Effects of Cover Crop Mixtures on Biological Indicators of Soil Health under Conservation Systems

by

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Abstract

Soil management practices affect the health and productivity of agroecosystems. Cover crops, which are grown between periods of cash crop production, play a critical role in building healthy soil. The most obvious benefit of cover crops is protection against soil erosion. Other benefits include nutrient retention, weed suppression, and increase of soil organic matter. Moreover, cover crops can alter soil habitat for living organisms by improving moisture status and soil aggregation. Although there is evidence that cover crops enhance soil microbial diversity and activity, limited information is available about the impact of cover crops on biological indicators of soil health. The use of multi-species cover crop mixtures has been promoted nationwide to enhance soil health; however, little data are available demonstrating the benefits of cover crop mixtures compared with a single species in the Southeastern U.S. This project examined the effects of three winter cover crop species grown singly and combined in multispecies mixtures on selected biological indicators of soil health and determined the changes of these indicators over a three-year period. The cover crops included single species and mixtures of cereal rye (*Secale cereale* L.), crimson clover (*Trifolium incarnatum* L.), and radish (*Raphanus sativus* L.) in a soybean (*Glycine max* L.)–cotton (*Gossypium hirsutum* L.) rotation system under strip tillage. The field experiment was a randomized complete block design with four replications and included the following treatments: fallow (no cover crop), rye, rye/clover, rye/radish, clover/radish, and rye/clover/radish (3-way mixtures) mixtures. Soil samples collected in the spring at the 0–15 cm depth were analyzed for microbial biomass C (MBC) and

arbuscular mycorrhizal fungi (AMF) colonization of cotton roots; those collected in the fall at 0–5, 5–10, 10–15, 15–30, and 30–45 cm depth were analyzed for active C, soil respiration, and glomalin-related soil protein (GRSP). In the first two years, significant treatment differences were observed only for the AMF colonization of cotton. In the third year, overall treatment effects were found for all of the parameters measured; however, there were no significant differences among cover crop types. There was an overall decreasing trend for active C, soil respiration, and GRSP with increasing soil depth. Winter cover crops did not influence metabolic quotients significantly. Active C was highly correlated with SOC ($r = 0.86$), soil respiration ($r = 0.91$), and GRSP ($r = 0.92$). Our results show that the beneficial effects of cover crops on biological soil health became more pronounced over time. Active C, soil respiration, and AMF colonization can be useful indicators of soil health reflecting short-term changes; AMF colonization of cotton responded to cover cropping most quickly. Within the three-year study period, biological indicators of soil health measured did not increase with the number of cover crop species used and a longer duration for cover crop treatments may be needed to detect effects of cover crops.

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List of Abbreviations

AMF	Arbuscular Mycorrhizal Fungi
BCA	Bicinchoninic Acid
C	Carbon
CBE	Chlorazol Black E
FAO	Food and Agriculture Organization
GRSP	Glomalin-related Soil Protein
HCl	Hydrochloric Acid
MBC	Microbial Biomass Carbon
N	Nitrogen
NRC	National Research Council
NRCS	Natural Resources Conservation Service
POM	Particulate Organic Matter
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
TON	Total Organic Nitrogen

I. LITERATURE REVIEW

Introduction

Soil has various functions besides being a medium for plant growth and biological activity. Soil functions, i.e., the capabilities of soil, are important for agriculture and the environment. Some of the key functions of soil include providing a medium for food and fiber production, recycling nutrients and wastes, controlling the flow and purity of water, providing a habitat for soil organisms, serving as a resource for building materials, acting as a carbon pool, and archiving archeological heritage (McBratney et al., 2014; Bünemann et al., 2018).

People have cultivated soil for centuries, resulting in soil degradation. Many societies have collapsed due to unsustainable soil management, such as the cultures of the Fertile Crescent in the Middle East, wherein the first agricultural reforms emerged 10,000 years ago (Magdoff and van Es, 2009). Since world population has expanded over time, the demand for food, drinkable water, and energy is on the rise as well, and it has been predicted that the demand for food and water will rise by 50% and 30%, respectively, by 2030 (Jónsson et al., 2016).

The entire world is undoubtedly paying attention to the global challenges arising with respect to food, water, and energy security, biodiversity, human health, and climate change (McBratney et al., 2017). It is commonly accepted that the supply of global food, water, and energy must be ensured to fulfill the world's demand. At the same time, it is believed that biodiversity and ecosystem services must to be managed as well to prevent their decline. The aforementioned challenges pose potential risks to the health of humans, animals, vegetation, and microorganisms. Consequently, the degradation of soil due to erosion, fertility and soil carbon decline, acidification, salinity, and compaction has significant consequences for agricultural productivity, provision of water, and biodiversity (Karlen and Rice, 2015).

Erosion is one of the key causes of soil degradation. Erosion is related to conventional tillage and lack of soil cover and leads to degradation of valuable soil and sediment build up in reservoirs, ponds, and lakes. It was estimated by the United Nations that about 2.5 billion hectares of land have encountered erosion since 1945 (Magdoff and van Es, 2009)**.** Even though people utilize new land for agriculture, it is clear that the proportion of arable land has shrunk, as the area of agricultural lands post-expansion is less than the area lost to erosion (Magdoff and van Es, 2009).

According to the Food and Agriculture Organization (FAO), approximately 1.5 billion hectares of arable land was available worldwide in 2014 (FAO, 2016). However, the size of agricultural lands expanded over the years with increased crop yield owing to conventional agricultural methods (usage of mechanization, irrigation, chemical fertilizers, pesticides), resulting in extensive soil degradation (Foley et al., 2005). As a result of human agricultural activities and natural factors, it is estimated that degraded soils can be found in approximately 12 million hectares of global arable every year (Rickson et al., 2015). It can be concluded that soil functions are negatively influenced by soil degradation, which may lead to a degradation of soil fertility and biodiversity (Jónsson et al., 2016).

Soil supplies food and clean water to humans, breaks down contaminants, stores remarkable amounts of carbon, and provides habitat to the greatest number of species in any ecosystem on Earth. Moreover, nutrients are recycled, and water flow is controlled by soil. Due to its many functions in the ecosystems of Earth, soil has been used in astronomical proportions, resulting in its degradation (Jónsson et al., 2016). Over the course of a few decades, the productive capacity of the soil on more than 10% of the world's arable land has shown signs of

intensive degradation resulting from soil erosion, air pollution, intensive tillage, over-grazing, salinization, and desertification (Sanders, 1992).

Total agricultural land covers about 40% of the earth's land surface (Foley et al., 2005). Even though soil covers a majority of the earth's land surface, it is considered a limited and largely non-renewable resource (Fangueiro et al., 2018). Since it is the source of nutrients for plants that in turn supply food to people and animals, soil is a crucial natural resource, and its effective usage is imperative for maintaining development and nurturing the increasing population (Sathish et al., 2016). Intensive agricultural practices, erosion, loss of nutrients, and air pollution threaten soil, and as a result, soil fertility has declined over the last decades. When soil does not function at its optimal levels of sustainable productivity, environmental quality and net farmer profits are at risk over the long term (Koch et al., 2013).

There is evidence of soil degradation that will signify us to employ adequate remediation to avoid or reduce soil degradation. The sustainable usage of resources in the ever-expanding world requires the protection of soil health, which is a major issue in intensive farming (Kinyangi, 2007). The concept of soil health has been systematically developed with better understanding of soil (Karlen and Stott, 1994)

Soil Health

Soil quality is also known as soil health in recent times, and it has been studied for years. The notion of soil health is generally used when assessing the sustainability of land management in agriculture and environment. Improvements in soil health are often a key consideration for producers incorporating conservation practices (Anderson et al., 1997). Since soil health directly impacts crop production, understanding soil health is crucial. Soil health assessment may yield an evaluation of the sustainability of soil (Andrew and Ruaysoongnern, 2011).

Assessment of soil health, as part of sustainable agriculture, provides knowledge about the capability of soil to promote the growth of vegetation. A few indicators have been recommended for the assessment of soil health, which can be achieved at certain times by evaluating different soil properties and using an applicable soil health index (Sharma et al., 2005).

The significance of soil health and organic matter for the sustainability of soil was recognized by early scientists, farmers, and gardeners. Scientists have indicated the importance of organic matter, which includes living organisms, in the soil. John Evelyn, a scientist in England in the 1670s, highlighted the significance of surface soil and described the loss of productive soil over time (Magdoff and van Es, 2009).

Various definitions of soil health can be found in literature. Soil health was broadly described as "the capacity of a specific kind of soil to function within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water quality, and support human health and habitation" (Karlen et al., 1997). It is also defined as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (Doran et al., 1996; Doran and Zeiss, 2000). According to the National Research Council (NRC) (1993), soil health is "the capacity of the soil to promote the growth of plants, protect watersheds by regulating the infiltration and partitioning of precipitation, and prevent water and air pollution by buffering potential pollutants such as agricultural chemicals, organic wastes, and industrial chemicals" (Sims et al., 1997). In addition, the Natural Resources Conservation Service (NRCS) briefly defined soil health as "the capacity

of the soil to function as a vital living ecosystem to sustain plants, animals, and humans" (NRCS, 2018).

Assessment of Soil Health

It is not easy to directly measure or assess soil health. The key is to understand the functional elements of soil that maintain biological activity and determine the indicators of these functions. Since merely evaluating crop yield, water quality, or any single outcome cannot comprehensively assess soil health, these indicators can enable the evaluation of soil health. Indicators refer to measurable properties of soil or plants that convey information about soil functions. Effective indicators must be sensitive to changes in management and climate and combine the physical, chemical, and biological characteristics of soil (Karlen and Stott, 1994; Powers et al., 1998; Allen et al., 2011).

Primary soil health indicators should be appropriate in different ecological areas. Indicators should be conveniently integrated with soil processes, combine the physical, chemical, and biological characteristics of soil, and function as main inputs required for the prediction of the soil characteristics or roles that are not easy to determine directly. The sensitivity of these indicators to changes in different managements and climates should be high (Fauci and Dick, 1994).

Describing soil health and reaching a consensus with respect to the precise standardized measurements required for its assessment is not an easy process, since various properties must be taken into consideration due to many options for the management of the soil resource, including production of agronomic, vegetable, and forestry crops. To aid soil health assessments, Larson and Pierce (1991) introduced a minimum data set and recommended using pedo-transfer functions to predict the indicators when current measurements were unavailable. Minimum data

set comprises adequate physical, chemical, and biological indicators to efficiently and effectively observe critical soil functions as selected by the particular management goals (Karlen et al., 2003). The NRCS and other organizations, such as the Soil Health Institute, coordinated and made a great effort to develop a standard set of methods, including soil organic carbon (SOC), water-stable aggregation, short-term mineralizable carbon, β-Glucosidase, N-acetyl-β-D-glucosaminidase, acid/alkaline phosphatase, arylsulfatase, permanganate oxidizable carbon, autoclaved citrate extractable protein and phospholipid fatty acid analysis, to assess soil health (NRCS, 2018). These methods could help measure changes induced by land and crop management applications used to protect surfaces and control erosion as well as groundwater impurity (Dumanski and Elliott, 1991).

Soil health depends on various properties of soil. Individual biological or chemical indicators would not be appropriate measures of soil health, as soil health involves a unique interaction among the physical, chemical, and biological properties of soil. All the measurements must be combined to produce an overall measure of soil health, alterations in soil quality, or alteration in quality over time in response to alternate management practices, according to Larson and Pierce (1991). They did not specify whether all the soil health indicator values should be equally weighed or how they might differ for various soil functions or issues (Dumanski and Elliott, 1991).

