Pedobiologia - Journal of Soil Ecology 81-82 (2020) 150666

Contents lists available at ScienceDirect

Pedobiologia - Journal of Soil Ecology

journal homepage: www.elsevier.com/locate/pedobi

Winter cover crops and no till management enhance enzyme activities in soybean field soils

Heather L. Tyler

Crop Production Systems Research Unit, USDA Agricultural Research Service, Stoneville, MS, United States
USDA Agricultural Research Service, Crop Production Systems Research Unit, PO Box 350, Stoneville, MS 38776, United States

ARTICLE INFO

Keywords:
Microbial biomass
Cover crops
No till
Soil enzymes
Soil health

ABSTRACT

Elimination of tillage and planting winter cover crops are two conservation practices employed in agricultural row crop production to promote the accumulation of plant residues on the soil surface, improve soil structure, reduce erosion, and decrease losses of excess nutrients in runoff. The purpose of the current study was to assess the effects of no till management and two different cover crop species, Elbon rye (*Secale cereal* L.) and Crimson clover (*Trifolium incarnatum* L.), on microbial aspects of soil health, namely microbial biomass and enzyme activities (phosphatase, β-glucosidase, N-acetylglucosaminidase, and fluorescein diacetate [FDA] hydrolysis) in order to determine potential impacts on the size and nutrient cycling capabilities of microbial communities in lower Mississippi Delta soybean (*Glycine max* L.) field soils over a three-year period. Microbial biomass in surface soil was elevated under no till and both cover crop treatments. Soil enzyme activities associated with organic matter breakdown were increased by cover crop more than tillage, while phosphatase and FDA hydrolysis were increased by both. The higher activities due to cover crop input appear to be the result of an enlarged microbial community as well as increased substrate availability. While these enhanced soil biological traits did not translate into higher soybean yields, longer evaluation periods may be necessary to fully appreciate the benefits of improved soil health in crop production systems.

1. Introduction

Agricultural production practices employed in the cultivation of crops such as corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and soybean (*Glycine max* L.) have numerous detrimental effects beyond the beneficial role they play in sustaining crop production. Tillage contributes to weed control and prepares soil for seed planting but can result in increased erosion (Prasuhn, 2012). Meanwhile, application of pesticides and fertilizers can eventually lead to these chemicals being transported into downstream ecosystems (Tilman, 1999; Kröger et al., 2012). These practices also have negative impacts on soil health, with sustained crop production resulting in decreases in soil structure (Prasuhn, 2012) and organic matter (Kumar et al., 2012), as well as in the size and activity of microbial communities in soils (Tyler et al., 2016). Such effects are of increasing interest, as the long-term impacts of continued soil degradation are unclear. Some soil health parameters, particularly microbial biomass and enzyme activities, have been associated with crop yield (Lupwayi et al., 2015), highlighting their potential impact on agricultural production and the need for research on how to maintain healthy soils in crop fields.

There are various management practices based on minimizing impacts of agriculture on soil health and the environment. Conservation tillage involves reducing tillage frequency or eliminating tillage altogether (no till), while cover crops are used to maintain vegetative ground cover over fields during the winter months rather than leaving them fallow. Both these practices leave plant residues on the soil surface, which helps preserve soil moisture by limiting evaporation (Locke and Bryson, 1997) and also decreases the loss of herbicides, nutrients, and sediments in runoff, thereby improving water quality in downstream ecosystems (Krutz et al., 2009; Knight et al., 2013; Locke et al., 2015). They can also increase aggregate stability (Blanco-Canqui et al., 2009; Mitchell et al., 2017) as well as promote the accumulation of organic carbon in surface soil layers (Kumar et al., 2012; Locke et al., 2013). Conversely, elimination of tillage and planting of winter cover crops can have negative impacts. No till management often necessitates increased application of herbicides for weed control (Tyler and Locke, 2019). Meanwhile, cover crops can serve as a host for pathogens that can be transmitted to summer crops if they are terminated too late in the spring (Bakker et al., 2016). As such, interaction of both positive and negative effects, such as reduced herbicide loss in runoff versus increased herbicide application in fields, must also be considered when implementing these conservation practices.

E-mail address: heather.tyler@usda.gov.

https://doi.org/10.1016/j.pedobi.2020.150666
Received 20 September 2019; Received in revised form 16 July 2020; Accepted 16 July 2020
Available online 17 July 2020
0031-4056/ Published by Elsevier GmbH.

Both no till and cover cropping lead to shifts in the soil microbial community (Jackson et al., 2003; Helgason et al., 2010) responsible for mediating nutrient cycling. As a result, factors that influence soil microbes can have lasting impacts on soil function and plant growth. No till management has been found to enhance microbial biomass (Feng et al., 2003; Helgason et al., 2010; Zhang et al., 2014), as well as soil enzymatic activities (Acosta-Martínez et al., 2007; Zhang et al., 2014; Mbuthia et al., 2015) in surface soils. Cover crops can have similar beneficial effects. For instance, rye (*Secale cereal* L.) can return an average of 1534 and 4095 kg C per ha to soil each year in cotton and corn systems, respectively, with the amount increasing over each successive year (Balkcom et al., 2013), and has been found to significantly enhance microbial biomass carbon and nitrogen in soils (Steenwerth and Belina, 2008a, b). Meanwhile, leguminous cover crops can also increase soil nitrate to levels twice as high as seen in rye (Zablotowicz et al., 2010).

While the general effects of tillage and winter cover crops are well documented, different cover crop species can have variable impacts on soil health and crop yield. Regional differences in soil and climate can also influence the efficacy of these practices. Prior work on cover crops in Mississippi Delta field soils has focused on their role in weed control, with only minimal microbial analyses that assessed total loads of soil microbes using culture-based methods that exclude microorganisms that do not grow under laboratory conditions and evaluated only one enzyme for shifts in general microbial activity and hydrolytic potential (Reddy et al., 2003). As such, the purpose of this study was to assess effects of two different cover crop species, Crimson clover (*Trifolium incarnatum* L.) and Elbon rye (*Secale cereal* L.), on microbial aspects of soil health, including microbial biomass and an expanded range of soil enzyme activities, over a three-year period in plots maintained under till and no till management for 14 years, with the goal of determining if either species was more beneficial for soil health and crop yield.

2. Materials and methods

2.1. Study location, experimental design, and crop cultivation

The study site is located on an experimental research farm in Stoneville, MS. Monthly precipitation and average daily soil temperatures from the Stoneville location were obtained from the Mississippi State University Delta Agricultural Weather Center and is presented in Fig. 1 (http://deltaweather.extension.msstate.edu/coop-stoneville). The field was set up as a randomized complete block with four blocks each of tilled and no till land. Each block contained three plots (32m × 8.4m) planted to either Elbon rye, Crimson clover, or no winter cover crop. Tillage treatments were previously established in October 2000 (Locke et al., 2013) and have been maintained since that time.

