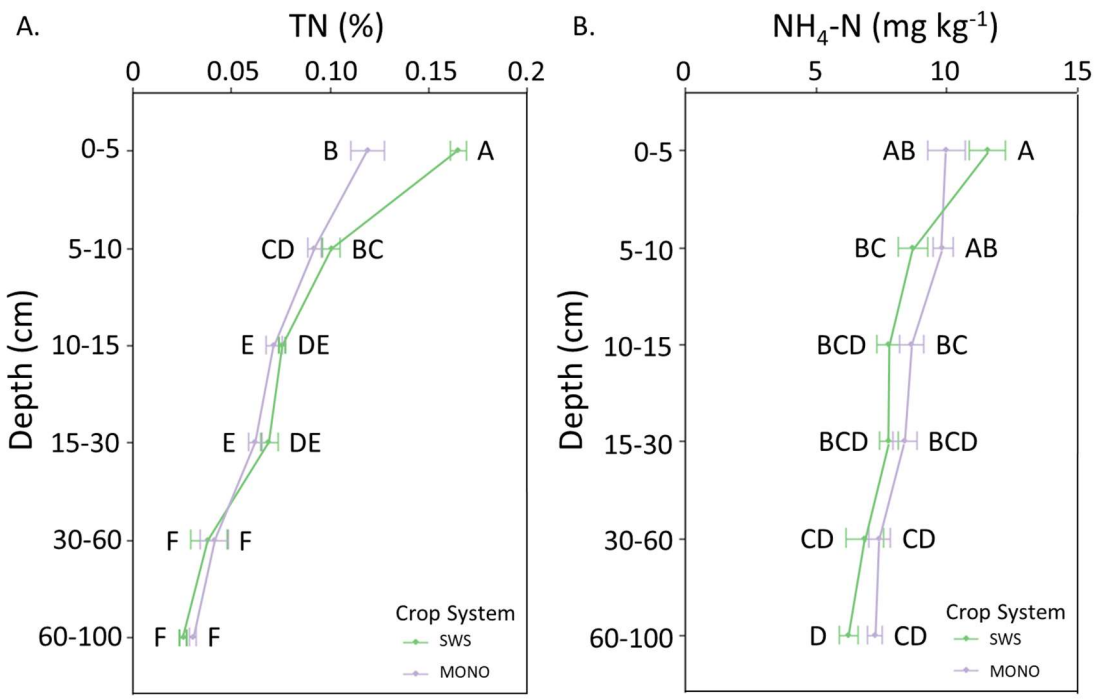

Figure 3.5. Total nitrogen (TN) and ammonium (NH_4-N) concentrations by depth for soil sampled following harvest of wheat in a rotated (SWS) and monoculture (MONO) cropping system evaluated in 2020 following more than 35 years of consistent management. Error bars indicate +/- standard error, colors indicate crop system and differing letters indicate a significant difference as determined by Tukey's HSD.

Comparable to TN, soil NH_4-N showed an interactive effect between cropping system and depth. The concentration of NH_4-N decreased with depth, and while Tukey's HSD did not reveal any differences between cropping systems at a particular depth, the Student's t test indicated that SWS had greater NH_4-N (11.6 ± 1.9 g kg^{-1}) than MONO (10.0 ± 2.02 g kg^{-1}) at 0-5 cm ($p = 0.0116$). The research plots used in this study are known to be N limited for agricultural production, but soil NH_4-N contents for these agricultural plots were comparable to those reported in native semi-arid Texas sites by Geesing et al. (2000). Management, minerology, and soil type influence soil NH_4-N, which is particularly dynamic in agroecosystems (Juang et al., 2001).

3.4 Conclusion

Between the two N fertilization rates, differences in biologically linked metrics (e.g., POxC and EEA) were generally limited to the 0-5 cm depth. The increase of SOC due to the higher N fertilization rate was notable, specifically at 0-5 cm (0.2%). Even these small gains in SOC can significantly improve soil function and health, and could be a tool in restoring soil function, especially in coarse-textured soils (Rawls et al, 2003). The increase of POxC at 0-5 cm due to the higher rate of N also suggests improved soil health, as it can be reflective of biologically active C. Soil BD was decreased under the higher rate of N fertilization, likely due to increased plant biomass (Li et al., 2018; Lin et al., 2019; Lan et al., 2020), again emphasizing the value of fertilization in reducing crop nutrient limitations. While increasing N fertilization to improve long-term C storage is not supported by this data, it is clear that N fertilization plays an important role in maintaining crop health and production.

