Crop Rotation Effect on Fungal Community Complexity and Soil Carbon Stabilization

Thesis

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By

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Abstract

Agricultural soils have considerable potential to mitigate climate change by sequestering substantial amounts of atmospheric carbon as soil organic carbon (SOC). This research hypothesized that adding winter wheat to corn-soybean crop rotations increases fungal abundance and diversity and the amount of more stabilized SOC. Corn-soybean (CS) and corn-soybean-wheat (CSW) treatments were established in 2012 under a no-till system with a randomized block design at two separate locations, the Northwest and Western Agricultural Research Stations (NWARS and WARS, respectively). Soil samples were collected in fall 2020 after corn harvest at a depth of 0-10 cm. Total fungal biomass and enzyme activity were measured in conjunction with long-read genetic sequencing of the mycobiome to provide fungal taxonomic abundance and diversity. Soil carbon (C) was examined using a modified Walkley-Black method along with total C analysis after size and density separation and chemical oxidation. Total fungal biomass was higher under CSW than the CS rotation (26% at NWARS, 9% at NWARS) with greater diversity at WARS but not NWARS. Concentrations of oxidatively resistant SOC were higher under CSW than the CS rotation (0.68% at NWARS, 13.1% at WARS). This study showed that diversifying crops can impact soil fungi and potentially increase the amount of stable SOC, but farmers should be aware of how rotations affect their operations. As methods to

monitor and increase SOC improve, C farming programs and markets will expand allowing yield uncertainty to be countered by generating sellable C credits.

Dedication

To my mother and father, and all of those affected by climate change. May you know I tried by best.

Acknowledgments

I would like to take a moment to recognize the following people, as they played an important role in my ability to complete the work reported in this document: Nick Basta, Shane Whitacre, Dave Tomashefski, Tim Frey, Leslie Taylor, Gabriela Silva-Pumarada, Melanie Medina, Fabiano Colet, Todd Peterson, Greg McGlinch, and Freddie Mayer.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Soil fungal community complexity

1.1. Introduction

A recent review that included key crops grown in Ohio identified and described some of the numerous benefits that conservative agricultural practices can have on agronomic, economic, and environmental outcomes (Shrestha et al., 2020). Understanding how soil microbes respond to these practices can allow farmers to manipulate their soil microbial communities knowingly and intentionally, in ways that can optimize crop yields or improve resiliency while maximizing climate change-mitigation potential of the soils involved.

The overall complexity of soil microbial communities has been recognized as relatively unknown, but is increasingly seen as a crucial component of agricultural systems, contributing to crop production through numerous functions (Fierer, 2017; Compant et al., 2019). Techniques and technologies have evolved and are developing to help scientists understand the effects of various land management practices on the collective components of soil microbial communities.

Under a corn and soybean rotation, crop type and tillage were shown to be factors that significantly affect bacterial species composition with little change to total overall richness or diversity (Smith et al., 2016). Continuous cropping of soybean has been shown to increase fungal abundance and diversity compared to a wheat-corn-soybean rotation, but the monocrop nature of this system created an environment that was ripe for

the growth of fungi that were antagonistic to plant health (Liu et al., 2019). Since plants grow in the ground, they are susceptible to such negative outcomes due to their direct interaction with soil microbiology, but there are also instances and conditions in which soil microbiology can benefit plants. For example, certain microbes have demonstrated an ability to outcompete various *Fusarium* species found on corn stubble that tend to cause head blight in wheat (Luongo et al., 2005). With enough knowledge of microbial community responses to crop rotations on a species level, catering land management practices to site specific needs can be used as a tool, in the sense of being able to alter microbial community composition as a natural way to offer protection to plants from pests, reducing the need for synthetic pesticides.

Bacterial communities have been shown to be more directly influenced by soil chemical characteristics, while fungal communities respond more to the timing of crops planted throughout rotations, and the types of crops included in those rotations (Suzuki et al., 2012; Ai et al., 2018). The relationships that exist between bacteria and fungi make them both indispensable to the soil ecosystem, but the larger and more direct response of fungi to crop type and planting date make them a better proxy for developing management plans that utilize the effects of crop rotations on the soil microbiology to combat climate change.

Being aware of the key enzymatic activities related to the carbon cycle taking place in the soil is critical when managing land for controlling carbon dioxide emissions and retaining soil organic carbon. Intercropping with legumes and grasses produced a greater response in enzymatic activity, related to organic matter degradation, compared to

forbs and woody vegetation (Curtright and Tiemann, 2021). Chemical fertilizers can enrich soils with microbial species associated with soil organic carbon (SOC) decomposition, resulting in boosted production of extracellular enzymes that work to breakdown the complex carbon found in plant litter when other sources are not readily available (Wu et al., 2021). When growing the fungus *Aspergillus oryzae* in submerged culture conditions, the enzymes produced associated with organic matter degradation were contained within the cell wall, but they were released in a free state when grown in solid-state cultures (Bouws et al., 2008). When the enzymes exist in a free state, they can be mobilized by forces enacted upon them by the air and water components of the soil volume, increasing their probability of coming in contact with organic matter.

Combining the analyses of total fungal biomass and genetic profiling of the fungal portion of the soil microbial community, referred to henceforth as the mycobiome, can help growers better understand the effect of their crop rotations on the biological components of their soil, and whether they result in propagation of beneficial or harmful species. This research hypothesized that adding winter wheat to a corn-soybean crop rotation increases the abundance of soil fungal communities while increasing diversity and raising enzymatic activity.

1.2. Methods to explore crop rotation effect on fungal community complexity

1.2.1. Field site setup and experimental design

The data collected and analyzed in this study came from soil samples taken out of experimental agricultural plots consisting of two different crop rotations, corn-soybean, and corn-soybean-wheat. The described planting and sampling setup was replicated four times at each location, demonstrated by Table 1.1. The plots are planted at two separate locations in the state of Ohio. Those locations are the Northwest and the Western Agricultural Research Stations (NWARS and WARS, respectively). The plots at NWARS are situated in the southwestern portion of the 247-acre piece of property located at 41°12'46.2"N 83°45'49.3"W. The plots at WARS are in the northwestern part of the property, which is 428-acres, and are located at 39°51'37.7"N 83°40'38.4"W. At NWARS the plots were 21.34 m long and 9.14 m wide, while at WARS the plots were shorter, at 9.14 m in length, and 9.14 m wide. The area focused on for this study was contained to the center portions of each plot. Mowed rows with a width of 6.3 m separated each replicate. Both research stations had weather systems that tracked various climate variables, and those abiotic factors are summarized in Table 1.2.

1.2.2. Soil sampling and processing

Soil extraction

Researchers took undisturbed push tube soil samples as cores using a probe with a diameter of 2.5 cm. Each sample was a composite of eight soil cores taken in a zig-zag

pattern in an attempt to represent whole-plot characteristics. The cores were taken from a depth of 0-10 cm. Cores were combined and homogenized by hand within their storage bags once all eight were pulled. Proximal plant litter and debris was cleared from the soil surface before pulling each core. Once homogenized, subsamples of approximately 20 g were taken by sieving the bulk sample with a #10 mesh sieve to retain particles < 2 mm in a plastic sample vial, and all vials were then temporarily stored on dry ice. They were transported to a laboratory and transferred to a freezer set to -80°C. During the process, samplers wore nitrile gloves which were washed using 70% ethanol between samples, along with the soil sampling and processing instruments. This was done to minimize cross-contamination between samples.

Soil frozen storage

It is recommended that priority be given to extracting fatty acids, soil enzymes, and DNA before storing any samples, but this cannot always be done due to the length and duration of transportation as well as lab work coordination. If storage must take place, it's been shown that -20°C is ideal for fatty acid and enzyme extraction, and -80°C for DNA extraction (Lee et al., 2007). It is important to be aware of the storage effects on various soil biological components and maintain consistency within a given study. For this research, all samples were handled and stored in identical fashion with an interest in noting differences across treatments, rather than identifying true *in situ* values of the biological indicators of interest. The samples used for fatty acid and DNA extraction were put directly under dry ice then transported and transferred to a freezer set to -80°C.

Soil processing, air-drying, and room temperature storage

Composite soil samples gathered in the field were transported back to the laboratory in their storage bags, set on a bench with the tops of the bags rolled open and allowed to air dry. That was followed with a roller-mill grinding procedure that included sieving with a #10 mesh (< 2 mm) to homogenize the samples and remove any larger stones or objects if present. The samples in this research, coming from managed agricultural plots, did not contain any large rocks but some had small amounts of plant material larger than 2 mm, which was removed. These samples were then stored at a room temperature of 25°C. Subsamples were taken for ß-glucosidase extraction.

1.2.3. Soil biological measurements

Phospholipid fatty acids and enzyme activity

Gas chromatography determined microbiological content of fungi after the extraction of ester-linked fatty acids called EL-FAME. These are different than phospholipid fatty acids (PLFA). It's been demonstrated that EL-FAME extraction costs less, takes less time, and provides more resolute profiles based on fungal biomarkers compared to PLFA and helped maximize the distinguishment of samples (Li et al., 2020). Extraction was performed by the Microbial Ecology Lab at Ohio State University using 2.5 g of sample, and by dissolving 0.01 M methylnonadecanoate (C 19:0) in hexane/MTBE as a 1:1 solution to be used as an internal standard, all while following the procedure described in a method-comparison paper (Schutter and Dick, 2000) which was modified and based off of previous work (Zelles, 1999). Particular FAME compounds served as biomarkers to quantify arbuscular mycorrhizal, saprotrophic, and total fungi

was calculated as their sum, all reported using the units nmol g^{-1} soil. For arbuscular mycorrhizal fungi the FAME compound 16:1ω5c as a biomarker (Olsson, 1999), and 18:2ω6c and 18:1ω9c served as fungal biomarkers for saprotrophic fungi (Kaur et al., 2005). Soil used for this extraction was stored at -80°C.