Physical Indicators

Several physical indicators have been noted and used across literature. The physical indicators of soil health must be valid, reliable, sensitive, repeatable, and accessible. Factors such as soil bulk density, porosity, particle size, aggregation, available water content, and penetration

resistance are some of the most suitable physical indicators for the assessment of soil health (Karlen and Stott, 1994; Bünemann et al., 2018).

Including cover crops in rotations, contribute to the improvement of the physical properties of soil. For instance, a winter cover crop, such as cereal rye, improves water-stable aggregation in surface soils (Villamil et al., 2006; Steele et al., 2012). Villamil et al. (2006) focused on the impact of cover crops on the physical indicators of soil health, and they found that increased aggregation in surface soils results in reduction of bulk density and an increase in the total porosity. A 34-year long-term study on hairy vetch (*Vicia villosa*) and winter wheat (*Triticum aestivum*) cover crops with no-tillage in continuous cotton production system showed that long-term incorporation of cover crops with no-tillage significantly improved and promoted the physical properties of soil by increasing the portion of crop residues on the soil surface and root activity in which the low quantity of cotton residues are returned to soil (Nouri et al., 2019).

Since cover crops can be left on the soil surface after termination, they might help retain soil water later in the season thereby decreasing evaporation and improving water infiltration due to rooting channels (Blanco-Canqui et al., 2011). Williams and Weil (2004) observed that soil treated with a rye cover crop retained more water than soil treated with radish because rye produced a thick mulch on the soil surface as compared to the thin mulch produced by radish.

Cover crops have several positive effects on aggregate stability, but the extent of this change may differ depending on the species of cover crops. In one study, winter vetch was compared with brown mustard (*Brassica juncea*), and the winter vetch treatment demonstrated a 62% higher soil aggregate stability index than brown mustard (Sapkota et al., 2012). Cover crops have also been shown to improve soil structure. Hubbard et al. (2013) evaluated the effects of sunn hemp (*Crotalaria juncea*) and crimson clover as the summer and winter cover crops,

respectively on the physical properties of a loamy sand soil from 2002 to 2005. They found that cover crops under conservation tillage improved soil structure in the surface layer (0–2.5 cm) due to increased C and N levels.

Chemical Indicators

Since soil functions are difficult to quantify directly, selected chemical properties of soil are used instead that are related to its basic functions, such as supporting biodiversity and productivity (Bünemann et al., 2018). Chemical indicators widely used are soil pH, organic C, total N, EC, available nutrients, and CEC (Cardoso et al., 2013; Bünemann et al., 2018). Most chemical indicators are less sensitive to alterations resulting from soil management practices, and the detection of significant differences among treatments requires long-term studies (Lagomarsino et al., 2009).

Cover crops can be beneficial for controlling N in agricultural systems as they influence N cycling and availability. Legume cover crops play a significant role in nitrogen processes, such as N uptake, mineralization, and fixation, which results in an increase in available soil N and primary crop yields. Vyn et al. (1999) showed that the inclusion of a red clover cover crop increased corn (*Zea mays*) biomass by 40.4 kg per hectare. Kuo et al. (2001) observed an increase in corn yields as well as available soil N when a hairy vetch cover crop was used. Some types of cover crops can help reduce N leaching from the soil (Kaspar and Singer, 2011). In a study conducted by Strock et al. (2004), the use of rye as a cover crop in a corn-soybean rotation resulted in the reduction of NO_3^--N leaching.

Cover crops associated with reduced tillage systems can be utilized to manage chemical properties of the soil. Cover crops can influence the content of plant nutrients by scavenging nutrients and avoiding the leaching of nutrients. Abdollahi and Munkholm, (2014) found that ten

years of using a cover crop (fodder radish) resulted in an increase in the availability of K in the surface layer of sandy soil.

Biological Indicators

Significant changes in physical characteristics of soil are difficult to detect in the short term, whereas even moderate changes in biological properties resulting from soil management practices can be detected. To understand soil health, soil biology should be understood as soil biota mediating soil processes such as aggregate formation, water flow, and nutrient cycling has critical role in shaping soil health (Sparling, 1997; Andrew and Ruaysoongnern, 2011).

Soil harbors the greatest microbial diversity. It accommodates diverse habitats, species, and genes, and functions as a living ecosystem (Ritz et al., 2009). Soil microbial biomass refers to the biologically active parts of soil, and their sensitivity to changes in soil management is high (Kennedy and Smith, 1995). Biological indicators reflect the overall number, type, and activities of microorganisms as well as the diversity of living organisms in soil, particularly the microbial population (Ritz et al., 2009; Muscolo et al., 2015).

Biological indicators give us information about the living components of soil. These indicators are more susceptible to management of land, natural deteriorations, and chemical pollution (Muscolo et al., 2014). Doran and Safley (1997) and Pankhurst et al. (1997) believe that an indicator must be interpretable, well-correlated with ecosystem processes, accessible to several users, and sensitive to changes. Biological indicators satisfy all these requirements and inform us about soil processes initiated by soil organisms. They inform us about soil functions as well.

Since soil health is strongly influenced by microbiologically mediated processes (nutrient cycling, soil aggregate stability, and plant nutrient capacity), the identification of biological

indicators of soil health is reported to be critically important by several authors (Pankhurst et al., 1997; Ritz et al., 2009). The most commonly used biological indicators are microbial biomass C, respiration, potential N mineralization capacity, ATP content, fatty acid profiles, enzymes, and the sizes, structures, and activities of microbial communities (Igalavithana et al., 2017).

Soil microorganisms and plant roots produce extracellular enzymes, influencing the decomposition of organic materials and plant nutrient cycling in soil (Gil-Sotres et al., 2005). Bending et al. (2004) demonstrated that biological indicators would be very effective when evaluating the impact of soil management on soil health.

Improvement of Soil Health

Crop Rotation

Monocropping systems may cause depletion of soil nutrients and promote populations of pathogens and pests. Intensive use of agricultural land, especially when employing monocropping and row crops under conventional tillage, causes degradation of organic matter and physical structure of soil (Ketcheson, 1980).

Crop rotation is considered to be an essential part of the conservation of agricultural systems where crops are generally rotated with grasses or legumes (Bullock, 1992). Crop rotation has benefits above and below the ground. Weed suppression is a good example of the benefits of crop rotation above the ground (Krupinsky et al., 2002). A diverse system to enhance soil biodiversity and physical structure can be established owing to benefits of crop rotation below the ground. A meta-analysis by McDaniel et al. (2014) shows an increase in soil C, N, and microbial biomass when microbial diversity was increased through crop rotation.

Crop rotation contributes to organic matter in the soil and breaks pest and disease cycles by increasing diversity in the soil. In a study by Benintende et al. (2008), four-year rotations of

rice (*Oryza sativa*) and pasture positively influenced soil properties as opposed to rice monocultures. Reddy et al. (2006) found that while continuous cotton production caused a reduction in SOC, six-year rotations of cotton and corn resulted in an increase in the organic carbon content of the soil (Reddy et al., 2006). In addition, there is a unique cotton rotation experiment, which is the Old Rotation, Auburn, AL (circa 1986). The Old Rotation is the longest cotton rotation study in the world, providing valuable information relative to long-term crop rotation and winter legumes in cotton and corn cropping systems. Several studies conducted at the Old Rotation showed that long-term crop rotations combined with winter legumes improve SOC levels and resulting in better crop productivity (Mitchell et al., 2008)

Crop rotation not only enhances soil biodiversity but also increases crop yields by providing benefits to soil health, such as aggregation. Diverse microbial communities resulting from crop rotation promote the development of soil aggregates (Tisdall and Oades, 1982; Riedell et al., 2009). Smith et al. (2008) studied 3-year annual crop rotation (corn, soybean, and winter wheat) on corn yields, and they found that corn yield increased over a 3-year period under this regime with cover crops (single species and mixtures of red clover, crimson clover, and cereal rye).

Tillage Systems

Conventional tillage can be defined as the cultivation of soil by disking, plowing, and harrowing to prepare a seedbed and control weeds. Conventional tillage systems have adverse effects on soil properties and remove plant residues from the soil surface (Koller, 2003). Reduced tillage systems leave more plant residues on the soil surface thus reducing the risk of soil erosion as compared to conventional tillage (Sapkota et al., 2012; Higashi et al., 2014; Van Eerd et al., 2014).

Conservation tillage aims to retain more than 30% coverage of crop residue on soil surface after planting. There are several types of conservation tillage systems, namely, no-tillage, ridge-tillage, mulch-tillage, and strip-tillage (Endale et al., 2002; Koller, 2003). Conservation tillage reduces soil erosion, increases organic matter, enhances soil aggregation, improves WHC and water infiltration, and promotes biological diversity (Gebhardt et al., 1985; Feng et al., 2003; Balkcom et al., 2013).

In no-tillage systems, crops are planted directly into the soil and the vegetative cover or plant residues of the previous crop. No-tillage establishment are more feasible options for crops through the use of modern equipment. For instance, modern no-till drills can penetrate surface residues and provide consistent seeding depth as well as appropriate seed-soil contact (Curran et al., 2006).

Lagomarsino et al. (2009) compared no-tillage and conventional tillage practices in a wheat and corn rotation system and found no-tillage systems to remarkably enhance microbial diversity by protecting microaggregates. This diversity of the microbial community results in an increase in soil organic matter (SOM) (Lagomarsino et al., 2009).

Sharma et al. (2016) concluded that minimum tillage influenced activity of microbial enzymes in a positive way (Sharma et al., 2016). One of the primary purposes of using no-tillage is the minimal disturbance of soil and the increase of crop residues on soil surfaces that can reduce erosion, improve organic matter, and enhance microbiological diversity (Balota et al., 2014). Balota et al. (2014) found no-tillage, accompanied by cover crops, to yield 96% higher microbial biomass C as compared to conventional tillage.

Cover Crops

Another key element for the maintenance or improvement of soil health is use of cover crops. A cover crop can be defined as plants grown between periods of normal crop production to suppress weeds, reduce soil erosion, improve soil fertility/quality, scavenge soil nutrients, and control diseases and pests (Wick et al., 2017).

Increased crop residues associated with certain crop management practices might support the growth of beneficial organisms such as earthworms, insects, and microorganisms that have the ability to improve soil health. Cover crops provide a better habitat for soil organisms by retaining living roots in the soil (NRCS, 2011). Living roots promote microbial activity that influence N immobilization and mineralization. Living roots enhance biodiversity and biomass and promote better physical, chemical, and biological properties of the soil. They also increase organic matter (Magdoff and van Es, 2009).

Soil management practices such as conventional tillage may result in the degradation of the soil, and this would limit the supply of adequate biomass to maintain major soil processes responsible for maintaining soil health. Since cover crops provide on-site resources of crop residues, they have excellent potential to sustain soil health by improving organic matter in the soil as well as biological activity. The use of cover crops for a long time improves soil health due to increased organic matter, which is obtained from crop residues, microbial biomass, and root exudates (Nakamoto et al., 2012). Xavier et al., (2013) evaluated changes in soil C caused by cover crops in Brazil using different leguminous species, such as calopo (*Calopogonium mucunoides*), butterfly-pea (*Clitoria ternatea*), and pigeon pea (*Cajanus cajan*), in different years of the study. They found that the inclusion of cover crops increased the organic C stock in soil. Higashi et al. (2014) examined the effects of cover crops (rye and hairy vetch) with different tillage systems on changes in SOC at different soil depths from 2003 to 2011. They found the rye

cover crop with no-tillage to be highly effective in increasing SOC at 0–30 cm depth. Increased organic matter resulting from cover crop exudates and residues can improve soil health (Kögel-Knabner, 2002).