The SWS cropping system was hypothesized to have greater SOC storage than MONO, which was confirmed in this study. This also translated into increased POxC, TN, and NH_4-N under SWS compared to MONO. However, the effects were confined to the 0-5 cm depth. This agrees with previous studies (Mazzoncini et al., 2011; McDaniel et al. 2014; Nyambo et al., 2020; Ryan et al., 2008) and further establishes crop rotation as an important tool in maintaining and building long-term soil health.

Depth was a factor in every metric assessed, and the NT management likely contributes to the concentration of C and N metrics at the soil surface and distribution in the soil column. As NT is increasingly being recommended for improving soil health (Nunes et al., 2020), it is crucial that land managers understand how this practice can impact C storage.

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APPENDIX A

Table A.1. P values (p) from a generalized linear mixed effects model for soil samples collected across two growing seasons under a sorghum-wheat-soybean-fallow rotation. For each depth (i.e., 0-5 cm and 5-10 cm) 128 samples were analyzed (n = 128). N fertilization rate, crop at sampling, and sampling time were considered fixed variables, where plot and year were treated as random variables. Three-way interactions were accounted for. Significance was defined as $p \leq 0.05$ and significant p-values are bold.

Effects	**df**	TN	NH_4-N	SOC	POxC	Beta	Nag	Phos	Alpha	CBH
0-5 cm										
N Rate	1	0.66	0.76	0.74	0.72	0.63	0.47	0.77	0.51	0.99
Crop	1	0.37	0.13	**0.01**	0.74	0.11	**0.01**	0.13	0.53	0.28
Sample Time	3	0.83	**<0.0001**	0.11	**0.05**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Crop x Sample Time	3	0.87	**0.002**	0.84	0.29	0.65	0.72	0.90	0.58	0.65
N Rate × Sample Time	3	0.06	1.00	0.08	0.34	0.12	0.13	0.48	**0.02**	0.06
N Rate × Crop	1	0.71	0.88	0.45	0.82	0.38	0.89	0.17	0.43	0.09
N Rate × Crop x Sample Time	3	0.46	0.99	0.80	0.76	0.38	0.82	**0.03**	0.53	0.34
Est. Variance										
Plot (%)		54.7	0.00	50.0	40.9	3.40	0.00	0.00	0.00	0.00
Year (%)		0.00	37.5	2.69	10.1	18.7	7.90	21.0	25.3	19.3
Whole Model (r^2)		0.60	0.39	0.60	0.58	0.47	0.39	0.46	0.39	0.42
5-10 cm										
N Rate	1	0.89	0.55	0.77	0.36	**0.03**	0.49	0.91	0.09	0.16
Crop	1	0.37	0.27	0.17	0.72	0.65	0.36	0.28	0.94	0.31
Sample Time	3	0.10	**<0.0001**	**0.004**	0.65	**<0.0001**	**<0.0001**	**<0.0001**	**0.001**	**0.002**
Crop x Sample Time	3	**0.001**	0.13	**0.03**	0.21	0.78	0.68	0.48	0.37	0.68
N Rate × Sample Time	3	0.35	1.00	0.61	0.94	**0.02**	**0.03**	0.12	0.23	0.44
N Rate × Crop	1	0.55	0.90	0.77	0.64	0.98	0.76	0.34	0.06	0.47
N Rate × Crop x Sample Time	3	0.92	1.00	0.80	0.89	0.81	0.56	0.80	0.33	**0.03**
Est. Variance										
Plot (%)		33.9	0.00	17.8	0.00	2.35	0.00	0.00	0.00	1.40
Year (%)		1.55	0.52	15.2	23.6	30.2	0.00	9.39	30.5	20.1
Whole Model (r^2)		0.44	0.16	0.45	0.19	0.44	0.33	0.11	0.36	0.34