ß-glucosidase was analyzed by measuring enzymatic activity through the release of *p*-nitrophenol (PNP). Air-dried samples were sent to the Ohio State University Rhizosphere Dynamics Lab in order to determine ß-glucosidase activity. The method used was the same as described in Methods of Soil Enzymology for carbohydrate hydrolases (Deng and Popova, 2011).

Long-read sequencing of mycobiome

Samples for this method were stored at -80°C. Fungal genomic DNA was extracted from each sample, purified, and amplified using PCR. This extracted genetic material was sent to Loop-Genomics for complete synthetic long-read sequencing of the 18S-ITS1-ITS2 region ("Mycobiome," 2022). The sufficiency of this method to provide reliable data describing fungal isolates has been compared and shown to be more accurate than older, short read sequencing methods (Callahan et al., 2021). The captured contigs representing individual amplicon sequence variants (ASVs) were cross-referenced with the genetic database UniteCommunity (Nilsson et al., 2018) to assign species level taxonomic information based on the ITS1 and ITS2 region, producing relative abundance values for those species in each sample. Results were then filtered to remove all bacteria, archaea, and unrecognized fungal species, leaving a species level description of the fungal communities found in each sample.

1.2.4. Statistical analysis

Descriptive and exploratory statistics were performed using R software and Microsoft Excel. Data assumptions were tested to verify the appropriate test for analyzing measured variables, which ended up being two-way ANOVA. The below equation indicates the formula used to run two-way ANOVA on each of those variables of interest:

$$Y \sim X_1 * X_2$$

with Y representing a given dependent variable, X_1 representing the location of the plots, either NWARS or WARS, and X_2 representing CS or CSW. The use of the $*$ operator in the equation denotes an interaction between the plot location and crop rotation. In the instance of the ANOVA test showing significant differences between crop rotation, post-hoc analysis, conducted using multiple comparison of means using Tukey Honest Significant Differences test, helped determine whether the crop rotation effect was significant between samples at individual locations.

Ecological diversity was measured by calculating the Shannon Diversity Index (H) and Equitability Index (E_H) utilizing the long-read sequencing data. (Magurran, 1988; Balezentis et al., 2020). In an attempt to quantify the amount of individual species, the biomass results provide by EL-FAME analysis and the relative abundances of each species, given in the long-read sequencing data, were used in conjunction. The relative abundances of an individual species represent the proportion of the total biomass found in a given sample, and act as a form of standardization to see whether an individual species had more biomass in one sample than another. The following example explains this general calculation for the first ASV in Table A.2 under CS:

The relative abundance (ra) for *Mariannaea punicea* of all four replicates of the CS rotation at NWARS were multiplied by the total fungal biomass value (nmol g^{-1} soil) determined by EL-FAME analysis. The mean of these four values represent the average amount of *Mariannaea punicea* biomass found under CS at NWARS. This information can be found for the top 20 ASVs in Tables A.2 and A.3.

Pearson correlation analysis was used to show the relationship between enzymatic activity and the levels of different fungal phyla in the soil.

1.3. Results

1.3.1. EL-FAME profiles

Total FAMES for each biomarker quantified the total amount of that particular microbiological component of the soil sample using the units nmol g^{-1} soil. There was more saprotrophic fungi (SAP) than arbuscular mycorrhizal fungi (AMF) in every sample. Table 1.3 shows the average and standard deviation of SAP, AMF, as well as their ratio and total fungal biomass for each crop rotation at both locations.

The average total amount of AMF was lower under CSW at both NWARS and WARS by 6.8% and 11.7%, respectively. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on total AMF. This revealed that there was not a statistically significant interaction between the effects of crop rotation and plot location ($F(1, 12) = 0.02$, $p = 0.90$). Simple main effects analysis showed that plot location did not have a statistically significant effect on total AMF ($p = 0.81$), neither did crop rotation ($p = 0.66$).

The average total amount of SAP was higher under CSW at both NWARS and WARS by 38.1% and 15.8%, respectively. Again, a two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on total SAP at the time of sampling. This revealed that there was not a statistically significant interaction between the effects of crop rotation and plot location ($F(1, 12) = 0.54$, $p = 0.48$). Simple main effects analysis showed that plot location did not have a statistically significant effect on total SAP ($p = 0.27$), but crop rotation did ($p = 0.04$) at $\alpha = 0.05$. While the two-way

ANOVA indicated that crop rotation had a significant effect on SAP, post hoc analysis using Tukey multiple comparison of means showed that isolating samples based on location, then comparing the average values of SAP found under CS to CSW, indicated no significant effect on the levels of SAP as a result of the wheat crop added to the rotation.

The average ratio of AMF to SAP was lower under CSW at NWARS and WARS by 39.9% and 25.9%, respectively. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on the ratio of AMF to SAP at the time of sampling. This revealed that there was not a statistically significant interaction between the effects of crop rotation and plot location ($F(1, 12) = 0.27$, $p = 0.61$). Simple main effects analysis showed that plot location did not have a statistically significant effect on the ratio of AMF to SAP ($p = 0.66$), neither did crop rotation ($p = 0.11$)

The average amount of total fungi was higher under CSW at NWARS and WARS by 26.4% and 9.3%, respectively. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on total fungi at the time of sampling. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1, 12) = 0.40$, $p = 0.54$). Simple main effects analysis showed that plot location did not have a statistically significant effect on total fungi ($p = 0.34$), neither did crop rotation ($p = 0.15$).

1.3.2. ß-glucosidase

Activity was higher under CSW at NWARS by 20.5%. Activity was lower under CSW at WARS by 11.5%. A visual boxplot representation of this information can be

found in Figure 1.1. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on ß-glucosidase activity at the time of sampling. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1, 12) = 1.50$, $p = 0.24$). Simple main effects analysis showed that plot location did not have a statistically significant effect on ß-glucosidase activity ($p = 0.11$), neither did crop rotation ($p = 0.87$).

1.3.3. Shannon Diversity and Equitability Index

Fungal taxonomic data was used to determine the total amount of different phyla represented in each sample. Figures 1.2 and 1.3 provide this information based on the crop rotation treatment for both locations. Ascomycota were the dominant phyla in all samples, with Basidiomycota and Mortierellomycota being the second and third most abundant. Tables A.2 and A.3 provide a more granular look into the most dominant species. Two metrics were calculated to represent fungal species level diversity within each sample, as well as the evenness of the abundance of each of those species. The Shannon Diversity Index (H) represents species diversity within samples, and the Equitability Index (E_H) represents the evenness of the spread of abundance of those species, and this information can be seen in Table A.1.

H was lower under CSW at NWARS compared to CS by 3.57%. The average value of H under CS at NWARS was 3.11, and 3.00 under CSW. Diversity was higher under CSW at WARS by 15.12% compared to CS, with average values of 2.68 under CS and 3.09 under CSW. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on species diversity within samples at the time they were

taken. This revealed there was not a statistically significant interaction between plot location and crop rotation ($F(1, 9) = 0.91$, $p = 0.37$).

When compared to CS, E_H was 7.7% lower under CSW compared to CS at NWARS, and 20.5% higher at WARS. A two-way ANOVA was performed and revealed no significant differences resulting from the interactions between plot location and crop rotation, or from either of the simple main effects when separately considered.

1.4. Discussion and concluding remarks

With the establishment of the plots in 2012, the 2-year corn-soybean rotation had gone through four full cycles, and the 3-year corn-soybean-wheat rotation had gone through two full cycles plus two thirds of another. The reason the center of the plots were focused on was to minimize edge effects. This made the contrast in plot lengths at each location a negligible difference. This was done as a way to reduce any effects that might be caused by different crops being grown in close proximity to each other. To help control for any cross contamination, all sampling supplies and gloves worn by those pulling samples were washed with ethanol before and after each sample was collected. Even after taking these precautions, adding winter wheat to the corn-soybean crop rotation showed no significant effect on the soil biological characteristics measured for this research.

While this study was focused on soil microbiological characteristics, an important agronomic aspect to consider when comparing these rotations, especially in the state of Ohio, is the effect that adding winter wheat has on corn and soybean crop yields. Several experiments have looked at this showing inconsistent results. In one instance, adding winter wheat had no significant effect on either of those crops (Lund et al., 1993). That project was conducted in the state of Wisconsin at a location with climate and soil conditions comparable to those found in Ohio. Another more recent study, utilizing the same experimental plots considered in this research, reported that adding winter wheat reduced overall corn yield, but offered some resilience to soybean (Huo et al., 2022). This

disparity should come as a caution when altering land management and consider whether adding different crops to rotations are worth the risk.

At both locations, prior to sampling, corn was the most recently harvested crop from both treatments out of all replicates. The lack of simple main effects significantly influencing the measured biological characteristics could indicate that the top 10 cm of the soil that were sampled are fast changing and reach a state of similarity during a single growing season when the same crops are scheduled in both crop rotation cycles. Other corn-soybean-wheat research looked at two different sampling depths, 0-5 cm and 5-15 cm, and showed that variation was temporally significant in soil microbiological characteristics (enzymatic activity, microbial biomass) within crop growth cycles and strongly impacted by the timing and growth stage of different crops, suggesting an influence of previous root litter and current root exudates on fungal communities (Hsiao et al., 2019). The suggested rate of change of these characteristics in the upper portion of the soil profile could classify them as fluid, making them difficult to analyze unless sampling is done with more temporal acuity.