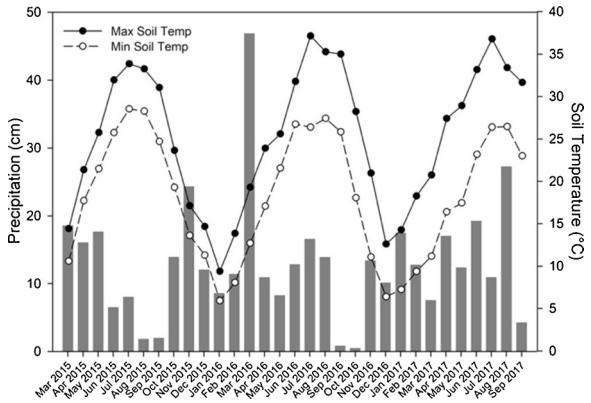

Fig. 1. Monthly precipitation (solid bars) and average monthly minimum (open symbol) and maximum (closed symbol) soil temperatures at the study location from March 2015 to September 2017.

The current study was initiated in the fall of 2014, with cover crops planted in mid-October of each year. Briefly, tilled plots were plowed to a depth of 15 cm in the fall after soybean harvest but before cover crop planting, while no till plots were not plowed during the entire year. Cover crops were allowed to grow through the winter and terminated by paraquat application in April of each year. Soybeans (Asgrow AG4632) were planted in early May and harvested in late September. The experiment was concluded after the fall soybean harvest in September 2017.

2.2. Sample collection

Cover crop biomass was collected in April 2016 and 2017 within one week before termination, using a 1 m^2 quadrat two times in each plot. Plant biomass was dried in the greenhouse for two months and recorded as the average dry weight of the two quadrats from each plot. Soil samples were collected each year prior to cover crop termination, at soybean planting, early and mid-summer, and after soybean harvest in the fall. Eight replicate surface soil cores were collected from the 0 − 5 cm depth from each plot using 1.8 cm diameter soil probes, and pooled to form one composite sample per plot. At the April 2016 and 2017 sampling times, both 0 − 5 cm and 5 − 15 cm soils were collected in order to monitor potential changes in the depth of soil disrupted by tillage. All soil samples were passed through a 2 mm sieve and stored at field moisture at 4 °C until analysis.

2.3. Enzymatic activity

All soil samples were assayed for the activities of β-glucosidase, cellobiohydrolase, N-acetylglucosaminidase (NAGase), and phosphatase in a 96-well plate format using pNP-linked substrates as previously described (Jackson et al., 2013). Fluorescein diacetate (FDA) hydrolysis was assayed using a protocol based on Schnürer and Rosswall (1982), modified for 96-well plate format. Briefly, 5 mL of 50 mM potassium phosphate buffer (pH 7.6) was added to 2 *g* of soil in 50 mL polypropylene centrifuge tubes. Tubes were vortexed and 150 µL were pipetted into six wells per sample in a deep well block, being sure to vortex between each well to keep soil particles suspended. Substrate (150 µL 12 µM FDA in phosphate buffer) was added to four sample wells. Phosphate buffer (150 µL) without FDA was added to the last two wells to serve as sample blanks. These 96-well blocks were incubated at 30 °C for one hour with shaking (200 rpm). Reactions were stopped by the addition of 300 µL acetone, shaken for an additional 3 min, and centrifuged at 3000 × *g* for 15 min. Supernatant (300 µL) from each well was withdrawn and transferred to a clean, 96-well plate, and absorbance was measured at 490 nm. Fluorescein concentration was calculated by comparison to a standard curve and FDA hydrolysis calculated as nmole fluorescein produced per g dry weight of soil per hour.

2.4. Microbial biomass and soil organic matter

Microbial biomass was determined for all soil samples using the chloroform fumigation extraction method (Horwath and Paul, 1994). Briefly, 12 *g* fresh weight of chloroform fumigated and unfumigated subsamples from each site were extracted with 50 mL of 0.5 M K_2SO_4 for 1 h with shaking (200 rpm). Extracts were gravity filtered through Whatman GF/F filter paper (GE Healthcare, Pittsburgh, PA). Total organic carbon (TOC) and total nitrogen (TN) concentrations were determined on a Shimadzu TOC-L analyzer with TNM-L module. Carbon and N concentrations in soil were calculated on a dry weight basis and microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were calculated using the following equations:

$$MBC = EC \div kEC$$

$$MBN = EN \div kEN$$

Where EC and EN are the difference in C and N between chloroform fumigated and unfumigated subsamples, and kEC and kEN are constants estimated at 0.35 and 0.68, respectively (Horwath and Paul, 1994). Triplicate soil samples (5 g fresh weight) were oven dried overnight at 105 °C and then weighed to determined soil moisture (SM), reported as percentage of the soil fresh weight. Soil organic matter (SOM) was then determined by ashing the oven dried soils at 500 °C for 2 h and reported as the percentage of dry soil burned off.

2.5. Statistics

Effects of tillage and cover crop on soil pH, SOM, SM, microbial biomass, and soil enzymes were determined by two-way repeated measures analysis of variance (ANOVA) of data from all timepoints for each depth in JMP version 11.2.0. (SAS Institute Inc., Carey, NC). The numerical differences between 0 – 5 and 5 – 15 cm soil pH, SM, SOM, microbial biomass, and enzyme values were calculated for the April timepoints and then compared by 2-way repeated measures ANOVA to determine how tillage and cover crops influenced distribution of these factors across depth within the tillage zone. Individual two-way ANOVA's followed by Tukey's Honestly Significant Difference (HSD) test were also performed on all parameters for each depth and timepoint. Variance inflation factors (VIFs) for MBC, MBN, SM, SOM, and pH were calculated in JMP and found to be less than 5, demonstrating no multicollinearity between these characteristics. Pearson correlations of data from all timepoints and depths were performed in JMP to determine associations between soil enzymes and pH, SM, SOM, and microbial biomass. Pearson correlations were also performed between cover crop biomass inputs and average annual enzyme activities and soybean yield from 2016 and 2017. Hierarchical partitioning analysis was performed using the "hier.part" package version 1.0–2 (MacNally and Walsh, 2004) in R Studio to determine the independent effects of pH, SM, SOM, and microbial biomass on soil enzyme activities. All analyses were assessed using an α of 0.05.

3. Results

3.1. Basic field plot and soil parameters

Elbon rye (rye) and Crimson clover (clover) winter cover crops produced approximately 3346 – 5972 kg dry weight per ha (kgdw ha^{-1}) plant biomass, significantly greater than the 1420–1935 kgdw ha^{-1} from natural vegetation that grew in unplanted (no cover) plots ($p < 0.0001$; Table 1). Neither cover crop produced significantly greater biomass between till and no till treatments in 2016 or 2017. Meanwhile, crop yields were variable, with few consistent differences between treatment, although two-way repeated measure ANOVA revealed lower yields from rye plots ($p = 0.015$), particularly in tilled soil (Table 1). There was a negative correlation between biomass of winter vegetation and crop yield of -0.380 ($R^2 = 0.144$; $p = 0.008$) across all treatments. When broken down by cover crop, negative correlations between winter biomass and yield were greater in rye ($r = -0.669$; $R^2 = 0.448$; $p = 0.005$) and clover ($r = -0.730$; $R^2 = 0.533$; $p = 0.001$) compared to no cover ($r = -0.043$; $R^2 = 0.002$; $p = 0.874$) plots.