The use of cover crops is favorable for subsequent crop production, as it provides a better cropping environment. For instance, there could be reduced light transmission for weeds, moderation of soil temperature, and increased water holding capacity (Araki, 2005). Lawley et al. (2011) compared forage radish with rye for winter weed control. They found no difference between the two winter cover crops for weed suppression, and winter annual weeds were suppressed by both cover crops.

Cover crops improve soil aggregate stability and increase the size of aggregates owing to the presence of bacteria and fungi (Roberson et al., 1991). Roberson et al. (1991) found a positive correlation between microbial biomass C and aggregate size and stability. Cover crop residues on the soil surface might protect soil particles from rain drops, reducing soil erosion. Cover crop roots can promote aggregation of soil particles, especially during winter compared with fallow (Dabney et al., 2001).

Cover crops can be planted as a single species or multi-species crop. It could be assumed that the use of a multi-species cover crop has advantages for soil health as compared to the use of single species, as each species contributes different characteristics. For instance, radish cover crops improve water infiltration, while legumes can biologically fix N levels (Curran et al., 2006; Clark, 2013). A short-term (2 years) cover crop study on 18 cover crop treatments, including monocultures of eight species, seven four-species polycultures, two eight-species cover crop polycultures, and fallow as a control in a corn production system was conducted by Finney and Kaye (2017). They evaluated the effects of increasing diversity in cover crop species through

five ecosystem services derived from agriculture. They found that weed suppression, N retention, and above-ground biomass N were positively influenced when species richness was increased, but cover crop polycultures had a negative impact on inorganic N supply, and subsequent corn yield was not influenced by diverse cover crop mixtures. Conversely, a study by Chu et al. (2017) observed the positive effects of cover crop multispecies on both crop yield and soil properties. They compared single and double cover crop species with a mixture of five species, including cereal rye, oat, daikon radish, purple top turnips, and crimson clover, in a corn-soybean rotation system. After three years of cover crop establishment, the composition of five species significantly increased moisture content and inorganic N in the soil and soybean yield, as compared to less-diverse cover crop treatments. Overall, many studies examining cover crops illustrate that the use of single and multi-species cover crops enhances the indicators of soil health (Veum et al., 2015; Chavarría et al., 2016; Brennan and Acosta-Martinez, 2017; Mitchell et al., 2017). Chen and Weil (2010) suggested that mixing rye with forage radish might provide bio-drilling and mulching benefits, and the use of forage radish itself may be useful in alleviating the impacts of soil compaction in no-till farming systems.

Characteristics of Cover Crops used in this Study

Cereal Rye (Secale cereal L.)

Cereal rye, as a nonlegume cover crop, has the ability to prevent soil erosion and suppress weed growth. In addition, it can increase organic matter content in the soil by producing a large amount of residue on the soil surface. Cereal rye can be planted successfully in many different locations and cropping systems (Clark, 2013; Liang et al., 2014). Most soils in the Southeast are considered to have poor quality because of soil erosion, runoff and leaching of nutrients, low organic matter, and lack of cover crops (Mitchell et al., 2015). Cereal rye is the

preferred cover crop in the Southeast, and it is used to build soil, reduce erosion, and suppress weeds and pests (Clark, 2013).

Cereal rye is generally used as a winter cover crop, since it is planted in the fall and it covers the soil during the winter until the period shortly before planting cash crops. Since cereal rye yields high biomass, it can protect the soil from erosion and prevent nutrients from leaching by covering the soil surface (Kaspar and Singer, 2011b). One of the most significant benefits of cereal rye cover crop is the reduction of N leaching, since it is cold-tolerant, grows rapidly, and produces a large amount of biomass. Ruffo et al. (2004) established that cereal rye as a winter cover crop takes up significant amounts of residual nitrate without affecting cash crop yield, providing a better environment to agricultural systems. In a 3-year short-term cover crop study conducted by Strock et al. (2004), rye reduced nitrate leaching by 13% as compared to no cover crop in a soybean and corn rotation.

Cereal rye as a cover crop improves and maintains the SOC level in the soil, which is an indicator of soil health, and provides additional residue C to the soil due to its large quantity of biomass. Moore et al. (2014) studied the effects of rye as a winter cover crop in a corn–soybean rotation in terms of SOM and particulate organic matter (POM). The results demonstrate that the presence of rye as a cover crop for 10 years significantly increased SOM and POM as compared to the absence of a cover crop. Kuo et al. (1997) confirmed that the cereal rye treatment resulted in higher amounts of SOC than other cover crop treatments such as *Pisum sativum*, *Vicia villosa*, and *Brassica napus*.

Crimson Clover (Trifolium incarnatum L.)

Unlike nonlegumes especially grass-based cover crops, most legumes do not produce as large an amount of biomass on the surface of soil. Therefore, legumes cannot provide as much

organic matter to the soil as nonlegumes. The low C:N ratio of legume cover crops may lead to faster decomposition of the residue, and as a result, the N and other nutrients coming from legume residues are more readily available to subsequent crops. However, the suppression of weeds by legume residues would not last as long as an equal quantity of nonlegume residue (Brennan et al., 2011; Clark, 2013).

Cover crops are sown for reduction of soil erosion, soil remediation, moisture conservation, and nutrient management (nitrogen in particular). Nitrogen management practices differ for legumes and nonlegumes. Legume cover crops can fix N_2 from the atmosphere, while nonlegume cover crops can only utilize N that already exists in the soil or is supplied by fertilizers. Residues left over from legume cover crops generally include more N that can be easily used by subsequent cash crops. Crimson clover acquires N from the atmosphere by establishing a symbiotic relationship with nitrogen-fixing bacteria (Winding et al., 2005; Wortman et al., 2012). Factors such as the type of legume species, climate, planting date, termination date, and residual soil N influence the N content of legumes and the N availability to subsequent cash crops (Clark, 2013).

Crimson clover has unique abilities among legume cover crops, such as rapid and robust growth that can provide early spring N. The amount of N released from crimson clover differs, as it depends on the sowing and termination date of the crop as well as its location (Curran et al., 2006; Clark, 2013). For instance, crimson clover produced 135 lb. N/A by April 21 in Mississippi as a winter cover crop and 60 lb. N/A by late November in Michigan as a summer cover crop (Clark, 2013). Varco et al. (1991) emphasized that crimson clover produces the largest amount of dry matter as compared to other legumes. Therefore, crimson clover can be planted to reduce soil erosion due to its high dry matter production. Torbert et al. (1996)

observed that crimson clover substantially increased corn yield as compared to winter fallow by providing a higher amount of N to the corn crop.

Forage Radish (Raphanus sativus L.)

Brassica cover crop species control soil erosion, scavenge nutrients, and limit weed growth with respect to their physical and chemical features (Magdoff and van Es, 2009; Martinez-Feria et al., 2016). Some *Brassica* species are important cash crops; there is increasing interest using *Brassica* as cover crops owing to their pest control features. Radish (*Raphanus sativus L.)* in the *Brassica* family, or forage radish, is known for its rapid growth in the fall (Dean and Weil, 2009; Clark, 2013).

Forage radish is an important cover crop and is being used by an increasing number of farmers (Weil and Kremen, 2007). It is a special cover crop due to its unique characteristics that provide multiple benefits to the soil, crop, and farmers. These benefits include reduction in soil compaction, less use of herbicide owing to greater weed control, lower cost of N and other fertilizers due to the release of N early, and enhancement of soil surface fertility. Moreover, it exudes chemicals that protect plants from pests and soil-borne diseases, improves soil health by increasing organic matter, prevents N-leaching by taking up N from topsoil and deep soil layers, reduces and prevents soil erosion and runoff by covering the soil surface rapidly, and increases the infiltration of rainwater due to the holes left behind by its long taproots (Williams and Weil, 2004; Chen and Weil, 2010; Lawley et al., 2011; White and Weil, 2011).

Despite its various benefits, the *Brassica* species have drawbacks. Many researchers have reported that members of the *Brassica* family do not host arbuscular mycorrhizal fungi. According to Schreiner and Koide, (1993), living roots of *Brassica* plants in the soil negatively

affect germination of arbuscular mycorrhizal fungi spores due to exudation of isothiocyanates from the roots.

Forage radish cover crops have excellent features as they can reduce the effects of soil compaction due to their deep/large taproots. After a forage radish plant dies and its root decompose, the remaining root channels can be used by the growing roots of subsequent crops to penetrate compacted deep soil layers (Williams and Weil, 2004; Chen and Weil, 2010).

Effects of Cover Crops on Biological Indicators of Soil Health

Active C

Soil organic matter or SOC alone cannot be an ideal indicator of soil health, because SOC consists of different forms of C that turn over either slowly or quickly. The evaluation of the biologically active parts of SOC that change rapidly over time, such as active C, can better assess changes in soil health resulting from soil management (Sainju et al., 2007).

Soil organic C is significant for soil health. It can be divided into labile C and recalcitrant C. Labile C is termed active C, and recalcitrant C is termed passive C. Active C is very sensitive to small changes in soil, such as changes in microbial activity (Islam and Weil, 2000), and it consists of short half-life C compounds such as microbial biomass and metabolites, soluble and particulate organic C, amino acid and sugars, simple carbohydrates, organic acids, and fine roots and root exudates (Culman et al., 2012, 2013). Sainju et al. (2007) assessed the influence of leguminous (crimson clover), leguminous mixture (crimson clover, balansa clover, and hairy vetch), and non-leguminous (rye) cover crop species on different soil C fractions at 0–15 cm depth over a three-year period. Cover crops have been found to significantly increase potential C mineralization and MBC, although slow fraction SOC was not influenced in the short period.

Microbial Biomass C (MBC)

Soil microorganisms are living and an important component of soil that is responsible for energy and nutrient cycling as well as the regulation of SOM transformation (Fauci and Dick, 1994). Soil microbial biomass is a component of SOM that contains living microorganisms not bigger than 5–10 μm^3 (Horwath and Paul, 1995; Rinklebe and Langer, 2013). Microbial biomass C consists of approximately 1–5% of the total SOC, and it is considered a source of available nutrients (He et al., 2003).

It is widely accepted that planting cover crops increases MBC. The carbon inputs and ratio of C:N of cover crops mainly affects the level of MBC. A study showed that a rye cover crop in combination with a no-till system enhanced the populations of fungi close to the soil surface where C inputs are high (Zhaorigetu et al., 2008).

Since the quantity of MBC in the soil is a function of soil C inputs into the system, MBC is strongly linked with SOM. Cover crops can provide crop residues that are high in quality and quantity on the soil surface and will be the main source of organic matter additions to the soil (Mbuthia et al., 2015; Frasier et al., 2016). Zhang et al. (2005) reported that when soil was amended with crop residues, there was an increase in MBC.

There is an important relationship between soil MBC and soil aggregate formation. Carbon contents in agricultural systems affect aggregate stability and size distribution (Verchot et al., 2011). Moreover, both bacteria and fungi play an important role in soil aggregate formation, because bacterial polysaccharides serve as agents to bind microaggregates, and macroaggregates and microaggregates are connected by fungal hyphae (Tisdall and Oades, 1982; Six et al., 2004). Nakamoto et al. (2012) confirmed that cover crops with no-tillage increase soil MBC as well as soil aggregate formation (Nakamoto et al., 2012).