The amount of fungi in each sample from both locations, representative of different phyla, can be found in Figure 1.2 and 1.3. As genetic technology improves and develops, databases cataloguing the many functions of various fungal phyla continue to grow and become more accurate. The qualities of two such databases (FungalTraits and FUNGuild) were recently compared, and it was shown that FungalTraits produced higher quality and quantity results and assigned a substantially higher number of saprotrophic ASVs than FUNGuild (Tanunchai et al., 2022). The phyla Ascomycota and

Basidiomycota, which consist of saprotrophic fungi, were described in terms of total function in a subsequent paper reporting results of classification using FungalTraits (Põlme et al., 2020). The samples from our research saw Ascomycota increase from 52.6% to 64.3% at NWARS and decrease from 85.0% to 52.3 % at WARS, while Basidiomycota decreased from 10.3% to 4.5% at NWARS and increased from 6.7% to 9.0% at WARS when winter wheat was added to the crop rotation. Mortierellomycota, also a saprotrophic fungi shown to increase with the addition of plant litter to the soil surface (Zhang et al., 2021), decreased from 31.5% to 25.5% in NWARS and increased from 6.8% to 34.6% in WARS. According to the previously mentioned works this indicates a shift in soil function. Ascomycota play a larger role as litter saprotrophs and smaller role as plant pathogens than Basidiomycota. Saprotrophic fungi are known to be responsible for the degradation of organic matter, breaking carbon bonds in the plant litter that gets deposited on the soil surface. The level of extracellular activity of ß-glucosidase has been shown to be higher in soils with greater amounts of saprotrophic fungi (Šnajdr et al., 2011). The correlation analysis conducted in this research showed no relationship between the levels of these different fungal phyla and enzymatic activity of ß-glucosidase. Percent change under CSW compared to CS of saprotrophic fungi biomass was much greater in proportion to the percent decrease of arbuscular mycorrhizal fungi biomass. Though the changes were found to be non-significant, the decrease in arbuscular mycorrhizal and increase in saprotrophic biomass under CSW compared to CS could indicate a shift in soil function as a response to the high carbon content of the wheat straw added to the rotation. The quick response time of the microbiological environment

in the upper portions of agricultural soils to changing above ground conditions could work in farmers' favor if they try to alter the mycobiome to suit their needs, such as when considering participation in emerging carbon credit markets.

While the insights gained by performing analysis of variance on each of the individual characteristics are important, being able to consider them all as co-variates and analyze the collective variance in a multivariate sense could be more telling. Such multivariate techniques for future work to consider are two-way multivariate analysis of variance followed by canonical discriminant analysis. This would help determine which characteristics contribute the greatest variance to the data, narrowing the focus by highlighting the variables that are most strongly affected by the addition of winter wheat. The current data violated the skewness component of the assumption of multivariate normality. This prevented a multivariate approach to looking at the data.

1.5. Tables

Table 1.1. The following crop rotation cycles of corn-soybean (CS) and corn-soybean-wheat (CSW) have been used at the Northwest Agricultural Research Station (NWARS) and the Western Agricultural Research Station (WARS) since establishment in 2012. Four replicates exist at each location.

Rotation	2013	2014	2015	2016	2017	2018	2019	2020
CS	Soybean	Corn	Soybean	Corn	Soybean	Corn	Soybean	Corn
CSW	Wheat	Corn	Soybean	Wheat	Corn	Soybean	Wheat	Corn

Table 1.2. Average values and their standard deviations for data of abiotic factors collected at Northwest Agricultural Research Station (NWARS) and Western Agricultural Research Station (WARS) from time before experiment establishment to soil sampling date (10/12/2012 - 10/10/2020).

	Yearly precipitation (mm)		Air temperature (°C)		GDD		Soil temperature (5 cm)	
Location	mean	sd	mean	sd	mean	sd	mean	sd
NWARS	889.29	178.66	10.54	10.78	8.85	10.34	11.73	9.33
WARS	998.40	121.15	11.27	10.47	9.33	10.27	13.20	8.54

Table 1.3. Northwest Agricultural Research Station (NWARS) and Western Agricultural Research Station (WARS) average values and their standard deviations for FAME results expressed as nmol g^{-1} soil, along with post-hoc p-values of Tukey multiple comparisons of means after performing ANOVA: Arbuscular mycorrhizal fungi (AMF), saprotrophic fungi (SAP), the ratio of AMF to SAP (AMF:SAP), and total fungi.

			AMF			SAP			AMF:SAP			Total fungi		
Location	Rotation	n	mean	sd	p-value	mean	sd	p-value	mean	sd	p-value	mean	sd	p-value
NWARS	CS	4	19.11	7.50	0.99	54.31	14.06	0.20	0.38	0.21	0.41	73.42	12.37	0.45
	CSW	4	17.81	8.41		75.01	11.82		0.23	0.07		92.82	20.21	
WARS	CS	4	20.71	10.86	0.98	67.26	19.28	0.70	0.32	0.14	0.82	87.97	24.50	0.91
	CSW	4	18.29	5.85		77.87	6.40		0.23	0.07		96.15	10.36	

1.6. Figures

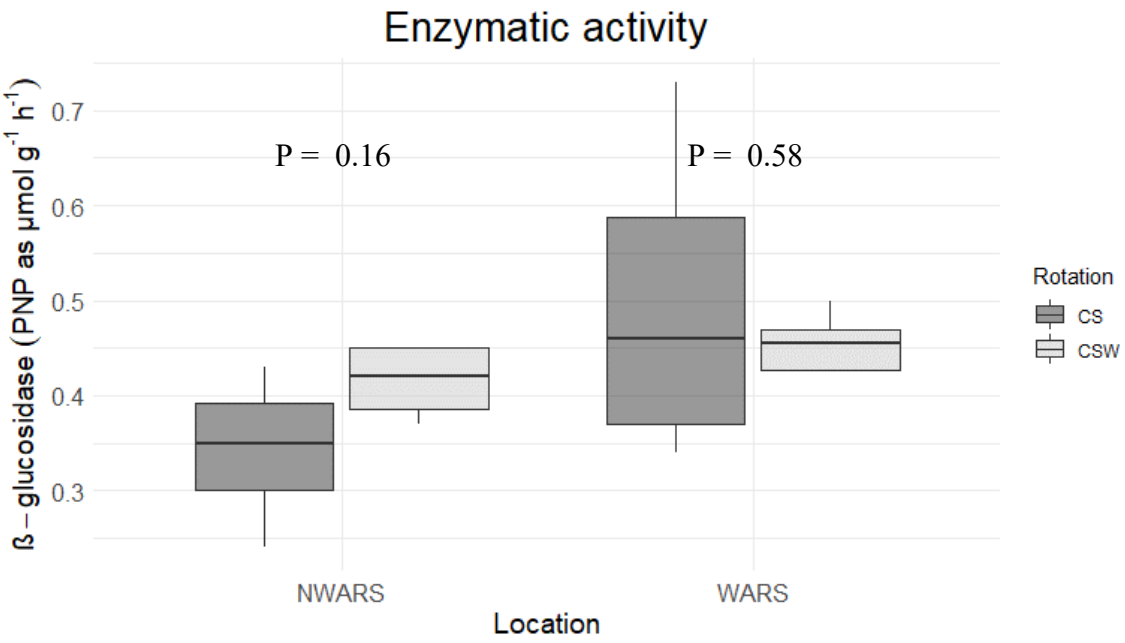

Figure 1.1. Box plot representation of the enzymatic activity of ß-glucosidase measured as the amount of *p*-nitrophenol (PNP) produced over the course of an hour. For each rotation at both locations, n = 4.

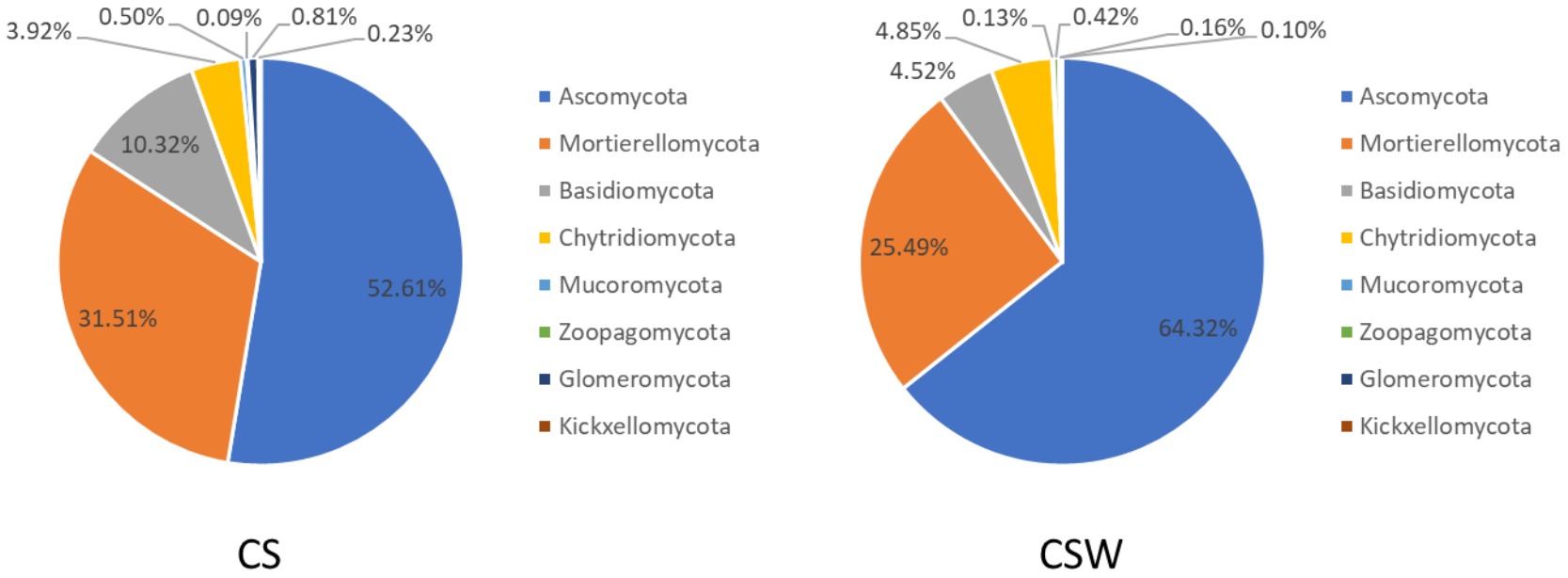

Figure 1.2. Total percentage of different fungal phyla at Northwest Agricultural Research Station under corn-soybean (CS) and corn-soybean-wheat (CSW) crop rotations, determined using the counts of ASV contigs returned after synthetic long-read sequencing of the mycobiome.