Soil pH ranged from 5.8 to 7, SM from 8.8 to 16.3%, and SOM from 4.8 to 6.6 % throughout the three-year study period, with SM being tending to be higher in 5 – 15 cm soils and SOM tending to be higher in 0 – 5 cm soils ($p < 0.001$, Table 2). The difference in SOM between depths was greater in no till soils (Table 2, $p < 0.0001$). However, tillage did not have a significant effect on soil pH, SM, or SOM in 5 – 15 cm in April soils (Table 2). In contrast, cover crop had a significant effect on pH, being lower in rye and clover than no cover plots in both 0 – 5 cm and 5 – 15 cm depths ($p < 0.001$), and SM, tending to be higher in cover vs no cover plots, with this difference increasing each year in 0 – 5 cm soil ($p < .0001$). Cover crops did not significantly impact SOM within either depth, but as with tillage, the difference in SOM between depths was greater in cover cropped compared to no cover plots ($p = 0.0004$).

3.2. Effects on microbial biomass

Two-way repeated measures ANOVA indicated there were no interactions between tillage and cover crop on microbial biomass, but both treatment types were significant independently of each other. MBC ($p < 0.0001$) and MBN ($p = 0.0003$) were higher in no till plots in 0 – 5 cm soil (Fig. 2a and c). An opposite trend was observed for MBC ($p = 0.024$) and MBN ($p = 0.003$) in 5 – 15 cm soils, being higher in till plots, particularly in 2016 (Table 3). As with SOM, microbial biomass tended to be higher in 0 – 5 cm than 5 – 15 cm soils, and the size of the difference was greater in no till soils for both MBC ($p < 0.0001$) and MBN ($p < 0.0001$). Rye ($p \leq 0.015$) and clover ($p \leq 0.041$) tended to have higher MBC than no cover at most time points in 0 – 5 but not 5 – 15 cm soils, and rye was occasionally greater than clover, in August 2015 ($p = 0.004$) and September 2017 ($p = 0.024$) (Fig. 2b). These cover crop effects were less notable in April 5 – 15 cm soils, although still significant ($p = 0.046$; Table 3). Similar cover crop effects were observed for MBN ($p = 0.001$), but with fewer timepoints where cover crops differed significantly from no cover plots (Fig. 2d), and no significant difference in 5 – 15 cm soils.

3.3. Effects on soil enzyme activities

There were no interactions between tillage and cover crop treatments for beta-glucosidase, cellobiohydrolase, NAGase, phosphatase, or FDA hydrolysis in 0 – 5 cm soils. As such, the 0 – 5 cm activities have been broken down and the effects of tillage and cover crop treatment examined individually. In deeper 5 – 15 cm soils, there were interactions between tillage and cover for cellobiohydrolase ($p = 0.038$) and NAGase ($p = 0.024$). Cellobiohydrolase in 2016 at this depth was clearly influenced by tillage, with elevated levels in tilled plots ($p \leq 0.010$). The interaction between cover and tillage on cellobiohydrolase was only observed in 2017, when tilled rye plots had higher

Table 1
Above ground cover crop biomass inputs and soybean yields*.

	Winter cover biomass (kg ha^{-1})		Soybean yield (kg ha^{-1})		
	2016	2017	2015	2016	2017
Till					
Rye	3977 ± 196	4668 ± 208	4100 ± 205	4048 ± 142	3643 ± 69
Clover	4416 ± 144	5972 ± 763	4283 ± 180	4335 ± 77	3986 ± 161
None	1935 ± 427	1537 ± 277	4501 ± 121	4716 ± 10	4196 ± 196
No till					
Rye	3346 ± 207	4334 ± 469	4353 ± 30	4476 ± 70	3950 ± 83
Clover	3735 ± 141	5744 ± 152	4693 ± 99	4915 ± 36	4190 ± 199
None	1498 ± 66	1420 ± 486	4204 ± 137	4446 ± 142	4166 ± 137

* Values represent mean ± standard error of all measurements taken per treatment (n = 4).

Table 2
Average soil pH, soil moisture (SM), and percent organic matter (SOM) values from 2015 to 2017 in 0-5 and 5-15 cm soils*.

Treatment	Depth (cm)	pH			SM (%)			SOM (%)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Till										
Rye	0–5	6.37 ± 0.04	5.9 ± 0.06	5.88 ± 0.05	10.3 ± 0.8	12.6 ± 1	12.4 ± 0.6	5.39 ± 0.21	5.78 ± 0.24	5.76 ± 0.19
	5–15	–	6.4 ± 0.06	6.48 ± 0.08	–	15.3 ± 0.4	14.3 ± 0.6	–	5.63 ± 0.46	5.15 ± 0.52
Clover	0–5	6.29 ± 0.06	5.96 ± 0.08	5.91 ± 0.05	9.5 ± 0.8	11.8 ± 0.9	11.1 ± 0.4	5.68 ± 0.19	5.46 ± 0.22	5.44 ± 0.18
	5–15	–	6.4 ± 0.08	6.35 ± 0.03	–	15.4 ± 1	12.1 ± 0.6	–	5.36 ± 0.44	4.95 ± 0.5
None	0–5	6.85 ± 0.04	6.61 ± 0.05	6.43 ± 0.05	8.8 ± 0.7	10.8 ± 1	9.7 ± 0.4	4.92 ± 0.21	5.26 ± 0.23	5.08 ± 0.21
	5–15	–	6.95 ± 0.1	6.8 ± 0.1	–	17.1 ± 0.3	12.4 ± 0.8	–	5.36 ± 0.41	4.88 ± 0.48
No till										
Rye	0–5	6.26 ± 0.05	6.09 ± 0.07	6.07 ± 0.06	11.6 ± 0.8	12.7 ± 0.9	13.5 ± 0.6	5.85 ± 0.2	6.15 ± 0.22	6.44 ± 0.18
	5–15	–	6.34 ± 0.12	6.47 ± 0.02	–	15.5 ± 0.4	14.3 ± 0.2	–	5.09 ± 0.5	4.97 ± 0.59
Clover	0–5	6.15 ± 0.06	6.08 ± 0.06	6.1 ± 0.05	11.2 ± 0.7	13.1 ± 0.9	13 ± 0.5	5.87 ± 0.24	6.38 ± 0.3	6.6 ± 0.23
	5–15	–	6.5 ± 0.09	6.5 ± 0.05	–	14.7 ± 0.5	11.2 ± 0.4	–	5.48 ± 0.78	5.44 ± 0.78
None	0–5	6.4 ± 0.04	6.3 ± 0.06	6.33 ± 0.06	10.8 ± 0.7	11.5 ± 0.8	10.6 ± 0.4	5.59 ± 0.24	5.77 ± 0.27	6 ± 0.23
	5–15	–	6.67 ± 0.07	6.72 ± 0.05	–	16.3 ± 0.4	14.7 ± 0.5	–	5.19 ± 0.54	5 ± 0.65

* Values represent mean ± standard error of all measurements taken per treatment for 0–5 cm each year (n = 20) and 5–15 cm each April (n = 4).

activity than no till rye, and both no cover treatments ($p \leq 0.027$) and tilled clover displayed higher activity than no till no cover plots ($p = 0.018$; Table 3). Tilled rye plots also had 1.4–1.6 times greater NAGase activity than no till rye and clover in 5–15 cm soil during both 2016 and 2017 (Table 3).