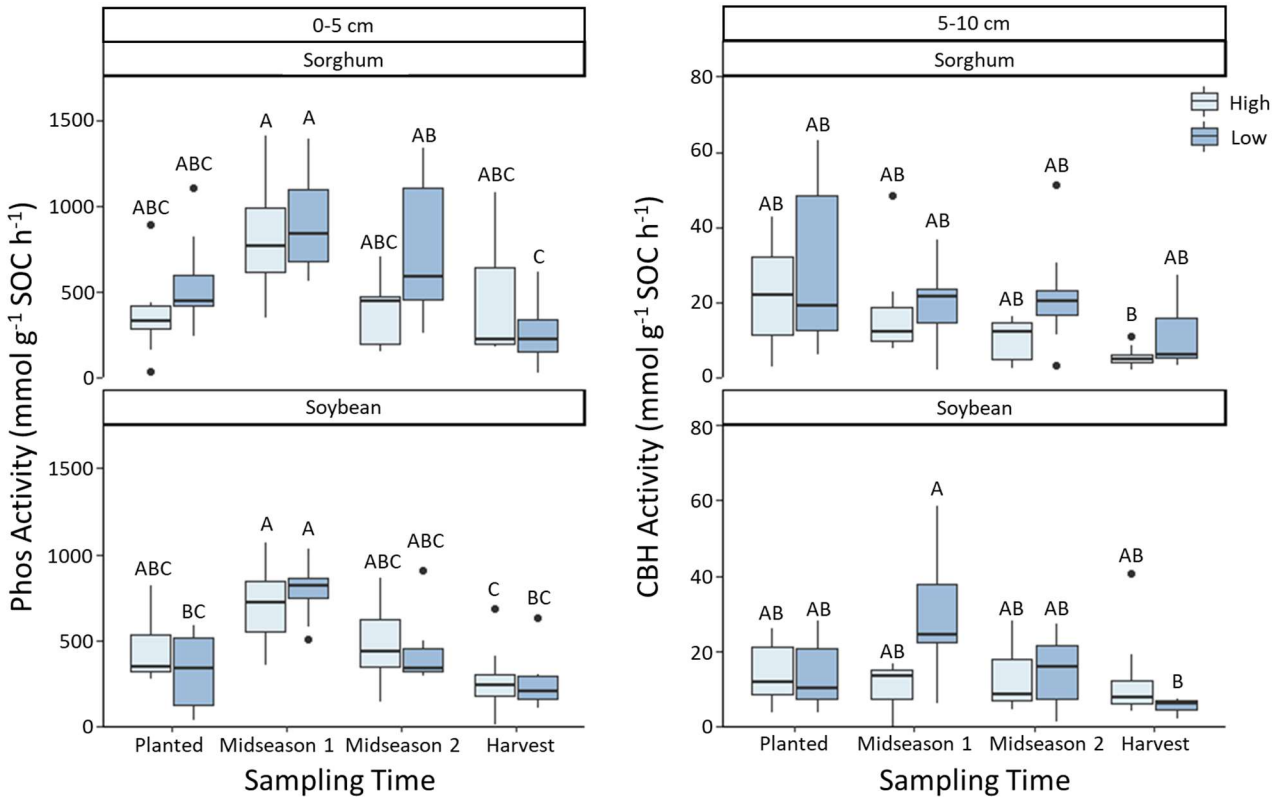

Figure A.1. Soil extracellular enzymatic activities, normalized by SOC, under a sorghum-wheat-soybean-fallow crop rotation system at 4 sampling times across two growing seasons. High and low fertilization rate are represented by box color. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Letters indicate significant differences as determined by Tukey's HSD