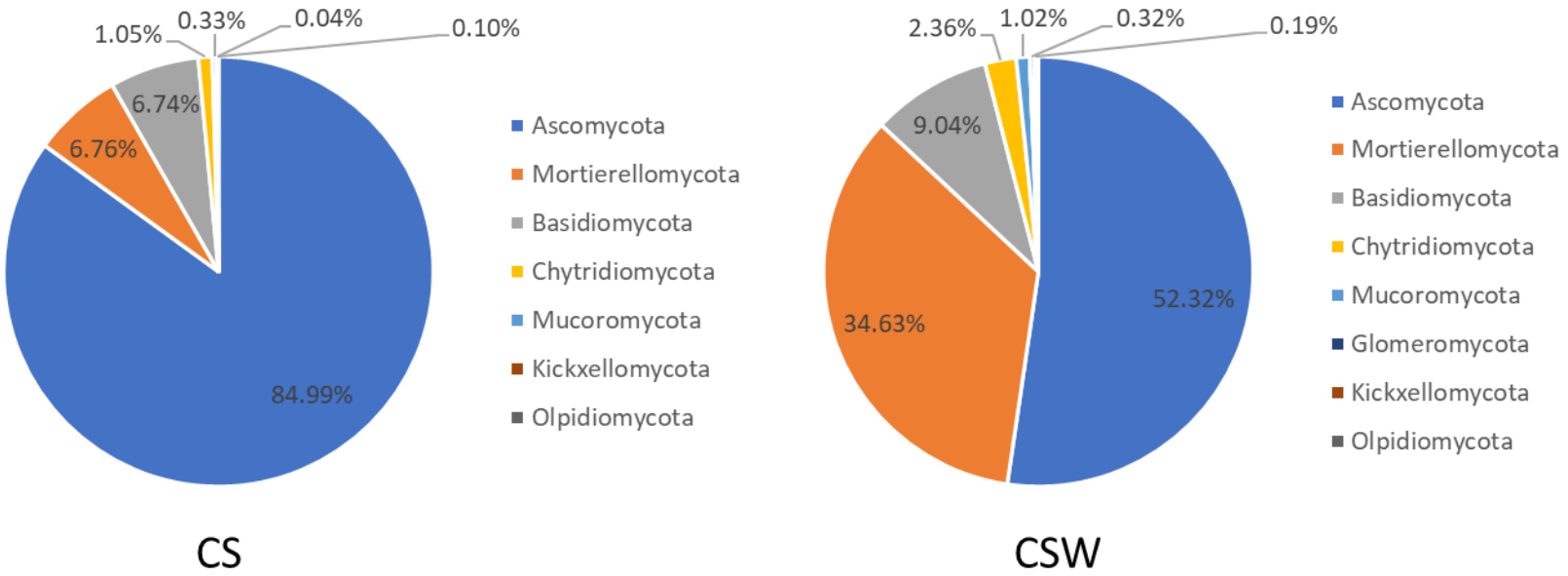

Figure 1.3. Total percentage of different fungal phyla at Western Agricultural Research Station under corn-soybean (CS) and corn-soybean-wheat (CSW) crop rotations, determined using the counts of ASV contigs returned after synthetic long-read sequencing of the mycobiome.

Chapter 2. Soil carbon permanence

2.1. Introduction

The pressure for food, fuel and fiber production on the available areas of cropland, pasture, and rangelands across the world grows as the number of dedicated parcels per capita shrinks with population growth (FAO, 2021). That said, agriculture is a large and actively managed system with an ability to contribute and engender effective results, in tandem with food production, to mitigate climate change by sequestering atmospheric carbon dioxide as soil organic carbon (SOC).

When using farmland as a tool for carbon sequestration, it is crucial that land managers understand how their systems and crop rotations impact carbon entering and exiting the soil. Both crops included in rotations and tillage decisions can affect those dynamics. Soil carbon stocks have been shown to be greater under grasses or rotation cropping systems such as corn and oats when compared to monoculture corn (Gregorich et al., 2001). Another experiment showed that significantly greater amounts of aggregate associated carbon were found in both large (2-4.75mm) and small (0.25-2 mm) aggregates under no-till treatments when compared to moldboard plow (MP), two-year no-till and one-year MP, and chisel-disk (Weidhuner et al., 2021). Organic carbon associated with aggregates is offered physical protection by the surrounding minerals and reduces the ability of biological and chemical processes from accessing it. This helps

lower the amount of carbon mineralization taking place in the soil. Finding ways to strengthen this protection, increasing the amount of stabilized soil organic matter, is crucial to building up stocks of soil carbon and allowing natural forces to transform the way it is being stored for stable yet accessible to a residual, more permanent state.

One framework suggests that the route to enhancing soils with this type of carbon is by incorporating vegetation that produces more labile plant litter that microbes decompose with greater efficiency, leading to a quicker and more efficient formation of stabile soil organic matter (Cotrufo et al., 2013). What this framework describes would not only increase the rate at which plant litter is incorporated into soil but could also increase soil microbial biomass to add to the carbon pool. In some instances, over 50% of total SOC in agricultural soils was derived from soil microbial necromass, with fungi accounting for 40% of that amount (Liang et al., 2019).

A majority of the research surrounding the relationship between winter wheat and SOC look at how carbon levels present in the soil impact the production and yield of the wheat without focusing on what adding wheat straw to the soil does to soil carbon levels. One study showed that as SOC content significantly increased, winter wheat experienced greater grain yields and larger amounts of above ground biomass (Ghaley et al., 2018). This research hypothesized that adding winter wheat to a corn-soybean crop rotation increases the amount of soil organic matter and residual SOC.

2.2. Methods to explore crop rotation effect on soil chemical and physical properties and organic carbon

2.2.1. Field site setup and experimental design

The data collected for this research was generated from soil samples taken in fall 2020 from two research farms located in the state of Ohio. Both farms are associated with the Ohio Agricultural Research and Development Center. The first location, Northwest Agricultural Research Station (NWARS) is north of Findley in Custar, which is in Wood County. The geographical coordinates for the plots are 41°12'46.2"N 83°45'49.3"W. The second location, Western Agricultural Research Station (WARS) is east of Dayton in South Charleston, which is in Clark County. The geographical coordinates for the plots are 39°51'37.6"N 83°40'38.3"W.

The soils found at NWARS are of the Hoytville series, typically a clay loam classified as a fine, illitic, mesic mollic Epiaqualf. The soils at WARS are of the Kokomo series, typically a silty clay loam classified as a fine, mixed, superactive, mesic typic Argiaquoll (Soil Survey Staff). The experiment was established in 2012 at both sites to observe the effect on crop yield and select soil characteristics after adding winter wheat to a corn-soybean rotation. Table 1.1 shows the yearly rotation cycle for each crop rotation, which was followed at both locations. All plots have been managed using a no tillage management system since their establishment. At both locations, all of the plots make up a complete randomized block design with four replicates, and all crops for both rotations are present in each replicate. The rotation cycles of each treatment happen to

align at the time of sampling, as samples from both CS and CSW plots were taken after corn harvest.

2.2.2. Soil sampling and processing

Undisturbed soil samples were collected in fall 2020 at both sites using a standard push tube probe. Surface plant litter was cleared away by hand before extracting a soil core that was 2.5 cm in diameter and 10 cm in length. A zigzag pattern was used to sample the center portions of each plot eight times, with all cores being combined in their respective storage bags to form a composite sample.

Samples were transported in their storage bags back to the laboratory and allowed to air-dry before being homogenized. Homogenization of each sample was accomplished through grinding by use of roller-mill and sieving through a #10 mesh screen with 2 mm openings. Any rock fragments, soil particles, or pieces of plant material larger than 2 mm were removed.

Once homogenized, the bulk samples were return to their storage bags and kept at room-temperature (approximately °25 C). Subsamples were taken as needed for each subsequent measurement and procedure.

2.2.3. Soil physical and chemical measurements

Soil texture through particle size analysis

Soil texture expressed through percent based on mass of sand, silt, and clay for each sample was obtained through a modification of the pipette method (Gee and Or, 2002) for conducting particle size analysis, using the salt sodium hexametaphosphate, prepared as 35.7 g sodium hexametaphosphate and 7.94 g of sodium carbonate per liter of

H_2O, as a dispersant. 10 mL of the solution were added to 10 g of soil in 450 mL sedimentation bottles, then were filled halfway with deionized water, sealed with rubber stoppers, and allowed to horizontally shake for 24 hours on a reciprocating shaker in an attempt to fully disperse any and all aggregates. Bottles were removed from the shaker and uniformly filled to 410 mL, so they all contained equal amounts of soil, dispersant, and deionized water. Dispersed particles were fully suspended in solution with the bottles in an upright position using a vertical, electric mixer inserted for 30 seconds, then set aside and allowed to settle for a set time so that the sand and silt fraction could fall out of suspension. After 3.5 hours of settling, aliquots were taken from each bottle using the calibrated, automatic pipette at a depth of 5 cm, then discharged into pre-weighed crucibles that were dried overnight in an oven set to 105°C. The bottles were then poured and thoroughly rinsed out through a #270 sieve with openings of 53 μm to wash out silt particles and remove salt from the dispersant. This retained the sand fraction of each sample, which was transferred to a glass beaker of known mass and allowed to dry overnight in an oven set to 105°C. After determining the mass of the sand and clay fractions, silt content was calculated by subtraction. A salt mass factor was determined for the dispersant and considered when determining clay content. No pretreatments for the removal of carbonates and soluble salts, organic matter, or iron oxides were administered.