Repeated measures ANOVA for all time points from 2015 to 2017 indicated phosphatase ($p < 0.0001$) and FDA hydrolysis ($p = 0.011$) activities were significantly higher in no till 0–5 cm soils (Fig. 3A, C). However, only phosphatase was consistently higher in no till plots over all three years of the study. Cellobiohydrolase activity in no till plots was elevated in early 2015 and late 2017, but indistinguishable from tilled plots in 2016 (Fig. 4C), while FDA hydrolysis was inconsistently elevated at various time points through 2015–2017 (Fig. 3C). Activities of beta-glucosidase (Fig. 4A) and NAGase (Fig. 4E) in no till plots were only elevated in April 2015 ($p \leq 0.020$) and September 2017 ($p \leq 0.025$). In contrast, deeper 5–15 cm tilled soils had higher levels of beta-glucosidase ($p < 0.0001$), cellobiohydrolase ($p < 0.0001$), NAGase ($p < 0.0001$), and FDA hydrolysis ($p = 0.006$; Table 3). Two-way ANOVA indicated that phosphatase activity was also elevated in 5–15 cm till soils, but only in April 2016 ($p = 0.008$; Table 3).

Cover crop had a significant impact on the activities of all enzymes assayed, with both rye and clover displaying elevated activities of beta-glucosidase ($p < 0.0001$), cellobiohydrolase ($p < 0.0001$), NAGase ($p < 0.0001$), phosphatase ($p < 0.0001$), and FDA hydrolysis

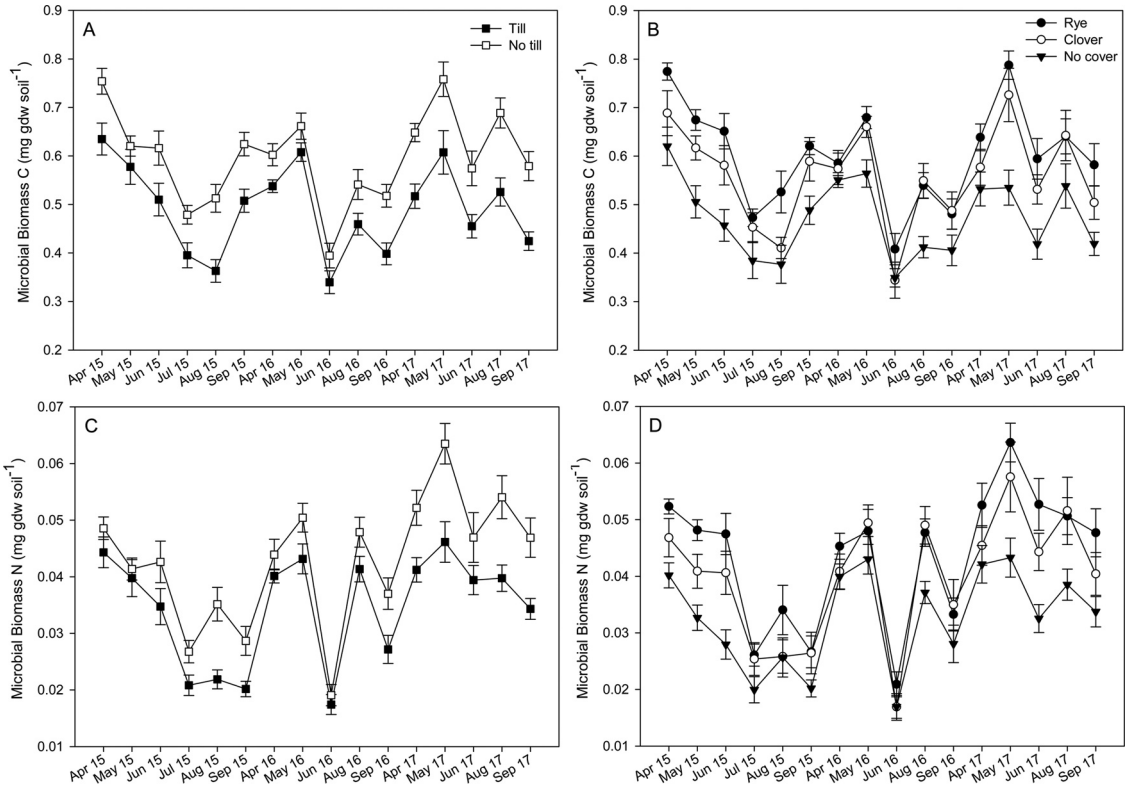

Fig. 2. Microbial biomass carbon (A and B) and nitrogen (C and D) levels in 0-5 cm soil in 24 field plots spanning all combinations of tillage-cover crop treatment from 2015 to 2017 with graphs broken down by tillage (A and C) and cover crop (B and D) treatments. The two tillage treatments (n = 12) include till (closed square) or no till (open square), and the three cover treatments (n = 8) include rye cover (closed circle), clover cover (open circle), or no cover (inverted triangle). Values represent mean ± standard error.

Table 3
Microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), and soil enzyme activities in 5-15 cm soils in April 2016 and April 2017*.