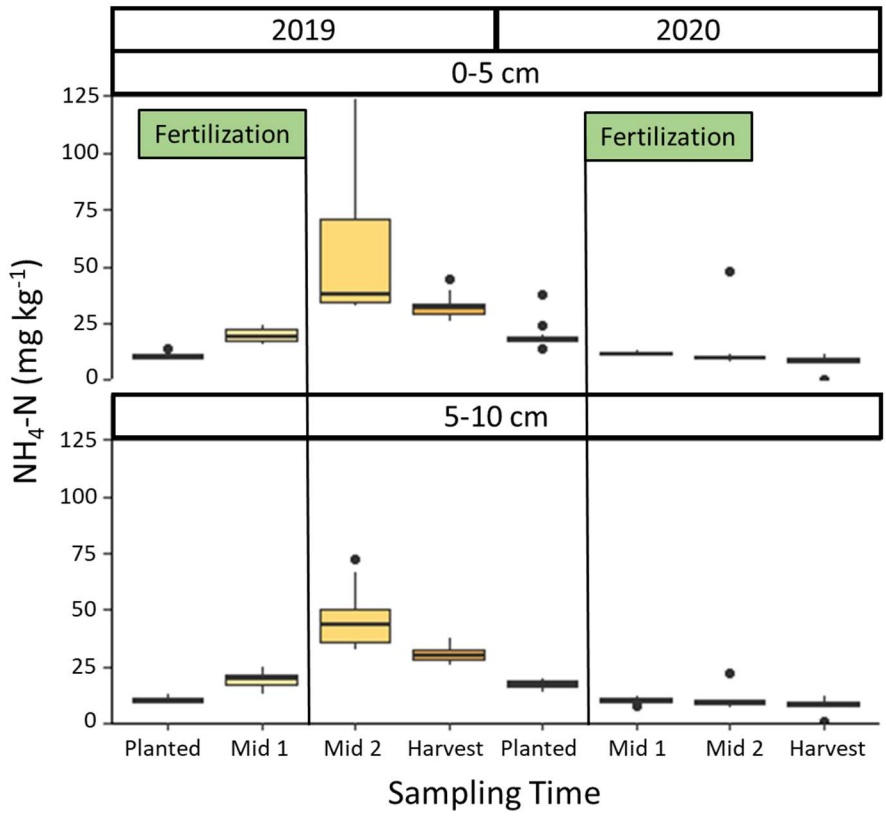

Figure A.2. Soil NH_4-N concentration under a sorghum-wheat-soybean-fallow crop rotation system at 8 sampling times across two growing seasons. Midseason 1 sample time is indicated by Mid 1, and midseason 2 sample time is indicated by Mid 2. Fertilization occurred at the times marked with urea broadcast at the surface.

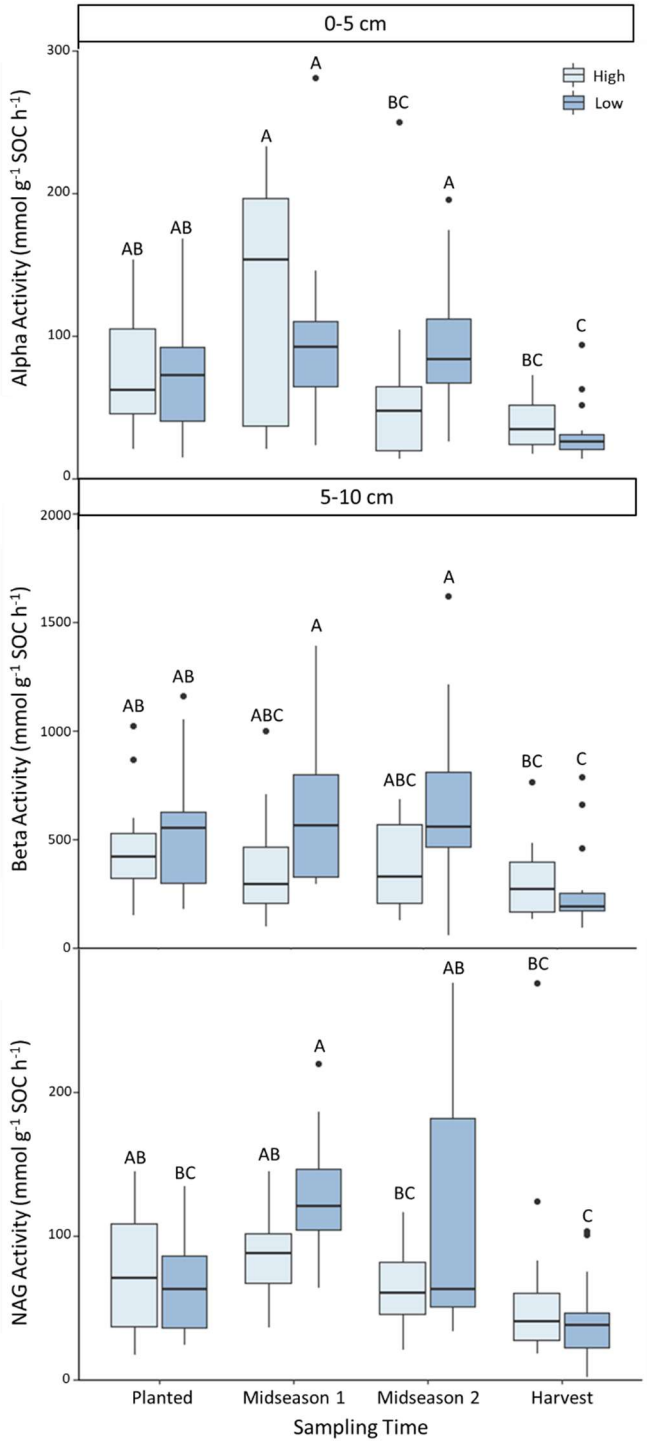

Figure A.3. Soil extracellular enzymatic activities, normalized by SOC, under a sorghum-wheat-soybean-fallow crop rotation system at 4 sampling times across two growing seasons. High and low fertilization rate are represented by box color. Boxplot whiskers indicate +/- standard deviation, colored area indicates the middle 50% of the data values, and the bolded center line indicates the mean of the values. Letters indicate significant differences as determined by Tukey's HSD.