Bulk density (ρ_b)

Soil cores were taken at a different time than soil sampling was conducted. These were taken in the winter of 2022 from each plot using an AMS slide hammer soil core

sampler to determine their dry bulk density. It is important to note that seasonal differences in bulk density have been shown to exist due to variation in soil moisture characteristics (Hu et al., 2012; Mora and Lázaro, 2014). The removable liners used to capture the cores were 10 cm in length and 4.78 cm in diameter. Plant litter was removed from the surface to expose the top of the soil before taking each core. Care was taken to not impose unnecessary compaction on the cores by drawing an indicator line on the outside of the core sampler cup and unscrewing the top cap of the sampler once the line was level with the ground. This was done to check if the soil surface was level with the top of the liner and prevent the core from being compressed. Once fully extracted, the liner was removed from the sampler along with the soil core, and the ends of the core were trimmed flush with the ends of the liner using a sharp, straight edge blade. The ends were then capped by placing a plastic sandwich bags flush against each end, wrapping excess material around the outside of the liner, then securing the bags in place with rubber bands. This was done to prevent any potential soil loss during transport. The cores were oven-dried at 105°C to remove moisture and determine dry soil mass. Bulk density was then calculated as dry soil mass divided by volume of the core liner.

Wet aggregate stability

The stability of wet aggregates was determined as a percentage following a wet sieving method using a benchtop oscillating wet-sieving device with sieves containing 53 µm openings (Kemper and Rosenau, 1986). 4.0 g of air-dry aggregates ranging 1 to 2 mm in size were used to complete the experiment. At the conclusion of the oscillation period, the unstable portion of the aggregates that passed through the sieve were transferred to a

glass beaker and dried overnight in an oven at 105°C. Deionized water was used to rinse the remaining stable aggregate fraction through the sieve, retaining the sand fraction, which was transferred to a souffle cup and then dried overnight. The mass of the stable fraction was determined by subtraction once the mass of the unstable and sand fractions were calculated. Water stable aggregates (WSA) were calculated as a percentage of entire sample.

pH and electrical conductivity (EC)

Acidity of soil solution and soil salinity were determined by preparing samples in a 1:1 soil to deionized water solution. Completion of this method was accomplished by using 2.5 grams of soil mixed with 2.5 mL of deionized water, following a procedure for determining pH (Thomas, 1996) as well as soil salinity (Rhoades, 1996).

2.2.4. Soil carbon measurements

Size and density fractionation

The following method is a modified version of the procedure described for quantifying organic carbon fractions by infrared-spectroscopy (Zimmermann et al., 2007). Samples were separated into five fractions of interest: dissolved organic carbon (DOC), particulate organic matter (POM), sand and stable aggregates (SA), oxidation-resistant residual carbon (rSOC), and silt and clay (s+c) particles.

For each sample, 10.0 g of bulk soil was weighed out on an analytical balance (already sieved < 2mm) and transferred to a glass beaker, which then had 70 mL of deionized H_2O (DI) added it. Once the sample was wet and in solution, it was exposed to

22 J mL^{-1} for 277 seconds by vertically submerging a sonification probe into the solution. This amount of energy was chosen to avoid the breakdown of sand-sized plant material while fully dispersing all other aggregates (Amelung and Zech, 1999).

Once dispersed, samples were poured through a 53 μm sieve, retaining SA and POM fractions, and catching the s+c content that passed. Wet sieving was used to ensure all s+c had passed through the mesh screen, taking care not to direct the flow of water directly at SA particles to avoid any further disruption. Upon completion, the s+c and water used for wet sieving was transferred to a large centrifuge bottle. The SA and POM fractions were transferred to a petri dish and dried overnight in an oven set to 40°C.

To obtain the s+c fraction, 4 mL of 0.75 $CaCl_2$ were added to the centrifuge bottle to aid in the flocculation, which was then centrifuged at 3240 rpm for 15 minutes. To capture DOC, 10 mL of the supernatant was pipetted into a 15 mL falcon tube and put into frozen storage. The supernatant was then decanted and the s+c contents were transferred to a Petri dish and oven-dried overnight at 40°C.

POM was separated from SA by density using sodium polytungstate (SPT) prepared to a density of 1.80 g cm^{-3}. The dried SA and POM fractions were transferred to a 15 mL falcon tube, and 5 mL of SPT was added. Samples were vortexed to ensure complete mixing with the SPT and then centrifuged at 3420 rpm for 15 min. The POM was transferred to a vacuum apparatus and rinsed with DI to remove any SPT from the fraction. DI was added to the SA in the falcon tube, which was vortexed, centrifuged, and decanted. This process was repeated three times to washout any SPT from the fraction.

SA and POM content was transferred to separate Petri dishes and dried overnight in an oven at 40°C.

In order to obtain the rSOC fraction, a subsample of 2.5 g was taken from s+c and oxidized using a 6% NaClO solution with pH adjusted to 9.5. The sample and 10 mL of solution were combined in 50 mL falcon tubes and placed in a water bath set to 95°C for 15 min before being centrifuged at 800 rpm for 8 min, and then decanted. This oxidation wash process was repeated three times, followed by three washes with DI water to remove any NaClO from the fraction. The s+c content was then transferred to a Petri dish and dried overnight in an oven set to 40°C.

After drying, the contents of each fraction and bulk samples were individually transferred to 20 mL scintillation vials and finely crushed by adding three cylindrical stainless steel grinding bars and being placed on an automatic rolling machine for 24 hours.

Active carbon (AC)

The method used for determining active carbon (AC), closely followed a procedure described for the purposes of soil quality assessment (Weil et al., 2003). A mass of 1.25 g of bulk sample was weighed out, then 9.0 mL of DI was added, followed by 1.00 mL of 0.2 M $KMnO_4$ containing 1 M $CaCl_2$. This combination diluted the $KMnO_4$ to 0.02 M (the recommended concentration from the previously mentioned method), and the $CaCl_2$ improved settling of soil particles. Samples were shaken for two minutes at a rate of 120 rpm, then set aside for 8 minutes to allow particles to settle out. Both the stock $KMnO_4$ and samples were covered throughout the process to prevent light

penetration from degrading the solution. An aliquot of 0.1 mL was taken and added to 9.90 mL of DI in a glass cuvette. Levels of AC were determined as mg AC kg^{-1} soil after taking absorbance readings of prepared $KMnO_4$ calibration samples with concentrations ranging from 0.000 to 0.020 M at 550 nm wavelength.

Organic carbon percentage

This measurement was taken after submitting bulk samples to chromic acid digestion and spectrophotometry in a modified version of the Walkley-Black method (Heanes, 1984). Samples were prepared for digestion by using 0.5 g of bulk soil, 10 mL of 0.1 M $K_2Cr_2O_7$ solution, and 20 mL of trace grade HCl. Digestion was carried out in a calibrated microwave set to maintain an average of 135°C for 30 minutes. Upon removal, 20 mL of DI was added, then samples and solution were inverted three times to ensure complete mixture, followed by an overnight settling period. Aliquots were then taken and added to glass cuvettes and absorbance of solution was measured at 600 nm.

Total carbon and nitrogen analysis

Total carbon and nitrogen was determined using an elementar UNICUBE© Trace organic elemental analyzer. Subsamples weighed out to the nearest ten-thousandth were taken of each finely crushed bulk sample and fraction from the rolled scintillation vials. Complete high-temperature combustion provided sensitive total carbon and nitrogen analysis to the determine the content of both elements in each sample.

2.2.5. Statistical analysis

Descriptive, exploratory, and predictive statistics were performed using R software and Microsoft Excel.

The mean and standard deviation of the soil physical and chemical characteristics were determined and displayed in Table 2.1. Bulk density was used in conjunction with carbon and nitrogen concentration to determine the total stock of each element, displayed in Figure 2.1 and 2.2. Stock values of carbon and nitrogen for each sample were determined by multiplying sampling depth in meters and bulk density as Mg m^{-3} by concentrations as g C or g N kg^{-1} soil, resulting in stock values with units of Mg C or Mg N ha^{-1}.

As part of a method verification, linear regression was used to compare the outcomes of the dichromate digestion and total carbon analysis, plotting the determined organic carbon percentages against each other.

Pearson correlation was determined to show the relationship between the silt and clay content of each sample with the amount of carbon found in those particles in order to discuss the idea of carbon saturation.

Analysis of variance was used to test for differences of dependent variables based on the independent variables of plot location and crop rotation. Assumptions of the data were checked to ensure appropriateness of the test. The below equation indicates the formula used to two-way ANOVA:

$$Y \sim X_1 * X_2$$

with Y representing a given dependent variable, X_1 representing the location of the plots, either NWARS or WARS, and X_2 representing CS or CSW. The use of the $*$ operator in the equation denotes an interaction between the plot location and crop rotation. In the instance of the ANOVA test showing significant differences between treatments, post-hoc analysis was conducted by multiple comparison of means using Tukey Honest Significant Differences.

2.3. Results

2.3.1. Particle size analysis

Particle size analysis results were consistent with the soil series descriptions for both locations provided by the USDA mentioned in the section 2.2.1. Samples from NWARS, being classified as a clay loam, had higher clay percentage than samples from WARS, with an average clay content of 36.22% and 35.24% in CS and CSW plots, respectively, compared to 28.48% and 30.35% at WARS. Samples from WARS, classified as silty clay loam, had higher silt percentage than samples from NWARS, with an average silt content of 54.42% and 52.43% in CS and CSW plots, respectively, compared to 42.28% and 41.96% at NWARS. Table 2.1 lists the average values and standard deviations for each crop rotation at each location.

Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on silt and clay content. For silt content this revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1, 12) = 1.51$, $p = 0.24$). Simple main effects analysis showed that plot location had a significant effect on silt content ($p < 0.001$) at $\alpha = 0.05$, but crop rotation did not ($p = 0.11$). For clay content the ANOVA revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1, 12) = 1.52$, $p = 0.24$). Simple main effects analysis showed that plot location had a significant effect on clay content ($p < 0.001$) at $\alpha = 0.05$, but crop rotation did not ($p = 0.71$).