	No till			Till		
	Rye	Clover	Unplanted	Rye	Clover	Unplanted
	2016					
MBC	0.355 ± 0.033	0.349 ± 0.024	0.354 ± 0.021	0.467 ± 0.033	0.379 ± 0.023	0.440 ± 0.027
MBN	0.018 ± 0.002	0.017 ± 0.002	0.016 ± 0.002	0.031 ± 0.005	0.022 ± 0.001	0.027 ± 0.001
Phosphatase	1.41 ± 0.37	1.56 ± 0.36	1.33 ± 0.31	2.02 ± 0.48	2.28 ± 0.15	1.71 ± 0.07
NAGase	0.113 ± 0.01	0.119 ± 0.004	0.106 ± 0.003	0.182 ± 0.021	0.131 ± 0.007	0.162 ± 0.008
beta-glucosidase	0.331 ± 0.035	0.312 ± 0.005	0.271 ± 0.01	0.535 ± 0.027	0.461 ± 0.03	0.54 ± 0.035
Cellobiohydrolase	0.070 ± 0.007	0.070 ± 0.004	0.055 ± 0.003	0.126 ± 0.010	0.104 ± 0.002	0.127 ± 0.006
FDA hydrolysis	83 ± 8.7	75.6 ± 8.3	75.6 ± 9.0	123.7 ± 6.6	107.7 ± 9.3	109.9 ± 9.4
	2017					
MBC	0.374 ± 0.019	0.333 ± 0.006	0.374 ± 0.014	0.402 ± 0.009	0.324 ± 0.023	0.372 ± 0.027
MBN	0.022 ± 0.002	0.018 ± 0.002	0.021 ± 0.002	0.024 ± 0.001	0.02 ± 0.001	0.022 ± 0.002
Phosphatase	1.71 ± 0.09	1.88 ± 0.11	1.55 ± 0.11	1.96 ± 0.16	1.77 ± 0.11	1.37 ± 0.13
NAGase	0.141 ± 0.01	0.127 ± 0.003	0.151 ± 0.016	0.206 ± 0.007	0.173 ± 0.026	0.135 ± 0.009
beta-glucosidase	0.395 ± 0.026	0.435 ± 0.016	0.384 ± 0.017	0.569 ± 0.033	0.48 ± 0.039	0.443 ± 0.019
Cellobiohydrolase	0.076 ± 0.008	0.090 ± 0.003	0.064 ± 0.004	0.118 ± 0.011	0.104 ± 0.009	0.080 ± 0.008
FDA hydrolysis	71.6 ± 11.3	68.3 ± 13.2	61.1 ± 11.5	95.0 ± 11.6	70.7 ± 9.9	86.4 ± 19.7

* Values for MBC (mg C gdw^{-1}), MBN (mg N gdw^{-1}), and enzymes (μmole substrate consumed gdw^{-1} hr^{-1}) represent mean ± standard error (n = 4).

($p \leq 0.0003$) in 0 − 5 soils across most time points (Fig. 4B, D, F, Fig. 3B, D). However, activities in clover plots often tended to be lower than in rye at this depth. In 5 − 15 cm soil, cover crop effects were seen on beta-glucosidase ($p = 0.042$), cellobiohydrolase ($p = 0.011$), NAGase ($p = 0.02$), being higher in cover cropped vs non-cover cropped in no till plots in 2016 and tilled plots in 2017 (Table 3). No significant cover crop effects were observed in phosphatase or FDA hydrolysis at this greater depth.

Effects of both tillage and cover crop on enzyme activities in soil, particularly beta-glucosidase, cellobiohydrolase, NAGase, and phosphatase, appear to be driven by the larger microbial communities present in tilled and cover cropped plots, as evidenced by significant correlations with MBC and MBN ranging from 0.401 to 0.696 ($p < 0.0001$, Fig. 5). Substrate availability also appears to contribute, but to a lesser extent, with correlations to SOM from 0.205 to 0.465 (Fig. 5). In contrast, FDA hydrolysis, had no significant correlation with SOM, but did correlate with microbial biomass, although to a lesser degree than the other enzymes, with correlations of 0.248 − 0.392 (Fig. 5). Hierarchical partitioning confirmed that MBN had the highest independent effect on the activities of all enzymes (26–34 %), while SM had higher independent effects on phosphatase and NAGase than the other enzymes (Fig. 6). Meanwhile, pH had greater independent effects than SOM (Fig. 6). As previously mentioned, cover crops have yet to result in significant differences in SOM levels between treatments (Table 1), but above ground cover crop biomass inputs in 2016 and 2017 correlated highly with average annual enzyme activities for those years (Table 4).

4. Discussion

Previous work conducted at the current study site assessed effects of Balansa clover (*Trifolium michelianum* Savi var. *balansae* (Boiss.) Azn.) and Abruzzi rye (*Secale cereal* L.) in till versus no till treatments in a cotton production system (Locke et al., 2013). The work conducted here aimed to assess effects of Elbon rye and Crimson clover cover crops in soybean fields, a crop that has grown more common in the region since conclusion of the original study. These prior cover crop treatments were terminated eight years before the current study was initiated. However, tillage treatments continued to be maintained during this time, providing the opportunity to assess how long-term differences in tillage can impact how soil responds to cover crop treatment. Prior analyses have demonstrated there are no residual effects from the earlier cover crop treatments on soil pH, SM, SOM, microbial biomass, or soil enzymes (Tyler, 2019). As such, enhanced enzyme activities observed here were attributed to the current Crimson clover and Elbon rye treatments.

The long-term differences in tillage appeared to have only minor effects on the soil biological response to new cover crop inputs, with few synergistic interactions between no till management and winter cover crop observed. Only two enzymes (cellobiohydrolase and NAGase) displayed a significant interaction between cover and tillage treatments, but only in 5 − 15 cm soils. At this depth, NAGase was higher in till *vs.* no till rye, but not till vs no till clover plots for both 2016 and 2017. Meanwhile, cellobiohydrolase only displayed significant interactions between till and cover treatment in 2017, when till rye had higher activity than no till rye and both no cover treatments while till clover was only higher than no till no cover. Given that tillage disrupts the top 15 cm of soil, any above ground biomass left on the surface in the fall was incorporated into the soils of tilled plots, while it was left on the surface in no till plots. As such, the treatment interactions on activities of enzymes involved with organic matter breakdown in 5 − 15 cm but not 0 − 5 cm soils were expected, since the cover crop residues were incorporated in 5 − 15 cm for till but not no till plots. The observation that rye was more effected by tillage than clover may be due the greater persistence of rye residues on the soil surfaces, with 67 % of rye biomass remaining nine weeks after soybean planting compared to only 46 % of crimson clover (Reddy, 2001). Residues incorporated into the soil by tillage are more accessible to soil microbes for degradation compared to no till plots where these residues sit on the soil surface for longer periods of time before the organic matter is gradually reintroduced to the soil profile. Therefore, it may take multiple years for the full effect of cover crop treatments to be realized, and a longer observation period with inputs from successive years of cover crop biomass may be necessary before tillage-based differences in residue incorporation results in notable effects in microbial activities. This theory appears to be corroborated by the delayed interaction between cover crop and tillage in cellobiohydolase activity until the final year of the study.