Soil Basal Respiration

It is known that soil biota drives many important processes involved in soil health. Therefore, the development of proper biological indicators is important. As soil respiration can be used to quantify alterations in the activity of the soil microbial community, it can be an adequate biological indicator of soil health (Winding et al., 2005; Bispo et al., 2009; Ritz et al., 2009). According to Pell et al. (2005), soil basal respiration can be defined as "the steady rate of soil respiration, which is generated from the decomposition of organic matter."

Soil basal respiration can be measured by calculating either the CO_2 amount released from soil microorganisms or the O_2 uptake by microorganisms. It can give insight into the ability of microorganisms to mineralize residues and make nutrients available to cash crops (Winding et al., 2005). Soil temperature, moisture, and structure as well as the availability of nutrients has a significant impact on soil basal respiration (Sparling, 1997).

Soil basal respiration is correlated with SOM as well as microbial biomass. Xavier et al. (2013) compared legume cover crops with volunteer plants to determine their impact on soil respiration. They found soil respiration to be highest in soil treated with a legume cover crop. There was also a positive correlation between soil respiration and microbial biomass (Xavier et al., 2013).

Soil basal respiration is an indicator of soil microbial activity. Schulz (2003) conducted a study to examine the effects of cover crops in no-till systems on soil microbial activity by measuring soil respiration. He found that introducing legume cover crops such as cowpeas, hairy vetch, sunn hemp, and soybean and nonlegume cover crops such as pearl millet and canola had positive effects on soil respiration.

Mycorrhizal Arbuscular Fungi

Arbuscular mycorrhizal fungi (AMF) form a symbiotic relationship with roots of most agricultural crops including row crop species such as cotton and wheat (Rillig, 2004). Arbuscular mycorrhizal fungi are obligate biotrophs that require living plant hosts to obtain C and energy (White and Weil, 2010). Many field studies have shown the advantages of mycorrhizal infection for plant roots. Immobile nutrients, such as P, Zn, Cu, and NH_4^+, are transported from the soil to the host plant by AMF. The roots uptake K^+ and NO_3^- by flow of water through the soil except in the occurrence of drought stress. Under drought stress, AMF may help carry K^+ and NO_3^- to the roots (Kabir, 2005; Hamel and Strullu, 2006).

Living roots enhance biodiversity and biomass content of soil organisms by providing substrates and habitat to soil microorganisms and improve physical, chemical, and biological properties of soil (Kibblewhite et al., 2008; Magdoff and van Es, 2009). Agricultural management practices such as tillage, cover crop, and crop rotation influence distribution and abundance of AMF in soil (Roger-Estrade et al., 2010).

Recent studies emphasize the role of AMF for plants as well as the soil. Mycorrhizal symbiosis is important for plant productivity and diversity due to its influence on almost all metabolic processes of plants. Therefore, AMF symbiosis has an important role in growth and development of plants (Wilson et al., 2009); (Borie et al., 2008; Wilson et al., 2009; García-González et al., 2016). Arbuscular mycorrhizal fungi not only enhance plant growth but also promotes the maintenance of soil aggregates. It produces glomalin, which helps aggregating soil particles within the hyphae network (Wright and Upadhyaya, 1996).

Agricultural management practices such as tillage, cover cropping, and crop rotation affect the distribution and abundance of AMF in soil. For instance, radish belongs to the *Brassica* family that does not host AMF due to the antifungal chemicals in their structure. Some

studies demonstrated a decline in the mycorrhizal population after the growth of a *Brassica* species (Njeru et al., 2014). However, White and Weil, (2010) found that radish cover crops did not have a negative influence on AMF colonization. Soti et al. (2016) reported that cover crop treatments have positive effects on mycorrhizal fungi diversity. Their study of cover crops on mycorrhizal fungi structure demonstrated the abundance of mycorrhizal fungi varieties among different cover crops, and that the diversity of mycorrhizal fungi is influenced by different cover crop treatments.

Glomalin-related Soil Protein

According to Wright and Upadhyaya (1996), "arbuscular mycorrhizal fungi (AMF) produce abundant and persistent extracellular protein." The protein, glomalin, remains in the soil after hyphal death (Wright et al., 1998; Driver et al., 2005). Singh (2012) reported that glomalin is a unique protein produced by AMF, which plays an important role in the formation of soil aggregates by affecting C inputs. Glomalin promotes soil aggregation (Wright et al., 1998).

Cover crops influence the glomalin component of AMF. Glomalin has a slow turnover and fast accumulation in soils where it augments the stabilization of soil aggregates (Borie et al., 2008). Glomalin-related soil proteins can be easily measured and compared with the measurement of AMF, which is complex and time-consuming. Due to this, glomalin-related soil protein can be used as a simple indicator of soil health (Singh et al., 2013).

Tillage and cover cropping systems affect AMF activity and glomalin, because they influence soil aggregate stability and C forms. Balota et al. (2014) showed that cover crops associated with no-till systems increase glomalin-related soil protein as compared to fallow after long term cover crop treatments (23 years). They also found that easily extractable glomalin-related soil protein correlated with microbial parameters such as MBC (Balota et al., 2014).

Objectives of this Study

There have been few studies that focused on the biological indicators of soil health such as glomalin-related soil proteins. There is insufficient knowledge regarding the benefits of using cover crop mixtures. This study aims to investigate the effects of conservation practices on soil biological properties using prescribed management practices of cover cropping, conservation tillage, and crop rotation. The objectives of this investigation are to:

- Determine the effects of cover crops and their mixtures on selected biological indicators of soil health.
- Determine of the changes of biological soil health indicators over time with soil depth.

II. EFFECTS OF COVER CROP MIXTURES ON BIOLOGICAL INDICATORS OF SOIL HEALTH UNDER CONSERVATION SYSTEMS

Introduction

Soil health is "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (Doran et al., 1996; Doran and Zeiss, 2000). Maintaining and advancing soil health is the key for sustainable agricultural production, which should be secured to meet increasing human food demand (Doran et al., 2002). Intensive management systems are used in food production to feed increasing human population. These management systems involve the use of fertilizer and pesticide in large amounts (Wingeyer et al., 2015) and high levels of mechanization (Foley et al., 2005), which can degrade the health of the soil and reduce soil productivity.

Due to key roles of soil health in crop production and ecosystem services, understanding soil health is crucial. Soil health can be evaluated by using different soil indicators, including physical, chemical, and biological attributes of soil. Effective indicators must be sensitive to changes in management and climate, and combine soil physical, chemical and biological characteristics (Karlen and Stott, 1994; Powers et al., 1998; Allen et al., 2011). Recently, there has been increasing attention to measuring biological parameters of soil health compared to in the past when measuring and interpreting biological indicators were very challenging (Sparling, 1997). Furthermore, biological indicators have been reported as more responsive to agricultural management practices and environmental changes in short periods of time (Aziz et al., 2013).

Soil health can be maintained or improved using various conservation management practices, including crop rotation, conservation tillage, and cover cropping. Crop rotation and

conservation tillage practices have been shown to improve soil health as increased crop diversity and reduced disturbance of soils result in better soil structure and microbial activity. Most studies have shown that crop rotation combined with conservation tillage leads to increased crop yield and improved physical and biological soil health compared with monocultures (Nivelle et al., 2016; Nunes et al., 2018). Cover crops have been known to sustain and increase soil health. Most of soil's physical, chemical, and biological attributes are related to soil organic carbon (SOC), which can be increased by incorporating cover crops (Rosolem et al., 2016). Cover crops have been shown as a key way to enhance and promote biological attributes, including microbial activity and microbial community diversity (Roldán et al., 2003). Monoculture of cereals has been the cover crop of choice in the southeastern USA due to its high biomass production. The use of cover crop mixtures has been widely promoted because increased plant diversity may enhance ecosystem services (Finney and Kaye, 2017). A study conducted in the state of New York by Nunes et al. (2018) evaluated effects of interseeding cover crops in corn production systems on soil physical, chemical and biological properties and showed that four years of cover crops (annual ryegrass, red clover, crimson clover, and hairy vetch) improved soil health and increased corn yield for a loamy sand soil. Despite all the above-mentioned advantages, along with many others, effects of cover crops and their mixtures on biological indicators of soil health has not been adequately documented, especially in the Southeast. Therefore, the objectives of this study were to evaluate cover crops and their mixtures on selected biological indicators of soil health and to determine the changes in biological indicators of soil health over time with soil depth.

Materials and Methods

Field experiment

This study was initiated in the fall of 2015 on a Compass loamy sand (coarse-loamy, siliceous, subactive, thermic Plinthic Paleudults) (Soil Survey Staff, 2017) located at Auburn University's E.V. Smith Research Center in Shorter, AL (32°25'33.4"N, 85°53'19.2"W). Mean monthly air temperature and precipitation during the study period are provided in Figure 2.1. Previous crops at this location were managed using conventional tillage practices.

The field experiment was a randomized complete block design with four replications. Each individual plot was 3.7 m wide by 12.2 m long with 12.2 m alleys between blocks. The experiment consisted of 12 different winter cover crop treatments in total. A subset of six treatments were selected for this study: no-cover control (weedy fallow), cereal rye grown in monoculture, and four mixtures composed of two or three species (Table 2.1). All cover crops were planted using a no-till drill. Crimson clover seeds were coated with rhizobial inoculant. Each year the rye treatment was fertilized with 13.6 kg N ha^{-1} as a solution of urea ammonium nitrate (28% N) to promote maximum biomass production. All cover crops were terminated chemically with glyphosate and glufosinate followed by rolling to flatten the cover crop residues. The cash crops consisted of a two-year cotton and soybean rotation under strip tillage. Planting and harvest dates for cotton and soybean are given in the Table 2.2. Management of cash crop production followed Auburn University recommendations. Average soil pH was 6.3 over years.

Soil sampling

Soil samples were collected in the fall of 2016, 2017, and 2018. Any crop residue around the sampling area was brushed aside to avoid mixing of crop residues with soil samples. Soil samples taken in the fall of each year were collected after harvest of the cash crop by using a hydraulic probe (3.8 cm in diameter) (Giddings Machine Company, Windsor, CO). For the fall

samplings, each one of the 24 plots was sampled to a depth of 45 cm. Four soil cores were acquired from each plot (two cores from between crop rows and two cores from within the crop row). Each core sample was then divided into five depths (0–5, 5–10, 10–15, 15–30, and 30–45 cm) and composited by depth. All composite soil samples taken in the fall were air-dried and then ground to pass through a 2-mm sieve. The air-dried, ground, and sieved soil samples were stored in plastic bags at 4°C until analysis within four weeks.

Spring soil samples were collected after termination of the cover crops in 2017 and 2018. The spring soil samples, down to a depth of 15 cm, were taken from each plot with a soil sampling probe (2 cm in diameter). Similar to the fall sampling, surface residues present around the sampling area were brushed aside, and large pieces of residues were removed during the sampling process. Ten cores were randomly taken within each plot, combined into one composite sample, and thoroughly mixed to ensure that the samples were homogenized. All soil samples were transported to the laboratory on ice, immediately sieved with a 4-mm sieve, and stored at 4°C prior to determining MBC.

Biochemical and microbiological analysis

Soil samples collected each fall were subsampled after passing through 2-mm sieve, ground further to pass a 0.5 mm sieve and analyzed for total C by dry combustion using a C/N analyzer (Leco Corp., St. Joseph, MI) (Yeomans and Bremner, 1991).