2.3.2. Bulk Density

Bulk density (ρ_b) at NWARS averaged 1.11 and 1.18 g cm^{-3} under the CS and CSW rotation, respectively. At WARS the averages under CS and CSW were 1.20 and 1.24 g cm^{-3}. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on ρ_b. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1, 12) = 2.63$, $p = 0.13$). Simple main effects analysis showed that plot location had a small effect on ρ_b ($p = 0.07$) at $\alpha = 0.05$, but crop rotation did not ($p = 0.74$). Table 2.1 lists the average values and standard deviations for each crop rotation at each location.

2.3.3. Water stable aggregates (WSA)

Samples were shown to contain a higher percentage of water stable aggregates on average at WARS compared to NWARS by 21.1%. Table 2.1 lists the average values and standard deviations of WSA for each crop rotation at each location. A two-way ANOVA was performed to further analyze the effect of plot location, as well as crop rotation, on water stable aggregate percentage. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.54$, $p = 0.47$). Simple main effect analysis showed that plot location did have a significant effect on WSA ($p < 0.001$) at $\alpha = 0.05$, but crop rotation did not ($p = 0.75$).

2.3.4. pH and electrical conductivity (EC)

Soils at both locations were determined to be acidic, with an average pH of 5.16 and 4.85 at NWARS and WARS, respectively, with very slight differences between crop rotations at both locations. Table 2.1 lists the average values and standard deviations of

both pH and EC for each crop rotation at each location. Soils were also determined to be non-saline at both locations, with average electrical conductivity readings of 0.60 and 0.78 dS m^{-1} observed for NWARS and WARS, respectively. Average EC at NWARS was 43.2% higher under CS than CSW (0.77 and 0.44, respectively) with a single sample in the CS rotation accounting for a majority of that variation (the third replicate of CS had an EC value of 1.35 dS m^{-1}). Average EC at WARS was 6.5% lower under CS compared to CSW (0.76 and 0.80, respectively).

A two-way ANOVA was performed to further analyze the effect of plot location, as well as crop rotation, on both pH and EC. For pH, this revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 1.67$, $p = 0.22$). Simple main effect analysis showed that plot location did have a significant effect on pH ($p = 0.01$) at $\alpha = 0.05$, but crop rotation did not ($p = 0.12$). For EC, the two-way ANOVA revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1, 12) = 2.66$, $p = 0.13$). Simple main effect analysis showed that plot location did not have a significant effect ($p = 0.15$) and neither did crop rotation ($p = 0.25$).

2.3.5. Active carbon

A strong linear relationship was seen in the calibration curve, showing an $R^2 = 0.9947$. Though levels were slightly lower under CS than CSW at both locations, the average amount of active carbon showed no significant difference when considering crop rotation or plot location. At NWARS, average amounts of AC were 514.5 and 537.0 mg kg^{-1} (CS and CSW, respectively) and at WARS those averages were 529.0 and 532.2 (CS

and CSW, respectively). A two-way ANOVA was performed to further analyze the effect of plot location, as well as crop rotation, on AC. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.07$, $p = 0.80$). Simple main effect analysis showed that plot location did not have a significant effect on AC ($p = 0.90$) and neither did crop rotation ($p = 0.74$).

2.3.6. Organic carbon by dichromate digestion

A strong linear relationship was seen in the calibration curve, showing an $R^2 = 0.9987$. Percent organic carbon (OC) was 4.21% lower under CS than CSW at NWARS, and 3.63% at WARS. A two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on OC. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.02$, $p = 0.96$). Simple main effect analysis showed that plot location did not have a significant effect on OC ($p = 0.69$) and neither did crop rotation ($p = 0.53$).

2.3.7. Total carbon and nitrogen stocks

Carbon (C) stock in bulk sample and each fraction

Total C stocks found in bulk samples and all other fractions are listed in Table 2.2. Bulk soil samples were shown to have an average C stock that was 9.6% higher under CSW than CS at NWARS, and 1.18% higher at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on bulk C stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.77$, $p = 0.39$). Simple main effect analysis

showed that crop rotation did not have a statistically significant effect on carbon stock ($p = 0.26$) but plot location did ($p < 0.001$) at $\alpha = 0.05$.

Silt and clay C stock minus the stock of residual C (s+c-rsoc) was shown to have higher amounts of carbon under CSW compared to CS by 4.7% at NWARS and 4.0% at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on s+c-rsoc C stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.00$, $p = 0.99$). Simple main effect analysis showed that crop rotation did not have a statistically significant effect on carbon stock ($p = 0.25$) but plot location did ($p < 0.001$) at $\alpha = 0.05$.

C stock in the residual SOC fraction was 7.4% higher under CSW rotations than CS at NWARS, and 8.6% higher at WARS. Two-way ANOVA revealed that there was not a statistically significant interaction between the plot location and crop rotation ($F(1,12) = 0.04$, $p = 0.84$). Simple main effect analysis showed that crop rotation had a slight effect on residual C stock ($p = 0.08$) as well as plot location ($p = 0.08$) at $\alpha = 0.05$. While the two-way ANOVA indicated that crop rotation had a significant effect on rsoc C stock, post hoc analysis using Tukey multiple comparison of means showed that isolating samples based on location, then comparing the average values of rsoc C stock found under CS to CSW, indicated no significant effect on the levels of rsoc C stock as a result of the wheat crop added to the rotation.

Sand and stable aggregate (SA) C stock was shown to have higher amounts of carbon under CSW compared to CS by 20.2% at NWARS but was lower by 36.3% at

WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on SA C stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 1.74$, $p = 0.21$). Simple main effect analysis showed that crop rotation did not have a statistically significant effect on C stock ($p = 0.50$) and neither plot location did ($p = 0.25$).

Particulate organic matter (POM) C stock was shown to have lower amounts of carbon under CSW compared to CS by 18.7% at NWARS and 14.6% at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on POM C stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.00$, $p = 0.99$). Simple main effect analysis showed that crop rotation did not have a statistically significant effect on C stock ($p = 0.22$) but plot location showed a slight effect ($p = 0.09$) at $\alpha = 0.05$.

Nitrogen (N) stock in bulk sample and each fraction

Total N stocks found in bulk samples and all other fractions are listed in Table 2.3. Bulk soil samples were shown to have an average N stock that was 3.7% higher under CSW than CS at NWARS, and 0.9% higher at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on bulk N stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.06$, $p = 0.82$). Simple main effect analysis

showed that crop rotation did not have a statistically significant effect on N stock ($p = 0.70$) and neither did plot location ($p = 25$).

Silt and clay N stock minus the stock of residual N (s+c-rsoc) was shown to have higher amounts of N under CSW compared to CS by 5.1% at NWARS and 6.8% at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on s+c-rsoc N stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.05$, $p = 0.82$). Simple main effect analysis showed that crop rotation did not have a statistically significant effect on N stock ($p = 0.25$), but plot location did ($p = 0.05$) at $\alpha = 0.05$.

N stock in the residual SOC fractions were 7.8% higher under CSW rotations than CS at NWARS, and 4.2% higher at WARS. Two-way ANOVA revealed that there was not a statistically significant interaction between the plot location and crop rotation ($F(1,12) = 0.28$, $p = 0.61$). Simple main effect analysis showed that crop rotation had no effect ($p = 0.17$), but plot location did ($p < 0.01$) at $\alpha = 0.05$.

Sand and stabile aggregate (SA) N stock was shown to have higher amounts of N under CSW compared to CS by 27.6% at NWARS but was lower by 35.5% at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on SA C stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 2.04$, $p = 0.18$). Simple main effect analysis showed that crop rotation did not have a statistically significant effect on N stock ($p = 0.50$) and neither plot location did ($p = 0.25$).

Particulate organic matter (POM) N stock was shown to have lower amounts of N under CSW compared to CS by 21.1% at NWARS and 18.3% at WARS. Two-way ANOVA was performed to further analyze the effect of plot location and crop rotation on POM N stock. This revealed that there was not a statistically significant interaction between the effects of plot location and crop rotation ($F(1,12) = 0.02$, $p = 0.88$). Simple main effect analysis showed that crop rotation did not have a statistically significant effect on C stock ($p = 0.13$) but plot location did ($p = 0.02$) at $\alpha = 0.05$.

2.3.7. Comparison of carbon analysis methods

pH values demonstrated that analyzed soils were strongly acidic. Anticipation of little to no inorganic carbon content due to the acidic nature of the soils supported the decision to skip any pretreatment of samples before organic carbon analysis. Figure 2.3 shows a linear regression model comparing the outcomes of organic carbon percentage by dichromate digestion and total carbon analysis. While this is not a perfect relationship, indicated by a slope of less than 1, the R^2 value of 0.75 indicates a strong positive linear relationship. The p-value being less than 0.001 shows the strength of the model describing this relationship and shows that the results from both methods generally support one another.

2.4. Discussion and concluding remarks

Adding winter wheat to the corn-soybean crop rotation did not have a significant effect on the amount of oxidatively resistance, residual SOC at either location. However, the presence of more stable organic matter at WARS than NWARS, indicated by greater water stable aggregates and carbon content in both the bulk and s+c fraction, show that certain soils are functionally better at storing and protecting carbon than others. Research looking at the capacity of soils to preserve carbon and nitrogen showed that soils with higher silt and clay content tend to contain greater amounts of associated carbon and nitrogen (Hassink, 1997). Figure 2.3 supports that claim for our research, showing that samples with a greater amount of silt and clay also contained a greater amount of carbon. Others have followed that idea up with more work, suggesting that soils have a limit to the amount of carbon and nitrogen they can store. The idea is that if carbon inputs were to continue increasing and the amount of particulate organic matter went up without any additional carbon moving into the silt and clay fraction, that would suggest that the soils had reached their capacity to store carbon in a stabile fashion (Chung et al., 2008; Stewart et al., 2008). This concept is key when making land management decisions, since knowing the capability and capacity farms have for sequestering carbon can help resource managers make best practice decisions and help transition their properties to effective conservation agricultural practices. In this research we saw lower amounts of carbon in the particulate organic matter fraction of the soil with higher amounts in the silt and clay fraction and the residual SOC fraction. To fully assess whether these soils are at capacity, future work should monitor the amount of organic matter inputs, the carbon content of

those inputs, and compare each of those to the amount of carbon in found in each fraction.