When considered individually, tillage and cover crop both contributed to shifts in soil health parameters over time. Tillage consistently lowered levels of microbial biomass in 0 − 5 cm soil throughout the entire study, while it tended to have an inhibitory effect on most soil enzyme activities. There are numerous instances where decreased tillage frequency resulted in elevated soil enzyme activities, including beta-glucosidase (Pandey et al., 2014; Sharma et al., 2014; Zhang et al., 2014), beta-glucosaminidase (Zhang et al., 2014), cellobiohydrolase (Pandey et al., 2014), phosphatase (Pandey et al., 2014; Sharma et al., 2014), protease (Zhang et al., 2014), and urease (Sharma et al., 2014). Unlike previous studies where tillage had a greater effect on soil

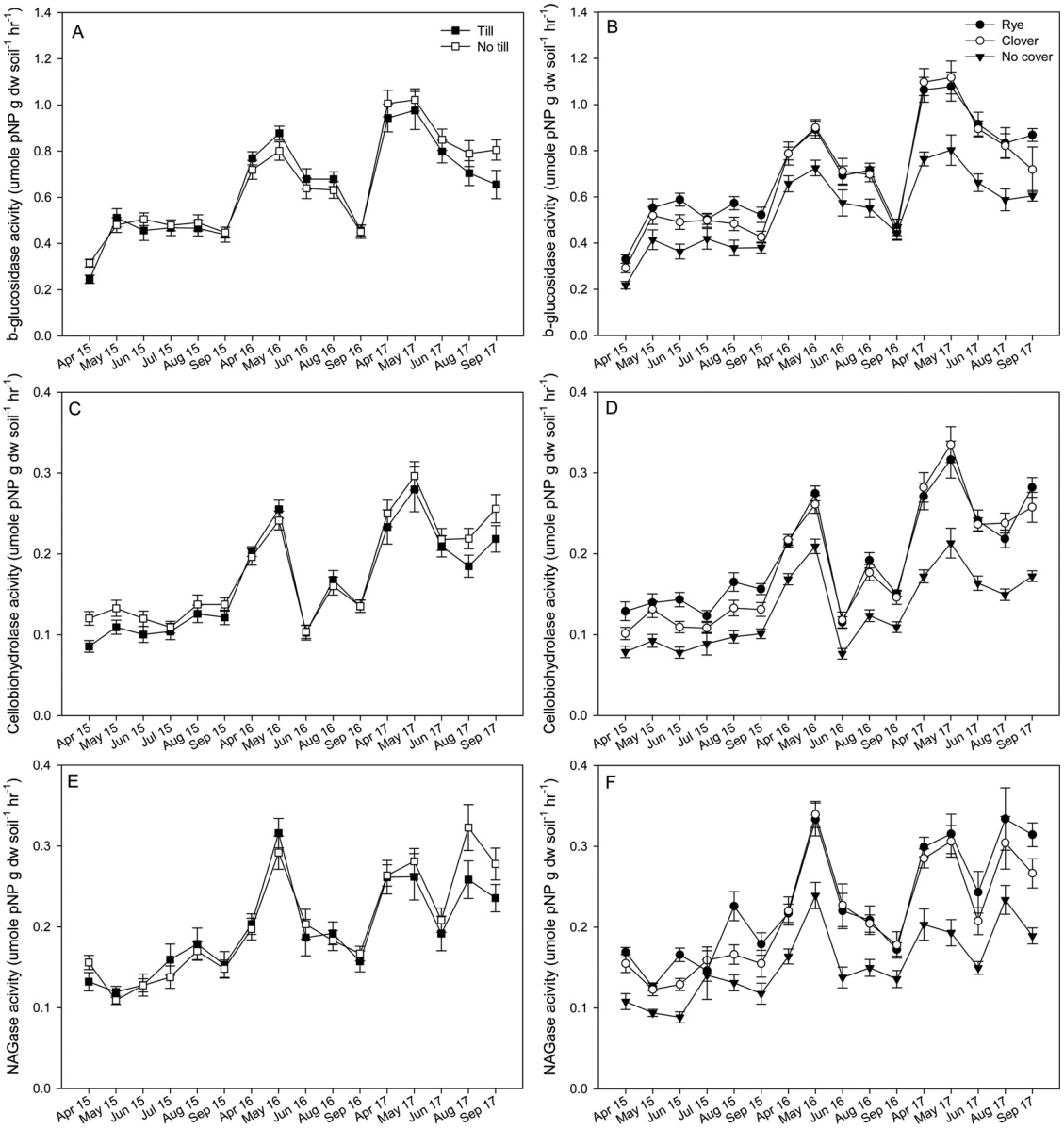

Fig. 3. Activities of beta-glucosidase (A, B), cellobiohydrolase (C, D), and NAGase (E, F) in 0-5 cm soils in 24 field plots spanning all combinations of tillage-cover crop treatment from 2015 to 2017 with graphs broken down by tillage (A, C, E) and cover crop (B, D, F). The two tillage treatments (n = 12) include till (closed square) or no till (open square), and the three cover treatments (n = 8) include rye cover (closed circle), clover cover (open circle), or no cover (inverted triangle). Values represent mean ± standard error.

enzymes than cover crops (Zablotowicz et al., 2010), the current study found that cover crops had a more pronounced and consistent impact on extracellular enzyme activities in soil. This difference may be due to timing of tillage, which was performed at the beginning of the growing summer season in Zablotowicz et al. (2010), but at the end of the growing season after soybean harvest in the current study. Fernandez et al. (2016) also found that winter rye significantly increased NAGase, beta-glucosidase, and phosphatase activities, although this effect was not consistent across sampling times and locations. While both rye and clover increased all enzymes, activities in clover were slightly lower than rye plots at several timepoints, particularly beta-glucosidase, cellobiohydrolase, and NAGase in 2015. This difference is not entirely unexpected, as rye tends to have a higher C:N ratio than Crimson clover (Ranells and Wagger, 1997) and the composition of plant litter (including the C:N ratio) can have an impact on the activities of such enzymes, with C-linked enzymes being positively correlated and N-linked enzymes negatively correlated with C:N ratios of plant residues added to soil (Tian and Shi, 2014). More generally, C:N ratios in soil are negatively correlated with C-cycling enzymes (Dai et al., 2019). The higher C content in rye residues could be responsible for the tendency for higher beta-glucosidase and cellobiohydrolase activities, which are both linked to C-cycling in soils.

Given the increased N-availability expected from a legume cover crop, NAGase (associated with N-mineralization; Ekenler and Tabatabai, 2004) was expected to be enhanced in Crimson clover relative to the other treatments. However, this was not the case, and may be due to the fact that the clover plots tended to have slightly lower levels of microbial biomass, as these enzymes are produced by soil bacteria and fungi in response nutrient inputs. Consistent with this observation, all enzymes had significant correlations with microbial biomass and the difference in microbial biomass between treatments may have confounded the effects of differences in litter composition. In fact, the only time when Crimson clover showed significantly higher activity was in April 2016 with phosphatase, an enzyme not involved in