Active C was measured by using a permanganate oxidation method modified after Weil et al. (2003). Two mL of 0.02 M $KMnO_4$ solution, which has dark purple color, was added to 2.5 g of air-dried soil weighed into a 50 mL plastic centrifuge tube that contained 18 ml of distilled water. Tubes were shaken for 2 minutes at 120 rpm on a platform shaker. After shaking, the tubes were allowed to stand at room temperature for 10 minutes; the dark color turned into light

purple color due to conversion of Mn (VII) to Mn (II) as a result of oxidizing soil organic matter. Supernatant (0.5 mL) from each tube was transferred to another tube containing 49.5 mL of distilled water (a 100-fold dilution). An aliquot of the diluted solution (0.2 mL) was transferred into a 96-well cell plate and each sample was replicated two times. The absorbance was measured at 550 nm using a spectrophotometric micro plate reader (Biotek MQX200, Winooski, Vermont). A standard curve was prepared with 0, 0.005, 0.01, 0.015, and 0.02 M $KMnO_4$ (Weil et al., 2003).

To determine soil basal respiration, a method modified after Alef (1995) was used. Four-day incubation of rewetted air-dried soil samples (20 g dry weight) were performed at room temperature (21 ± 2 ℃) in 1 L Mason jars. Soil moisture contents of the samples were brought to 50% of the water holding capacity. Then the rewetted soil samples were incubated for 4 days in the Mason jars containing 5 mL of 0.5 M NaOH in 20-mL of scintillation vials. Also, six blank jars (without soil) with the base trap were prepared. After four days of incubation, the Mason jars were opened one by one and adsorbed CO_2 was precipitated as insoluble $BaCO_3$ by adding 1 mL $BaCl_2$ (1.5 M). Hydrochloric acid (HCl) (0.25 M) was used to titrate unreacted NaOH. The amount of HCl used for the control and sample was used to calculate CO_2-C produced.

Easily-extractable glomalin-related soil proteins were analyzed following the procedure modified after Wright and Upadhyaya (1998) and Reyna and Wall (2014). Briefly, 3 g air-dried soil were placed in 50 mL plastic centrifuge tubes containing 24 mL of 20 mM sodium citrate (pH 7). The tubes were shaken for five minutes at 180 rpm. The tubes containing soil-sodium citrate mixture were autoclaved at 121 ℃ and 15 psi for 30 min. After cooling, the solids were resuspended by shaking the tubes for one minute. Then, approximately 1.7 mL of mixture from each tube was transferred into a 2-mL microcentrifuge tube in duplicate and centrifuged at

10,000 x g for three minutes to settle soil particles. Then, 1 mL of supernatant was transferred into a 1.5 mL tube for protein quantification. The concentration of dissolved proteins in the clarified extract was determined using the bicinchoninic acid (BCA) assay with bovine serum albumin as standards. Duplicate extracts (0.01 mL) from each tube were transferred into a 96-well cell plate. Then, the BCA working reagent (0.2 mL) (50:1 ratio of reagent A and B, respectively) was added to the samples and the plate containing samples and standards was incubated at 37 °C for one hour. The samples were quantified, against a standard curve (0-2000 µg of protein per well), by colorimetry using a 96-well Biotek MQX200 micro plate reader at 562 nm.

To determine MBC, the chloroform fumigation incubation method of Jenkinson and Powlson (1976) was used. Briefly, fresh soil samples equivalent to 25 g dry weight were weighed into 150 mL beakers. Soil moisture was adjusted to 50% of water holding capacity by adding distilled water and samples were preincubated in the dark for five days at room temperature (21 ± 2 ℃) in closed 1 L Mason jars. After preincubation, two replicates of the samples were fumigated at room temperature for 24 hours in a desiccator containing ethanol-free chloroform while the third sample served as unfumigated control. Fumigated and unfumigated samples were transferred to Mason jars containing a scintillation vial with 5 mL of 0.5 M NaOH. Six jars that contained everything except soil were included as blanks. The jars were incubated in the dark for 10 days. Carbon dioxide released from soil samples during the incubation period was determined by titration. Soil microbial biomass C was calculated from the difference between CO_2–C evolved from the fumigated and blank samples using a conversion factor of 0.41 (Voroney and Paul, 1984).

Cotton root sampling and AMF colonization

Cotton plants were sampled for AMF root colonization at the fourth leaf stage (V4, four leaves fully extended) in 2016 and 2018. The sampling was done by uprooting 10 plants from two randomly selected spots in each plot. The plants were placed in plastic bags and transported to the laboratory on ice. Cotton roots were washed to remove soil gently under a stream of water on a 0.5-mm sieve. The roots were then preserved in 50% ethanol in tightly sealed glass vials until assessing AMF colonization rates.

To determine AMF colonization of cotton, roots were cleared and stained using a method modified after Brundrett et al. (1996). Before clearing, roots were cut into 2–4 cm long pieces and placed in plastic biopsy cassettes (Sakura Finetek USA, Inc, Torrance, CA). The cassettes including the roots were put into a large glass beaker containing 10% KOH and the roots were autoclaved at 121 ℃ and 15 psi for 17 minutes. If the roots were not clear; they were soaked in $NH_4OH+H_2O_2$ (3 ml of 30% NH_4OH, 10 ml of 30% H_2O_2, and 587 ml distilled water) for 10 minutes. Then, the roots were rinsed with water and 0.25 N HCl four times each. The cleared roots were stained with 0.03% chlorazol black E (CBE) by autoclaving at 121 ℃ and 15 psi for 15 minutes. After staining, roots were destained in 50% glycerol for several days (at least 2 or 3 days) prior to quantification of colonization rates. Mycorrhizal colonization of the roots was observed under a dissecting microscope at 30X magnification. To determine colonization rates, the grid line intersect method (Giovannetti and Mosse, 1980) was used in which roots were randomly dispersed in a 9-cm diameter petri plate with grid lines on the bottom. Intersections between grid lines and roots were quantified by scanning along grid lines both horizontally and vertically. A total of at least 100 intersections were used. Samples were re-randomized and counted three times to improve accuracy.

Statistical analysis

All data were analyzed using mixed models in R studio (RStudio Team, 2016). All data were examined for normal distribution and homogeneity of variances. Block was considered as random effect. Cover crop, depth, year, and the interactions among these three variables were considered fixed effects. The effects of cover crop treatments, depth, and year on the biological indicators of soil health were evaluated by using ANOVA ($p \leq 0.05$). Post-hoc mean separations were performed using the Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$). Relationships among soil chemical and biological properties were determined using Pearson's correlation.

Results

Soil physiochemical parameters

In 2016, SOC, total organic N (TON) and bulk density across the cover crop treatments ranged from 3.5 g kg^{-1} to 12.1 g kg^{-1}, 0.23 g kg^{-1} to 0.86 g kg^{-1} and 1.38 g cm^{-3} to 1.70 g cm^{-3} from 0 to 45 cm, respectively (Tables 2.3, 2.4, and C.1). Soil organic C, TON, and bulk density were not significantly affected by cover crop treatments at any depth. Significant differences among depths were observed; SOC and TON contents were the highest at the 0–5 cm depth and bulk density was the highest at 30–45 cm ($p < 0.0001$). The interaction between the cover crop treatments and depth was not significant for SOC, TON, and bulk density (Table 2.8).

In the second year (2017), SOC, TON and bulk density across treatments varied from 2.7 g kg^{-1} to 11.8 g kg^{-1}, 0.27 g kg^{-1} to 0.91 g kg^{-1} and 1.35 g cm^{-3} to 1.81 g cm^{-3} from 0 to 45 cm, respectively (Tables 2.3, 2.4, and C.1). The cover crop treatments did not have any significant effect on SOC, TON, and bulk density within a given depth. There was a depth effect on the SOC, TON, and bulk density; the SOC and TON in the 0–5 cm was significantly higher than

other lower depths and the 30–45 cm depth had highest bulk density ($p < 0.0001$). Similar to 2016, the interaction between cover crop treatments and depth was not significant on the SOC, TON, and bulk density (Table 2.8).

Overall, when the two-year data were analyzed together, SOC and TON were not influenced by winter cover crops in 2016 and 2017 (Figures 2.2, 2.3). However, SOC significantly decreased across all treatments in 2017 compared to 2016 ($p = 0.0283$, Figure 2.2). In addition, the depth effect on SOC, TON and bulk density was apparent ($p<0.0001$). The cover crop treatment x depth x year interaction (3-way interaction) was not significant (Figures 2.2, 2.3, C.4).

Active C

In 2016, no significant differences among cover crop treatments were found for active C at any soil depth. The active C averaged from 80.1 mg kg^{-1} to 442.2 mg kg^{-1} from 0 to 45 cm (Table 2.5). The depth effect was significant for active C; the active C significantly decreased with increasing depth ($p < 0.0001$). There was no significant interaction between cover crop treatments and depth for the first year (Table 2.8).

Similar to 2016, active C was not significantly affected by winter cover crops at any depth in 2017. However, there was a gradually increase in active C at the 0–5 cm depth for all treatments, and active contents C varied from 37.6 mg kg^{-1} to 527.8 mg kg^{-1} from 0 to 45 cm (Table 2.5). The depth effect was significant on the active C; the 15–30 cm depth was not significantly different from the 30–45 cm depth ($p < 0.0001$). Like the first year, no interaction between cover crop treatments and depth was observed in the second year (Table 2.8).

In the third year, significant differences among cover crop treatments were detected for active C (Table 2.5). Active C ranged from 71 mg kg^{-1} to 603 mg kg^{-1} across all treatments and

depths (Table 2.5). Fallow had the lowest active C content among treatments at the 0–5 cm depth ($p< 0.0001$). Active C was highest under rye/clover mix; soil under rye/clover had 43% higher active C than soil under fallow (Table 2.5). Active C decreased with increasing depth ($p< 0.0001$). The interaction between cover crop treatments and depth was significant ($p= 0.0006$).

The active C was significantly influenced by cover crop treatments after three years of cover crop adoption ($p= 0.0005$, Figure 2.4). Significant depth and year effects ($p<0.0001$) were also observed; however, the 3-way interaction was not significant. When the three-year data were analyzed together, active C was significantly higher under the rye, rye/clover mix, and rye/radish mix treatments than the fallow at the 0–5 cm depth in 2018 ($p< 0.0001$, Figure 2.4). Overall, there was a gradual increase in active C from first to third year for each cover crop treatment in the 0–5 cm depth except the fallow treatment; this increase was significant for rye alone and rye/clover mix (Figure 2.4).

Microbial Biomass C

Microbial biomass C was determined in 2017 and 2018. In 2017, use of cover crops for two years did not affect MBC significantly at the 0–15 cm depth. Microbial biomass C ranged from 161.4 mg kg^{-1} to 211.9 mg kg^{-1} and MBC was numerically the highest under the rye/radish mix (Figure 2.5A). In 2018, cover crop treatments significantly ($p = 0.035$) affected MBC, which ranged from 151.4 mg kg^{-1} to 220.2 mg kg^{-1} (Figure 2.5B). Microbial biomass C under the rye/clover and 3-way mixes were significantly higher than under the fallow. Microbial biomass C under the rye/clover mix was the highest numerically among all treatments and was 42% and 45% higher than under the rye/radish mix and the fallow, respectively (Figure 2.5B). Although significant cover crop treatment effects were observed for MBC in 2018, MBC did not show significant changes when two-year data were analyzed together (Figure 2.6).

Soil Respiration

Soil respiration was determined for the top three soil depths since respiration levels were barely detectable at the two lower depths. In 2016, soil respiration was not significantly affected by cover crop treatments, but differed significantly by depth ($p< 0.0001$, Table 2.8). Soil respiration varied from 16.3 CO_2-C mg kg^{-1} to 51.0 CO_2-C mg kg^{-1} at the top three depths (0–15 cm) (Table 2.6). Higher soil respiration values were observed at the 0–5 cm depth while the lowest values were observed at the 10–15 cm depth (Table 2.6). There was no significant interaction between cover crops and depth for soil respiration in 2016 (Table 2.8).