The bulk density values being slightly lower at NWARS than WARS is consistent with the higher clay content in NWARS soils than WARS soils. The significance of plot location on water stable aggregates is likely due to this variation in particle size distribution. The lack of any significant differences in the amount of carbon found in the residual fraction of the soil indicates that either more time than just a few crop rotation cycles is needed for soil carbon levels to build up, or that more than a single additional crop is needed to make a significant difference. A 17-year experiment, compared to the 8 years that these experimental plots have existed, showed that no-tillage practices increased carbon stocks, with effects more noticeable in the top 0-10 cm layer of the soil profile than deeper layers (Liu et al., 2014). While there may not be a difference in carbon stocks when comparing crop rotations for the samples in this research, other work suggests that even small changes in stocks as a result of diversifying crop rotations can result in a significantly different soil organic matter composition, highlighting the importance of molecular level carbon assessments to monitor land management impacts on soil carbon stabilization (Man et al., 2021).

These experimental plots have already been planned and managed for future research by adding additional cover crops to the rotations in the form of cereal rye and red clover. Proper planning to take soil samples at appropriate times will increase the sample size, providing time series data to allow us to look at changes of soil carbon over time. It would be beneficial to incorporate soil sampling at various depths as well to look

at the effects of crop rotations on the positioning of SOC, determining whether any content is being moved vertically down into the soil profile.

2.5. Tables

Table 2.1. Average values and their standard deviations for the soil physical and chemical characteristics for Northwest Agricultural Research Station (NWARS) and Western Agricultural Research Station (WARS): Bulk density (BD), water stable aggregates (WSA), pH, electrical conductivity (EC), sand content, silt content, and clay content.

			BD (g cm^{-3})		WSA (%)		pH		EC (dS m^{-1})		Sand (%)		Silt (%)		Clay (%)	
Location	Rotation	n	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
NWARS	CS	4	1.11	0.06	0.69	0.06	5.01	0.21	0.77	0.41	21.50	0.53	42.28	0.48	36.22	0.62
	CSW	4	1.18	0.09	0.68	0.07	5.32	0.10	0.43	0.04	22.80	0.80	41.96	1.42	35.24	0.90
WARS	CS	4	1.24	0.10	0.81	0.07	4.83	0.28	0.76	0.18	17.10	2.57	54.42	1.90	28.48	3.93
	CSW	4	1.20	0.04	0.84	0.02	4.87	0.22	0.81	0.13	17.23	2.06	52.43	1.26	30.35	2.19

Table 2.2. Carbon stocks at Northwestern Agricultural Research Station (NWARS) and Western Agricultural Research Station (WARS) for bulk sample and each fraction under both corn-soybean (CS) and corn-soybean-wheat (CSW).

		Mg bulk C ha^{-1}		Mg sc-rsoc C ha^{-1}		Mg rsoc C ha^{-1}		Mg sa C ha^{-1}		Mg pom C ha^{-1}	
Location	Rotation	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NWARS	CS	22.78	1.57	12.78	0.99	5.40	0.33	1.49	0.29	3.00	0.58
	CSW	24.97	2.48	13.38	1.51	5.80	0.71	1.80	0.56	2.44	0.86
WARS	CS	28.37	2.70	15.47	0.68	5.81	0.48	2.73	1.81	3.78	1.03
	CSW	28.71	1.42	16.09	0.61	6.31	0.27	1.74	0.49	3.24	0.86

Table 2.3. Nitrogen stocks at Northwestern Agricultural Research Station (NWARS) and Western Agricultural Research Station (WARS) for bulk sample and each fraction under both corn-soybean (CS) and corn-soybean-wheat (CSW).

		Mg bulk N ha^{-1}		Mg sc-rsoc N ha^{-1}		Mg rsoc N ha^{-1}		Mg sa N ha^{-1}		Mg pom N ha^{-1}	
Location	Rotation	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NWARS	CS	2.53	0.38	1.20	0.13	0.83	0.05	0.13	0.03	0.16	0.04
	CSW	2.63	0.21	1.26	0.15	0.89	0.10	0.17	0.05	0.13	0.04
WARS	CS	2.75	0.34	1.32	0.12	0.71	0.03	0.25	0.15	0.23	0.06
	CSW	2.77	0.21	1.41	0.10	0.74	0.06	0.16	0.05	0.18	0.05

2.6. Figures

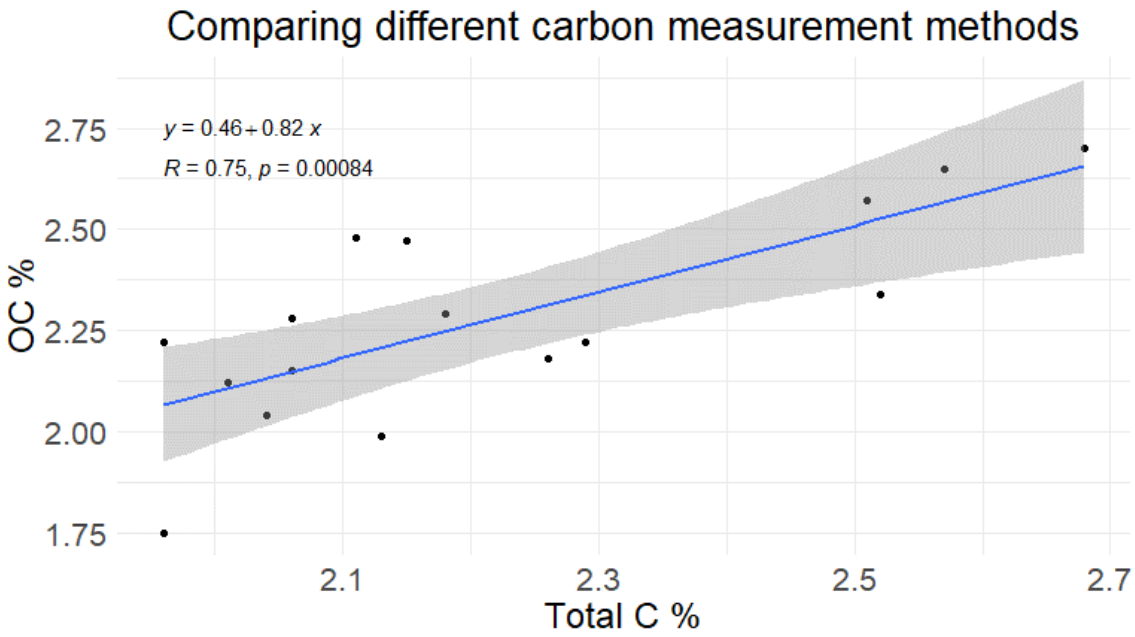

Figure 2.1. A linear regression model comparing the organic carbon percentages determined by dichromate digestion on the y-axis and total carbon analysis on the x-axis.

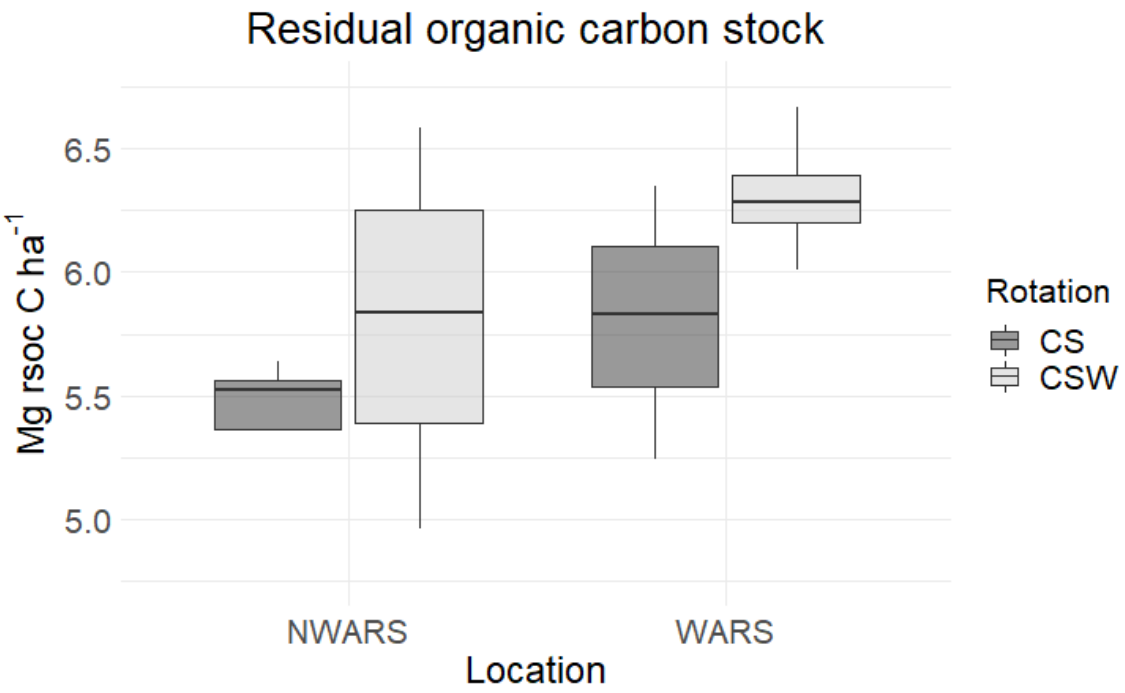

Figure 2.2. Carbon stocks determined in the residual SOC fraction of the samples for both CS and CSW at both locations, reported in Mg rSOC-C ha-1. Tukey multiple comparisons of means after performing ANOVA was used to show level of significance at $\alpha = 0.05$: $p = 0.64$ at NWARS and $p = 0.47$ at WARS.