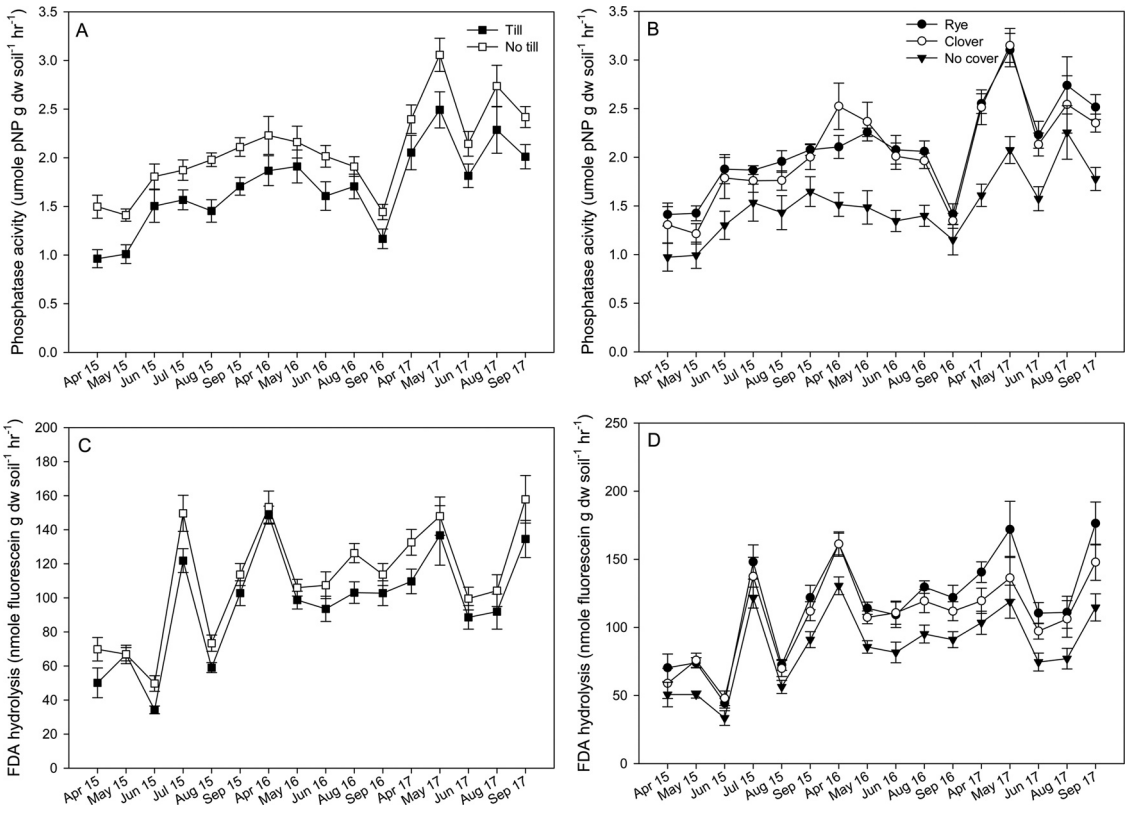

Fig. 4. Activities of phosphatase (A, B) and fluorescein diacetate (FDA) hydrolysis (C, D) in 0-5 cm soils in 24 field plots spanning all combinations of tillage-cover crop treatment from 2015 to 2017 with graphs broken down by tillage (A and C) and cover crop (B and D). The two tillage treatments (n = 12) include till (closed square) or no till (open square), and the three cover treatments (n = 8) include rye cover (closed circle), clover cover (open circle), or no cover (inverted triangle). Values represent mean ± standard error.

catalyzing the hydrolysis of C and N bonds. However, this timepoint was preceded by unusually heavy rains causing regional flooding in March that could have potentially impacted soil enzyme responses to cover crop inputs, as precipitation can have indirect effects on soil phosphatase activities (Margalef et al., 2017). Consistent with these observations, the independent effects of soil moisture were greater on phosphatase than other enzymes, again highlighting the advantage of longer observation periods in order to distinguish consistent responses to management practices from variation in soil conditions due to climatic differences between years.

Differences in enzyme activities between treatments appear to be driven, in large part, by the increased size of the microbial community in cover cropped plots, with the independent effects of microbial biomass contributing 29–41 % of the variability in enzyme activities observed in soil. Similar to the current study, Brennan and Acosta-Martinez (2017) found that MBC and MBN were impacted by cover crop frequency, with higher levels observed in plots planted with cover crops annually compared to only planting a cover crop every four years. This shift in microbial biomass can have substantial impacts on several key soil processes catalyzed by the microbial contingent of soils, including organic matter decomposition, N-fixation, P solubilization, nitrification, pesticide degradation, denitrification, and C cycling (Gonzalez-Quiñones et al., 2011).

Increased substrate inputs from winter cover crops also appears to play a role in stimulating soil enzyme activities, with high correlations between above ground biomass and average annual enzyme activities. While cover crop treatments have yet to significantly increase organic matter levels in soil, the activities of beta-glucosidase, cellobiohydrolase, NAGase, and phosphatase also displayed positive correlations to SOM, consistent with their functions in cellulose degradation (Woodward, 1991), N mineralization (Ekenler and Tabatabai, 2004), and organic phosphate mineralization (Turner et al., 2002), respectively. However, the independent effects of SOM (7–12 %) are small compared to those of microbial biomass (29–41 %). As such, the stimulation of soil enzymes with increasing cover crop biomass may be due to compounds in root exudates excreted while rye and clover were alive, since soil enzymes can remain viable for several months (Schimel et al., 2017).

Of the enzymes assayed in the current study, FDA hydrolysis has been the preferred tool in previous studies assessing microbial activity in Mississippi Delta soils. This assay serves as an indicator of total microbial activity that occasionally correlates with hydrolytic activity in soil (Schnürer and Rosswall, 1982), as FDA serves as a substrate for multiple classes of enzymes, such as proteases, lipases, and esterases (Guilbault and Kramer, 1964; Rotman and Papermaster, 1966). The lower correlations between FDA hydrolysis and SOM and microbial biomass relative to the other enzymes included in the current study confirm the importance of using multiple assays to assess the impacts of land management practices on microbial activities in soil. As these assays assess different processes, relying solely on one assay could result in missing potential effects on soil function.

Interestingly, while rye plots tended to display enhanced soil microbial traits, this did not equate to higher soybean yields. Such observations are not unheard of, and rye can decrease yields due to allelopathic effects (Kessavalou and Walters, 1999). As negative correlations to yield were observed for both clover and rye, two different species, allelopathic effects against soybean may not be the only factor at play. This observation illustrates another concern raised about cover crop implementation: cover crop biomass can tie up soil nutrients during the growing season when they are needed for development of summer cash crops, with time of cover crop termination impacting nutrient release to soil and crop yield (Sainju and Singh, 2001). Cover