Similar to 2016, cover crops did not show any significant differences on soil respiration within a given depth (Table 2.8) in 2017. Soil respiration ranged from 29.4 CO_2-C mg kg^{-1} to 92.4 CO_2-C mg kg^{-1} from 0 to 15 cm depth (Table 2.6). A depth effect was observed (Table 2.8); the soil respiration was significantly greater in the surface depth (0–5 cm) than the lower depths ($p< 0.0001$). An interaction between cover crop treatments and depth was not significant (Table 2.8).

In 2018, soil respiration was significantly influenced by cover crop treatment ($p< 0.0001$) and depth while their interaction was not significant (Table 2.8). Soil respiration levels varied from 23.5 CO_2-C mg kg^{-1} to 92.7 CO_2-C mg kg^{-1} (Table 2.6), slightly lower than in 2018. Soil respiration was highest under the rye/clover mix; soil under rye/clover mix produced 35% more CO_2 than soil under fallow (Table 2.6). Soil respiration was significantly higher at the 0–5 cm depth than at the lower depths ($p< 0.0001$) in 2018.

Overall, cover crop treatment ($p= 0.0007$), depth ($p< 0.0001$), and year ($p< 0.0001$) effects were significant for soil respiration when the three-year data were analyzed together; in 2017 and 2018, soil respiration levels under all cover crop treatments was significantly higher

than those in 2016 at the 0–5 cm depth (Figure 2.7). In addition, soil respiration under all cover crop treatments except fallow was significantly higher in 2018 at the 5–10 cm depth compared with 2016 (Figure 2.7). However, the 3-way interaction was not significant.

Arbuscular mycorrhizal fungi colonization

Arbuscular mycorrhizal fungi colonization rates were determined in the cotton phase of the rotation in 2016 and 2018. In 2016, arbuscular mycorrhizal fungi colonization of cotton roots was significantly impacted by cover crops ($p < 0.0480$, Figure 2.8A). Colonization rates varied from 28.3% to 50.8% (Figure 2.8A). The colonization rates under the rye/clover mix was 80% higher than the fallow; however, the other cover crop treatments were not significantly different from the fallow (Figure 2.8A).

In 2018, colonization rates were similar to those observed in 2016 and were significantly affected by winter cover crops ($p = 0.0013$, Figure 2.8B). The infection rate varied from 24.2% to 52.8% (Figure 2.8B). The colonization rate under the rye/clover mix ($p < 0.0001$), clover/radish mix ($p = 0.0018$), and the 3-way mix ($p = 0.0005$) was 118%, 81%, and 95% higher than the fallow, respectively. However, the other two treatments were not significantly different from the fallow for AMF colonization (Figure 2.8B).

In general, winter cover crops significantly affected the mycorrhizal colonization of cotton roots over three years ($p = 0.0001$), but the year effect and interaction of treatment and year were not significant (Figure 2.9). The colonization rates in the rye/clover mix were the highest numerically for both years.

Glomalin-related soil proteins

In 2016, GRSP varied from 144.3 mg kg^{-1} to 634.8 mg kg^{-1} from 0 to 45 cm (Table 2.7). Glomalin-related soil protein was not significantly affected by winter cover crops within a given

soil depth (Table 2.7) while the depth effect was significant ($p < 0.0001$). The GRSP declined significantly with increasing soil depth. No significant interaction between cover crops and depth was found (Table 2.8).

In 2017, GRSP data were similar to the previous year. The GRSP across all cover crop treatments ranged from 163.3 mg kg^{-1} to 679.2 mg kg^{-1} from 0 to 45 cm (Table 2.7). Winter cover crops did not have any significant effect on the GRSP within a given depth in the second year; yet, GRSP showed significant differences by depth (Table 2.8). Glomalin-related soil protein significantly decreased with increasing depths ($p < 0.0001$). There was no significant interaction between cover crop treatments and depth (Table 2.8).

In 2018, GRSP varied from 158.8 mg kg^{-1} to 754.8 mg kg^{-1} from 0 to 45 cm (Table 2.7). The cover crop treatment ($p = 0.0001$) and depth ($p < 0.0001$) effects were significant in the third year (Table 2.8). However, their interaction was not significant for GRSP (Table 2.8). The ANOVA results showed that the fallow, clover/radish mix and 3-way mix had lower GRSP than other treatments and rye/clover mixture was either numerically or statistically greater than the fallow ($p = 0.0012$), the clover/radish mix ($p = 0.0043$), and the 3-way mix treatments ($p = 0.0468$), regardless of depth.

Glomalin-related soil protein at the surface depth gradually increased over the three years except for the fallow treatment (Figure 2.10). An overall decreasing trend in GRSP was observed from the 0–5 cm to 30–45 cm depth over the three years (Figure 2.10). However, GRSP did not show significant differences among cover crop treatments when three years data were analyzed together (Figure 2.10).

Correlations

Correlations among biological parameters measured are given in Table 2.10. Some biological indicators showed significant correlations among each other and with SOC. Glomalin-related soil protein was significantly correlated with SOC, but AMF did not show significant correlation with SOC. Also, soil respiration was significantly correlated with SOC, while MBC was not (Table 2.10). Active C was highly correlated with SOC ($r = 0.86$), soil respiration ($r = 0.91$), and GRSP ($r = 0.92$), but not with MBC.

Ratios and metabolic quotient

In 2016 and 2017, ratios of active C (Table 2.11), soil respiration (Table 2.13), and GRSP (Table 2.15) to SOC were not significantly affected by cover crop treatments; however, ratios decreased with increasing depth (Table 2.9). Similarly, cover crop treatments did not significantly affect ratios of MBC to SOC (Table 2.12) and metabolic quotients (Table 2.14) in 2017. Since SOC data were not available in 2018, only the metabolic quotient was calculated. Similar to 2017, there was no treatment effect on the metabolic quotients in 2018; the metabolic quotient under cover crop treatments did not change significantly (Table 2.14).

Discussion

Cover crops have been shown to alter soil properties significantly, which leads to increased agro-ecosystem functioning by providing both above-ground and below-ground residue inputs into soil over long-term periods. Despite having this knowledge, relatively few cover crop studies have been conducted to assess how cover crops and their mixtures affect biological attributes of soil health under conservation tillage with crop rotations, especially in the southeastern USA. Our experiment aimed to improve understanding of the responses of

biochemical and microbiological indicators of soil health to single and multi-species cover crops under conservation tillage in a two-year cotton and soybean rotation system.

In the first two years, we did not observe treatment differences for most of the parameters measured; this is likely caused by heterogeneity of the soil at the study site and previous management practices. The study site was used for continuous corn production under conventional tillage causing diminishment in C pool and biodiversity of soils. Arbuscular mycorrhizal fungi colonization was the only parameter increased under cover crop treatments compared with the fallow during the experimental period. Our results are in agreement with previous studies, which noted one- and two-year cover crop treatments did not lead to discernable changes in MBC, labile parts of SOC (Sainju et al., 2007), and soil respiration (Liang et al., 2014). However, we found differences between cover crop treatments and the fallow for all biological indicators in the third year. We observed that there was an increasing trend for levels of active C, soil respiration, and GRSP at the surface soil (0–5 cm) under cover crop treatments. Similar trends were observed for MBC and AMF colonization at the 0–15 cm depth. These findings may result from increased C input from cover crop residues and live roots, and this increase may stimulate microbial activity and increase labile fractions of SOC pool and microbial diversity. Rye, which was the only monoculture in the experiment, did not differ from cover crop mixtures used in terms of measured soil biological attributes. This may be because rye can produce as much biomass as cover crop mixtures (Murrell et al., 2017). In addition, the rye/clover mix performed better than other cover crop treatments consistently for all biological parameters over the three-year period, which is likely due to special characteristics of the plant species in the mixture and C:N ratios of the mixed biomass.

SOC and active C

Soil organic C, a common soil health indicator, has been known to be unresponsive to short-term management practices, while showing significant differences over the long-term. In this study, there were no significant differences in SOC between cover crop treatments and the fallow treatment regardless of soil depth two years after adoption of winter cover crops. Similar to our results, Duval et al. (2016) reported that cover crops did not alter SOC dynamics compared with no-cover crop three years after establishment of cover crops. It has also been reported that SOC is positively and significantly correlated with active C, soil respiration, and GRSP, as reported in previous studies (Sainju et al., 2007; Balota et al., 2014; Wang et al., 2017). However, SOC and MBC were not significantly correlated in this study. This result contrasts with Feng et al. (2003) and Sainju et al. (2007), who found significant correlations between SOC and MBC.

Many studies have shown active C as a sensitive biological attribute for changes in agricultural management practices and a useful indicator for soil health assessment. In our study, no significant differences in active C levels were found under cover crop treatments compared with fallow treatment in 2016 and 2017. In the first two years of the study, small changes were expected in active C levels because it takes time for SOC to build up. A previous study conducted by Sainju et al. (2007) indicated that labile fractions of SOC were not affected by use of cover crops for two years. Our findings in 2018 revealed that there was a clear difference in active C between cover crop treatments and fallow at the surface soil layer (0–5 cm). These results confirm the findings by Duval et al. (2016), which found that cover crops increased reactive fractions of SOC compared with the no-cover crop treatment. Greater active C has been previously observed in soils under cover crops than in soils under fallow on a Bertrand silt loam soil in Wisconsin and on a Matapeake silt loam, a Mattepex silt loam, and a Manor/Chester loam

soils in Maryland, USA (Jokela et al., 2009; Steele et al., 2012). In this study, active C under 3-way and clover/radish mixes were not significantly higher than under fallow, while differences were significant under rye alone, rye/clover mix, and rye/radish mix compared with fallow. This finding may result from higher C inputs from rye and its blends with other cover crops (Brennan et al., 2013). Increasing cropping system diversity can produce higher biomass (Wortman et al., 2012), which provide better ecosystem functioning (Finney and Kaye, 2017); surprisingly, the 3-way mixture did not change active C. This finding presumably results from less biomass production of the 3-way mixture, which is likely due to seeding rate and competition among three species in the mixture. Moreover, since the fallow had less crop residues, the lowest active C was expected under fallow treatment.

In our study, there was a trend of higher active C for surface soils compared to sub-surface soil layers across all treatments, which is consistent with previous studies (Wang et al., 2017). In general, surface depth had higher concentrations of active C relative to lower depths, which could be attributed to the existence of higher organic matter (Liu et al., 2013) resulting from cover crop residues in the surface soil layer. Active C content in deeper soil layers (5–10, 10–15, 15–30, 30–45 cm depths) did not show changes between cover crop treatments. A similar finding was obtained by Souza et al. (2016) who reported no differences in amounts of active C between cover crop treatments under no-tillage in the 5–10 and 10-20 cm depths.

The proportion of SOC as active C ranged from 1.9% to 4.5% (Table 2.11) under cover crop treatments for the 0–45 cm depth and the active C/SOC ratio was not influenced by cover crop treatments and its interaction with depth. Higher ratios between active C and SOC indicate higher soil organic matter quality that may have resulted from quality and quantity of crop

residues (Balota and Chaves, 2011). Our results found a positive correlation between active C and SOC, in agreement with Culman et al. (2012) who found similar relationships.