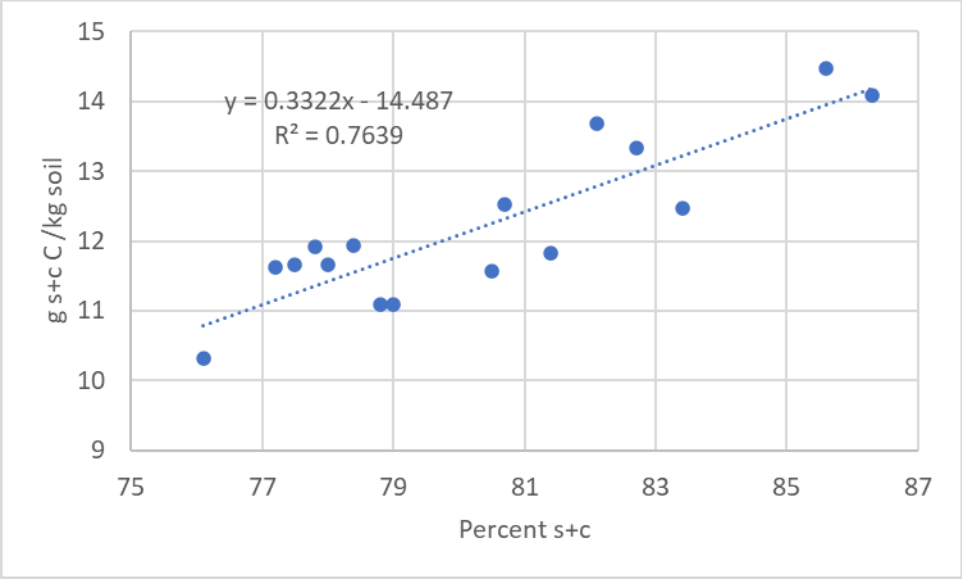

Figure 2.3. Relationship between C in the particle size fraction < 53 µm (silt and clay in g kg^{-1} soil) and the percentage of soil particles < 53 µm.

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Appendix

Table A.1. Total number of contigs identified for each sample (N), the number of individual species associated with those identifications (S), Shannon diversity index (H), and equitability index (E_H). Reps with * were removed before calculating H and E_H due to the low number of contigs read during the long-read sequencing process.

Rep	Location	Rotation	N	S	H	E_H
1	NWARS	CS	496	42	3.254	0.871
2	NWARS	CS	740	50	2.992	0.765
3	NWARS	CS	940	50	3.089	0.790
4*	NWARS	CS	42	8	1.899	0.913
1*	NWARS	CSW	10	2	0.673	0.971
2	NWARS	CSW	438	36	2.358	0.658
3	NWARS	CSW	839	51	2.918	0.742
4	NWARS	CSW	1785	85	3.726	0.839
1	WARS	CS	565	52	3.506	0.887
2	WARS	CS	1041	37	2.259	0.626
3	WARS	CS	2968	61	2.568	0.625
4	WARS	CS	648	32	2.402	0.693
1*	WARS	CSW	44	10	2.162	0.939
2	WARS	CSW	247	43	3.376	0.898
3	WARS	CSW	149	23	2.777	0.886
4	WARS	CSW	1131	56	3.116	0.774

Table A.2. Top twenty ASVs at the Northwest Agricultural Research Station (NWARS) in terms of average biomass found in each rotation. Average biomass for each species was calculated by multiplying relative abundance by total fungal biomass in each sample and taking the average across replicates.

CS			CSW		
Genus	**Species**	**nmol g^{-1}**	**nmol g^{-1}**	**Genus**	**Species**
Mariannaea	punicea	18.02673	29.54471	Mortierella	elongata
Mortierella	elongata	5.733855	13.13734	Fusarium	oxysporum
Neocosmospora	rubicola	5.437204	3.401393	Neocosmospora	rubicola
Trichoderma	viride	4.658206	3.400061	Fusarium	oxysporum
Fusicolla	aquaeductuum	4.221321	2.496408	Gibellulopsis	piscis
Mortierella	elongata	3.010324	1.689257	Condenascus	tortuosus
Condenascus	tortuosus	2.969904	1.655744	Preussia	flanaganii
Mortierella	minutissima	2.739329	1.594339	Fusicolla	septimanifiniscientiae
Fusarium	oxysporum	2.588365	1.440154	Mortierella	gamsii
Gibellulopsis	piscis	2.485631	1.431004	Corynespora	cassiicola
Tausonia	pullulans	2.400153	1.411113	Mariannaea	punicea
Humicola	nigrescens	2.132239	1.359146	Chalara	heteroderae
Fusarium	oxysporum	1.974784	1.3116	Neocosmospora	falciformis
Fusicolla	aquaeductuum	1.827633	1.282544	Fusarium	waltergamsii
Trichoderma	viride	1.761384	1.054946	Cladosporium	delicatulum
Fusicolla	septimanifiniscientiae	1.761219	0.866716	Operculomyces	laminatus
Fusarium	waltergamsii	1.49039	0.854672	Mortierella	hyalina
Mortierella	gamsii	1.414238	0.840837	Setophoma	terrestris
Setophoma	terrestris	1.294183	0.788928	Apodus	deciduus
Neocosmospora	rubicola	1.19996	0.755138	Exophiala	equina

Table A.3. Top twenty ASVs at the Western Agricultural Research Station (WARS) in terms of average biomass found in each rotation. Average biomass for each species was calculated by multiplying relative abundance by total fungal biomass in each sample and taking the average across replicates.

CS			CSW		
Genus	**Species**	**nmol g^{-1}**	**nmol g^{-1}**	**Genus**	**Species**
Mariannaea	punicea	15.5026	6.428677	Mortierella	minutissima
Trichoderma	viride	10.68282	5.343598	Mortierella	elongata
Humicola	nigrescens	7.320585	3.912941	Fusarium	oxysporum
Condenascus	tortuosus	6.902459	3.782703	Tausonia	pullulans
Mortierella	elongata	5.163057	3.332516	Fusarium	oxysporum
Neocosmospora	rubicola	5.136707	3.076169	Hymenoscyphus	menthae
Fusicolla	aquaeductuum	4.636464	2.8998	Neocosmospora	rubicola
Fusarium	oxysporum	3.554284	2.795914	Mortierella	hyalina
Trichoderma	viride	3.297845	2.655419	Fusicolla	aquaeductuum
Tausonia	pullulans	3.1983	2.352619	Gibellulopsis	piscis
Fusicolla	aquaeductuum	2.039155	2.340121	Mortierella	exigua
Gibellulopsis	piscis	1.866926	2.282333	Mariannaea	punicea
Alternaria	angustiovoidea	1.843347	1.958734	Condenascus	tortuosus
Mortierella	minutissima	1.75917	1.941423	Operculomyces	laminatus
Setophoma	terrestris	1.750214	1.853195	Solicoccozyma	terrea
Setophoma	terrestris	1.372893	1.653055	Mortierella	gamsii
Mortierella	gamsii	0.981846	1.153563	Mortierella	elongata
Solicoccozyma	terricola	0.974715	1.067832	Exophiala	equina
Fusarium	kurunegalense	0.864781	1.065585	Humicola	nigrescens
Mortierella	hyalina	0.848065	0.998984	Arthrobotrys	arthrobotryoides

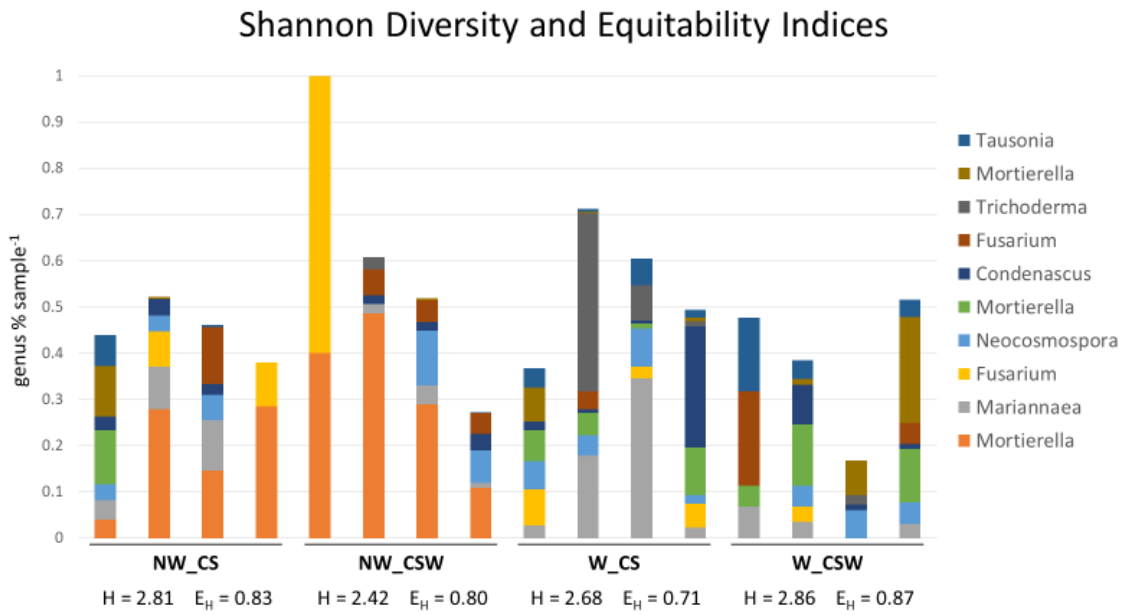

Figure A.1. Diversity (H) and evenness of the distribution of species abundance (E_H) for both corn-soybean (CS) and corn-soybean-wheat (CSW) at the Northwest Agricultural Research Station (NW) and the Western Agricultural Research Station (W).