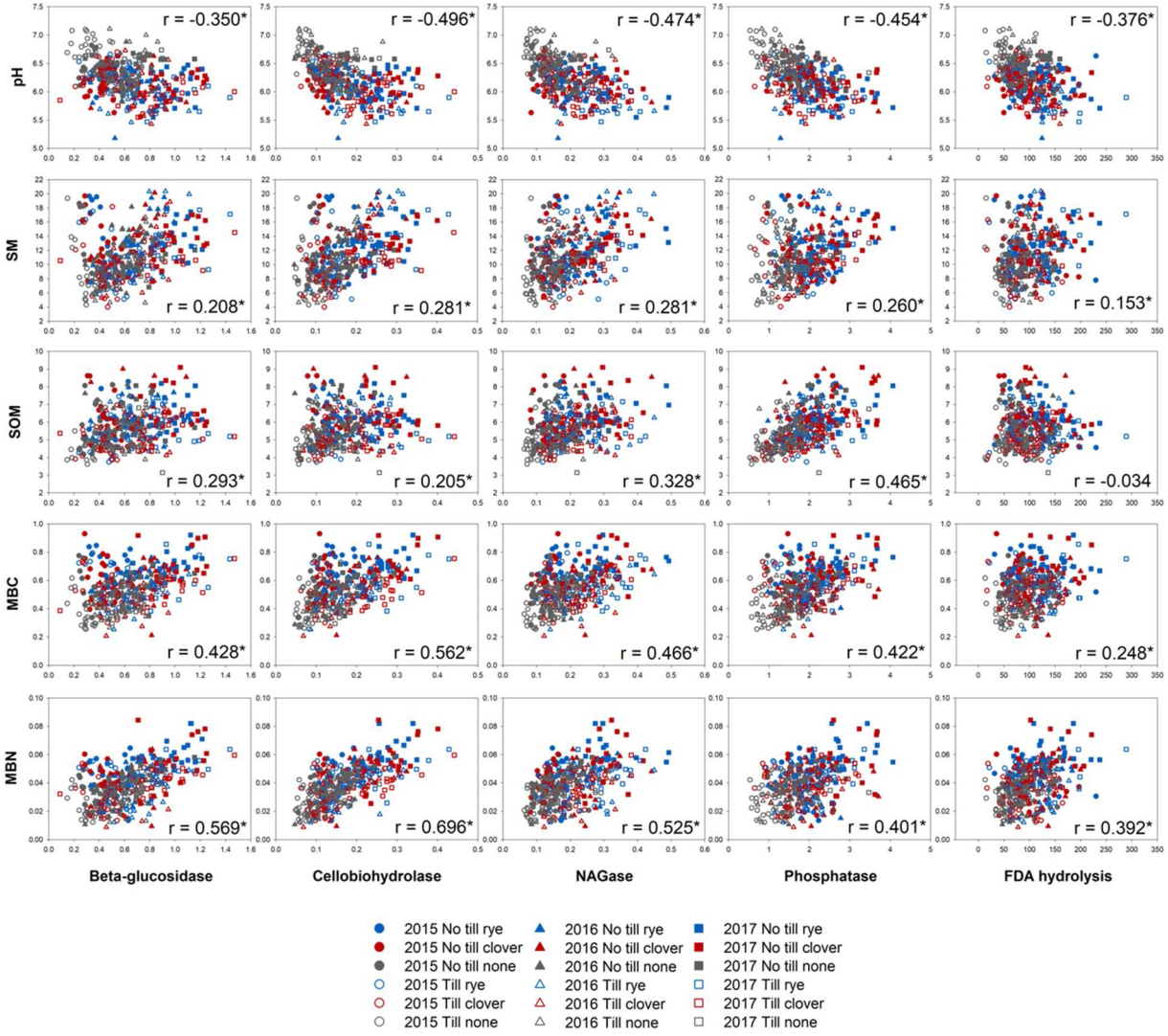

Fig. 5. Scatterplots and Pearson correlation coefficients between enzyme activities (beta-glucosidase, cellobiohydrolase, N-acetylglucosaminidase (NAGase), phosphatase, and fluorescein diacetate (FDA) hydrolysis) and soil pH, soil moisture (SM), soil organic matter (SOM), microbial biomass C (MBC), and microbial biomass N (MBN) from no till (closed symbol) and till (open symbol) plots planted with rye (blue), clover (red), and no winter cover (grey) in 2015 (circles), 2016 (triangles), and 2017 (squares). *Significant correlations ($p \leq 0.0002$).

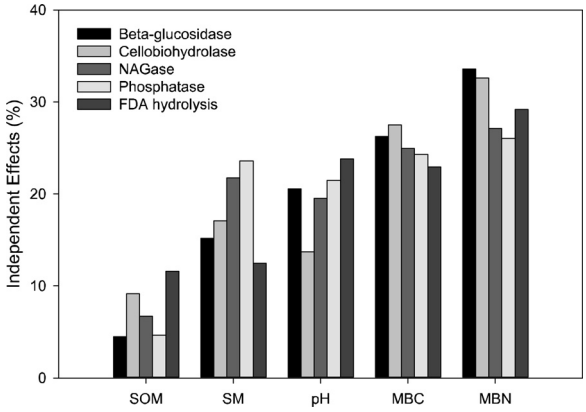

Fig. 6. Independent effects (%) of soil organic matter (SOM), soil moisture (SM), pH, microbial biomass C (MBC), and microbial biomass N (MBN) on beta-glucosidase, cellobiohydrolase, N-acetylglucosaminidase (NAGase), phosphatase, and fluorescein diacetate (FDA) hydrolysis activities in soils.

Table 4
Correlation coefficients between average annual soil enzyme activities and cover crop biomass~.

	Cover crop biomass
Phosphatase	0.513*
NAGase	0.617*
beta-glucosidase	0.643*
Cellobiohydrolase	0.730*
FDA hydrolysis	0.513*

~Abbreviations: NAGase (N-acetylglucosaminidase), FDA (fluorescein diacetate).
* Significant correlations $p \leq 0.0002$.

crops in the current study were all terminated in mid-April, and it is possible earlier or later times for spring termination may influence the cover crop impact on soil properties and crop yield.

5. Conclusion

Both no tillage and cover crop treatments significantly enhanced soil health parameters in surface soils spanning the tillage depth of

soybean field plots. These enhancements appear to be driven, in part, by a larger microbial community in no till and cover cropped plots, and to a lesser extent, substrate availability. Given that tillage incorporates and distributes above ground biomass from cover crops into the top 15 cm of soil, the response of microbial communities to cover crops were expected to differ between the till and no till treatments. This trend was largely unobserved in the current study, indicating more than three years of cover crop biomass input may be needed to observe how interactions between tillage and winter crop influence soil health. Overall, individual treatments resulted in increased microbial biomass and soil enzymes in surface soils, although these benefits were counter balanced by slightly lower yields. This observation highlights the importance of careful selection of cover crop species, as farmers may be hesitant to undertake the added expense of winter cover crops without an appreciable economic benefit (Bergtold et al., 2019). However, preserving soil health is a long-term investment in the sustainability of crop production for future generations. As such, further studies examining effects of differing combinations of plant species as well as variation in termination date, on an expanded range of soil health parameters and crop yield is called for to better determine optimal cover crop implementation for soils in the lower Mississippi Delta region.

Declaration of Competing Interest

None.

Acknowledgements

The author would like to thank Paige Goodlett for assistance in collecting and processing soil samples. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The USDA is an equal opportunity employer.

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