MBC

Microbial biomass C as a biological soil health indicator is related to soil organic matter content and environmental conditions. With additions of cover crops contributing more labile C into the SOC pool, favorable soil moisture and temperature may result in higher MBC. However, MBC changes under cover crop treatments were insignificant compared with fallow in the second year of cover crop treatments in our study. Frasier et al. (2016) reported that cover crops tended to increase MBC after three years. Microbial biomass C under rye/clover mix and 3-way mix was greater than under fallow in this study. This could be due to the cover crop mixtures supplying more C and energy to microorganisms. A study conducted in Argentina by Frasier et al. (2016) showed marginal improvement in MBC by a vetch/rye cover crop mix compared with a single rye cover crop and no-cover crop under conservation tillage systems in the third year of cover crop treatments. Also, the C:N ratio of crop residues returned to the soil surface by cover crops may play a role for higher MBC; rye/clover and 3-way mixes, having a narrower C:N ratio (Duval et al., 2016), add more labile C to the soil (Brennan et al., 2013), providing better microbial growth and activity (Balota et al., 2003).

When two-year data were analyzed together, cover crop treatments did not increase MBC compared with fallow after three-years of cover crop treatment, which is consistent with previous studies (Mendes et al., 1999; Abdollahi and Munkholm, 2014; Mbuthia et al., 2015). Nevertheless, there was a general increasing trend in MBC under cover crop treatments except the rye/radish mix. This increase could be attributed to several factors: favorable soil temperature and moisture (Dabney et al., 2001b; Zhou et al., 2016), increased soil aggregation (Delgado et

al., 2004; Balkcom et al., 2007), and higher C inputs (Liu et al., 2005; Steenwerth and Belina, 2008), owing to inclusion of cover crops. Our results infer that small increases in the easily decomposable fraction of SOC components (MBC) might be interpreted as an early indication of alterations in SOC pools due to the short-term effect of cover crop treatment under conservation tillage systems (Powlson et al., 1987).

Microbial biomass C comprised between 1.8 and 2.3% of SOC under cover crop treatments at the 0–15 cm depth (Table 2.14). Similar to our results, Sainju et al. (2007) noted values from 1.8 % to 3.3% under various cover crop treatments. Cover crop treatments did not affect the ratio between MBC and SOC, in agreement with Sainju et al. (2007) who found lack of cover crop effects on the MBC/SOC ratios. Higher ratios of MBC/SOC, indication of higher microbial activity under cover crop treatments, may lead to more accumulation of labile C, which eventually improve soil health.

Soil respiration

Soil respiration, which reflects microbial activity in the soil, is considered an important biological attribute for evaluation of soil health. In 2016 and 2017, we did not observe significant cover crop effects on soil respiration compared to fallow, which could be attributed to the land management history of the study site. The study site was managed using conventional tillage systems that disturb soil causing declines in both SOC pools and soil respiration. Recovery of SOC levels and soil respiration via soil management practices including implementation of cover crops has been postulated, but the time for recovering is not clear. Our results from the first two years suggest that converting conventional tillage to conservation tillage in high biomass cropping systems (cover crops and crop rotation) may need more than one year to recover the soil biota’s size and diversity for higher soil respiration. That could explain lower values for soil

respiration in 2016 compared with the 2017 and 2018. Nevertheless, there were significant differences in soil respiration under cover crop treatments compared with fallow in 2018 (third year of cover crop treatments); soil respiration under rye/clover mix in the surface soil layer (0–5 cm) was much higher than under fallow (Table 2.7). This finding may result from higher biomass production by the rye/clover mix serving as substrate for microbial activity. Our study is in agreement with Mbuthia et al. (2015) who found higher soil respiration under a vetch cover crop compared with no-cover crop at the 0–7.5 cm depth.

Our results also showed that there was a clear difference in soil respiration at the surface compared with the lower soil depths of 5–10 and 10-15 cm; the greatest amount of soil respiration occurred in the surface soil layer while decreasing with increasing depth. Higher values obtained for soil respiration at the surface layer may be attributed to more substrates in the top layer of soil, which would come from cover crop residues.

Metabolic quotient (qCO_2) has been widely used as a good indicator for changes in management practices. Lower values were observed under cover crop treatments (Table 2.16). Soil with lower qCO_2 values suggest that most of the C may be deposited into MBC while small C amounts are used in soil respiration. This means that C use by microorganisms is more efficient (Anderson and Domsch, 1989).

AMF colonization

Arbuscular mycorrhizal fungi are known as an important and beneficial portion of soil biota, stimulating plant growth and improving soil physical characteristics. Since AMF are obligate biotrophs, they need living host plants to obtain energy and C (White and Weil, 2010). Furthermore, fallow in farming rotations may reduce AMF growth and population, thus decreasing mycorrhizal colonization in subsequent crops. Many studies have shown that

implementing cover crops in cropping systems are favored to maintain development of AMF (Lehman et al., 2012; Benitez et al., 2016; García-González et al., 2016). This corroborated with our findings that AMF colonization of subsequent a cotton crop under cover crop treatments increased compared with that under fallow.

Radish is a member of the *Brassica* family, which is not mycorrhizal and may decrease AMF colonization in subsequent crops (Schreiner and Koide, 1993). In contrast, our findings showed that radish mixtures with rye and clover did not tend to decrease AMF colonization in the subsequent cotton crop. This is consistent with other studies that noted neither positive nor negative effects of a radish cover crop for AMF colonization (Pellerin et al., 2007; White and Weil, 2010). Overall, our results suggest that replacing fallow with cover crops is favorable for maintaining development of AMF in subsequent crops.

GRSP

Arbuscular mycorrhizal fungi produce a glycoprotein termed, glomalin, which can be released to the soil after hyphal death, as it is strongly glued to the walls of hyphae (Wright and Upadhyaya, 1996). Glomalin plays a remarkable role in soil aggregate stability (Rillig, 2004), and it is considered a portion of the SOC pool (Ferrero et al., 2016). In our study, we found that cover crops did tend to increase GRSP levels compared with fallow, but this increase between cover crop treatments and fallow was not significant within a given depth over the three-year time period. Also, our results showed a clear difference in GRSP concentrations between soil layers; the surface layer (0–5 cm) had the greatest GRSP compared with other sub-soil layers. These results can be attributed to introducing cover crops into conservation systems that produce high crop residue on the soil surface resulting in accumulation of SOM and higher microbial activity. The effect of cover crops on GRSP has not been studied extensively. Balota et al. (2014)

reported that long-term inclusion of cover crops under conservation tillage systems increased GRSP in Brazil.

Glomalin-related soil protein accounted for 1.9-8.8% of SOC at the 0–45 depth in our study (Table 2.15), similar to values ranging from 3.8% to 7.8% under different land management reported by Rillig et al. (2003). Ratios of GRSP to SOC can be used to identify quality of organic matter; higher values of the ratio indicate higher quality organic matter. We did not find significant correlations between GRSP and AMF, as reported by previous studies (Lutgen et al., 2003; Lovelock et al., 2004). This lack of correlation is likely due to the differences in turnovers of GRSP and AMF (García-González et al., 2018).

Many studies have reported that biological attributes of soil are more susceptible to changes of land management practices in short time periods compared with chemical and physical properties of soil (Doran and Zeiss, 2000; Aziz et al., 2013; Liang et al., 2014; Raiesi and Beheshti, 2014). Microbial biomass C has been known as a sensitive indicator of land-use management changes (Feng et al., 2003; Motta et al., 2007); yet, we found that MBC did not change under cover crop treatments compared with the fallow when two-year data were analyzed together. Similar to MBC data, GRSP did not respond significantly to cover crop treatments over three years. Unlike MBC and GRSP, active C, soil respiration and AMF colonization were more sensitive to adoption of cover crop treatments and these indicators under cover crop treatments were higher than under fallow over the three-year period. This result confirms Weil et al. (2003) and Culman et al. (2012), who proposed active C serves as a sensitive indicator of changes in soil health relative to agricultural management practices. In addition, AMF colonization was the only indicator higher under cover crop treatments compared with under fallow in the first year. This is likely because maintaining mycorrhizal colonization is highly dependent on living host

plants and cover crops are good host plants for mycorrhizae. Also, our findings revealed that plant species did not influence AMF colonization; AMF colonization rates did not differ across cover crop treatments significantly. Overall, our results show that active C, soil respiration, and AMF colonization were more sensitive to cover crop treatments than GRSP and MBC over the three-year period.

Conclusions and Future Directions

Residues returned to the soil by cotton and soybean crops can decompose in between harvesting and planting periods in the southeastern USA, but cotton and soybean crops are considered low residue crops, which is not ideal for soil health. Although there is no single formula to improve and maintain soil health, cover crops are widely known as a viable way for advancing and sustaining soil health. Our results in this study showed that the adoption of cover crops tended to enhance soil health indicators under conservation tillage in a cotton-soybean rotation.

Soil organic C has been widely used as an indicator of soil health and considered a fundamental factor for maintaining sustainable agriculture. However, observing significant cover crop-induced changes in SOC contents requires long-term studies. Therefore, labile fractions of SOC pools in the soil can be adequate for assessment of soil health over short-term periods. We observed overall cover crop treatment effects for active C and soil respiration three years after inclusion of cover crops, and these indicators were highly correlated with SOC. Thus, active C and soil respiration seem to be reliable indicators for SOC based assessment of soil health over short-term periods. Even though MBC and AMF colonization were not significantly correlated with SOC, they showed increases under cover crop treatments. Arbuscular mycorrhizal fungi

colonization was more sensitive to cover crop-induced changes compared to other indicators used in this study, as AMF colonization showed significant differences under cover crop treatments compared with fallow in the first year of the experiment. Taken together, active C, soil respiration and AMF colonization can be useful indicators of soil health as they showed significant differences to cover crop management in a relatively short period of time.

Cover crop species richness did not affect soil health indicators since no differences were observed between bi- and tri-species mixtures and the rye monoculture over the three-year period. Nevertheless, the rye/clover mix appeared to be the most effective cover crop combination in enhancing both biochemical and microbiological indicators of soil health compared with the rye monoculture and other mixtures. We recommend the use of additional single-species cover crop treatments (i.e., clover and radish alone) to determine the effect of increased plant diversity and to pinpoint the impact of individual species in the mixture.

The surface soil layer (0–5 cm) had the highest active C, soil respiration, and GRSP relative to lower soil depths. This was likely due to the less pronounced impact of cover crop residue on soil at lower depths. Our results also showed that below the 0–15 cm soil depth, active C, GRSP, and microbial activity (soil respiration) dramatically declined, suggesting that the 0–15 cm depth was the most suitable soil sampling depth to measure microbial activities.

Responses of biological indicators of soil health to cover crop treatments can be affected by the duration of cover crop treatments and sampling time. Since biological indicators are susceptible to soil moisture, temperature, and substrate availability, more than one soil sampling in a year is suggested for future research. Termination type and date, sowing date, seeding rates of cover crops, as well as soil type should be considered in future research. In addition, more

biological indicators such as soil enzymes can be added to detect cover crop effects on soil health over short-term.

Overall, biological soil health indicators used in this study showed improvement under cover crop treatments compared with the fallow. This infers replacing fallow with winter cover crops is favorable for agro-ecosystems and enhancing biochemical and microbiological indicators of soil health.

Table 2.1 Species composition and seeding rates of cover crop monocultures and mixtures planted in 2015, 2016, and 2017.

	Seeding rate (kg/ha)		
Treatment	Cereal Rye	Crimson Clover	Forage Radish
Cereal Rye	101 (90)		
Cereal Rye + Crimson Clover	34 (30)	22 (20)	
Cereal Rye + Forage Radish	34 (30)		8.97 (8)
Crimson Clover + Forage Radish		11 (10)	8.97 (8)
3 Way Mix	34 (30)	11 (10)	4.48 (4)

Note: Numbers in parenthesis indicate seeding rates as lb./ac.

Table 2.2 Dates of field operations.

Operation	**2015**	**2016**	**2017**	**2018**
Cover crop planting	16-Nov	27-Oct	17-Nov	-
Cover crop termination	-	20-Apr	19-Apr	16-Apr
Cotton planting	-	12-May	-	8-May
Soybean planting	-	-	14-Jun	-
Soil sampling in the fall	-	18-Oct	15-Nov	17-Oct
Soil sampling for MBC	-	-	9-Jun	25-May
Cotton root collection	-	31-May	-	6-Jun

Table 2.3 Effects of cover crop treatments on soil organic carbon (SOC) (g/kg) by soil depth and year.

	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	10.9 (±1.0)	9.4 (±1.0)	8.3 (±0.9)	6.2 (±1.2)	4.3 (±1.3)
Cereal rye	11.9 (±1.1)	9.4 (±1.3)	8.4 (±1.0)	5.7 (±1.8)	3.6 (±1.3)
Rye+Clover	10.6 (±1.4)	9.4 (±1.1)	8.3 (±1.2)	5.7 (±1.7)	3.9 (±1.1)
Rye+Radish	11.3 (±0.8)	9.5 (±0.9)	8.9 (±1.1)	5.0 (±1.2)	3.7 (±1.4)
Clover+Radish	12.1 (±2.2)	9.3 (±1.2)	8.4 (±1.7)	6.4 (±1.9)	5.5 (±2.3)
3 Way mix	10.8 (±0.6)	9.5 (±0.3)	8.1 (±0.7)	5.0 (±1.0)	3.5 (±0.7)
Mean	*11.3 (±0.3)A*	*9.4 (±0.03)AB*	*8.4 (±0.1)B*	*5.7 (±0.2)C*	*4.1 (±0.3)C*
2017					
Fallow	10.2 (±0.4)	8.2 (±0.7)	8.1 (±0.5)	5.7 (±1.0)	4.2 (±1.0)
Cereal rye	11.8 (±1.1)	8.8 (±1.1)	7.0 (±1.2)	5.3 (±1.7)	3.1 (±0.8)
Rye+Clover	11.2 (±1.2)	9.3 (±1.2)	7.7 (±1.1)	5.8 (±1.9)	3.6 (±0.8)
Rye+Radish	11.6 (±1.3)	8.2 (±0.3)	6.8 (±0.7)	4.5 (±1.4)	3.0 (±0.8)
Clover+Radish	10.9 (±1.5)	8.8 (±1.4)	7.6 (±1.3)	6.1 (±1.8)	3.0 (±0.7)
3 Way mix	10.7 (±0.9)	8.5 (±0.7)	7.2 (±0.9)	4.7 (±1.1)	2.7 (±0.5)
Mean	*11.1 (±0.2)A*	*8.6 (±0.2)B*	*7.4 (±0.2)B*	*5.4 (±0.3)C*	*3.3 (±0.2)D*

Note: Value in the parenthesis is the standard error of the mean (n= 4). Different capital letters within each row indicate significant differences by depth according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments.

Table 2.4 Effects of cover crop treatments on total organic nitrogen (TON) (mg/kg) by soil depth and year.

	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	744.0 (±69.7)	606.0 (±53.2)	516.8 (±92.2)	328.3 (±33.0)	248.0 (±22.1)
Cereal rye	864.0 (±73.0)	658.0 (±58.4)	552.3 (±18.7)	308.5 (±58.4)	319.5 (±37.6)
Rye+Clover	825.5 (±105.5)	675.3 (±62.5)	596.8 (±24.4)	341.8 (±32.3)	310.0 (±32.3)
Rye+Radish	828.5 (±53.8)	669.8 (±38.8)	587.8 (±44.6)	281.8 (±29.7)	231.8 (±41.3)
Clover+Radish	858.5 (±162.3)	661.5 (±79.3)	594.8 (±92.9)	356.8 (±52.4)	338.5 (±69.6)
3 Way mix	812.8 (±56.7)	711.8 (±29.3)	584.3 (±32.8)	344.3 (±34.7)	275.8 (±49.9)
Mean	*822.2 (±17.6)A*	*663.7 (±14.0)B*	*572.1 (±12.9)C*	*326.9 (±11.2)D*	*287.3 (±17.3)D*
2017					
Fallow	784.5 (±26.2)	598.8 (±15.5)	530.5 (±52.4)	316.8 (±17.6)	318.3 (±41.0)
Cereal rye	909.3 (±53.7)	675.8 (±34.8)	495.8 (±32.4)	327.8 (±30.9)	330.3 (±28.5)
Rye+Clover	856.3 (±43.5)	680.8 (±57.2)	538.0 (±47.5)	340.0 (±43.3)	335.8 (±23.8)
Rye+Radish	902.0 (±68.4)	618.5 (±32.1)	487.8 (±43.8)	278.5 (±46.0)	296.0 (±20.3)
Clover+Radish	818.0 (±84.4)	670.8 (±63.6)	529.0 (±50.7)	342.0 (±39.5)	277.3 (±36.3)
3 Way mix	853.0 (±59.8)	675.5 (±45.0)	500.0 (±51.3)	273.5 (±24.3)	300.0 (±49.5)
Mean	*853.8 (±19.6)A*	*653.3 (±14.4)B*	*513.5 (±8.7)C*	*313.1 (±12.3)D*	*309.6 (±9.1)D*

Note: Value in the parenthesis is the standard error of the mean (n= 4). Different capital letters within each row indicate significant differences by depth according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments.

Table 2.5 Effects of cover crop treatments on active carbon (mg/kg) by soil depth and year.

	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	385.8 (±22.9)	338 (±17.1)	284.4 (±15.7)	144.4 (±8.9)	91.3 (±18.8)
Cereal rye	412.2 (±43.2)	319.1 (±37.3)	306.9 (±24.9)	133 (±27.3)	140.6 (±28.7)
Rye+Clover	403.3 (±28.0)	341.0 (±40.8)	282.1 (±21.9)	157.1 (±37.3)	93.1 (±9.6)
Rye+Radish	442.2 (±30.3)	388.2 (±16.7)	328.9 (±24.9)	156 (±19.2)	80.1 (±17.3)
Clover+Radish	428.8 (±36.2)	344.7 (±24.3)	273.9 (±34.3)	132.8 (±32.7)	98.6 (±16.6)
3 Way mix	416.7 (±26.0)	336.4 (±29.9)	292.6 (±37.3)	128.9 (±16.5)	96.2 (±9.0)
Mean	*414.8 (±8.0)A*	*344.6 (±9.4)B*	*294.8 (±8.2)C*	*142.1 (±5.1)D*	*100.0 (±8.5)E*
2017					
Fallow	440.2 (±10.5)	339.2 (±10.1)	270.9 (±15.6)	91.8 (±25.0)	59.7 (±22.2)
Cereal rye	507.8 (±39.7)	333.6 (±28.1)	225.3 (±32.2)	96.9 (±21.9)	58.4 (±22.2)
Rye+Clover	512.6 (±38.6)	362.8 (±35.6)	285.3 (±27.6)	99.7 (±21.1)	91.9 (±15.0)
Rye+Radish	527.8 (±28.1)	391.3 (±11.1)	247.5 (±21.6)	94.5 (±15.1)	37.6 (±12.2)
Clover+Radish	490.0 (±33.9)	338.9 (±40.4)	267.7 (±39.0)	106.0 (±36.1)	45.7 (±16.1)
3 Way mix	518.5 (±44.3)	361.3 (±14.5)	238.1 (±16.0)	104.4 (±29.7)	69.2 (±31.4)
Mean	*499.5 (±12.9)A*	*354.5 (±8.9)B*	*255.8 (±9.2)C*	*98.9 (±2.3)D*	*60.4 (±7.8)D*
2018					
Fallow	419.1 (±20.2)b	322.1 (±17.5)cdef	246.8 (±6.4)fg	156.0 (±12.5)gh	77.1 (±8.8)h
Cereal rye	561.2 (±19.7)a	375.2 (±21.3)bcd	264.2 (±29.7)ef	137.9 (±29.1)h	82.7 (±12.1)h
Rye+Clover	603.0 (±13.6)a	413.5 (±30.5)bc	318.3 (±22.2)def	144.4 (±16.3)h	81.9 (±9.0)h
Rye+Radish	564.2 (±22.0)a	362.8 (±18.4)bcd	299.2 (±12.7)def	159.4 (±11.8)gh	78.5 (±8.0)h
Clover+Radish	542.0 (±21.5)a	346.5 (±15.4)bcde	267.4 (±22.9)ef	144.5 (±17.0)h	71.0 (± 8.6)h
3 Way mix	548.2 (±29.2)a	351.9 (±23.3)bcde	265.8 (±11.8)ef	129.6 (±14.8)h	76.5 (±2.3)h

Note: Value in the parenthesis is the standard error of the mean (n= 4). Different capital letters within each row for 2016 and 2017 indicate significant differences by depth according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments. Different lowercase letters for 2018 indicate significant differences by treatment and depth.

Table 2.6 Effects of cover crop treatments on soil respiration ($mgCO_2$-C/kg) by soil depth and year.

	Depth (cm)		
Treatment	**0–5**	**5–10**	**10–15**
2016			
Fallow	36.9 (±4.4)	26.6 (±1.9)	16.3 (±3.3)
Cereal rye	48.4 (±5.0)	30.4 (±4.0)	18.0 (±3.1)
Rye+Clover	38.3 (±9.1)	22.9 (±7.7)	19.5 (±4.2)
Rye+Radish	49.5 (±8.0)	36.2 (±2.5)	24.7 (±3.8)
Clover+Radish	51.0 (±4.9)	33.4 (±7.8)	18.9 (±3.9)
3 Way mix	42.8 (±5.1)	28.1 (±5.0)	21.4 (±3.9)
Mean	*44.5 (±2.5)A*	*29.6 (±2.0)B*	*19.8 (±1.2)C*
2017			
Fallow	82.2 (±1.3)	41.8 (±4.8)	29.4 (±3.6)
Cereal rye	88.8 (±7.8)	47.6 (±2.2)	33.1 (±2.9)
Rye+Clover	89.6 (±5.2)	47.0 (±6.4)	35.2 (±5.1)
Rye+Radish	92.4 (±6.4)	44.6 (±4.3)	31.3 (±3.5)
Clover+Radish	83.4 (±7.9)	43.6 (±5.0)	35.4 (±5.7)
3 Way mix	87.7 (±6.9)	42.3 (±4.2)	30.7 (±3.2)
Mean	*87.3 (±1.6)A*	*44.5 (±1.0)B*	*32.5 (±1.0)C*
2018			
Fallow	68.7 (±2.7)bcd	38.13 (±1.1)efg	23.5 (±1.1)g
Cereal rye	78.3 (±3.7)ab	57.4 (±6.6)d	29.5 (±2.6)g
Rye+Clover	92.7 (±6.5)a	56.5 (±1.9)de	34.4 (±3.8)fg
Rye+Radish	79.8 (±3.5)ab	52.0 (±0.5)def	26.7 (±1.8)g
Clover+Radish	76.9 (±5.4)abc	50.9 (±2.4)def	37.7 (±4.1)fg
3 Way mix	77.3 (±1.8)ab	58.4 (±5.7)cd	30.4 (±3.4)g

Note: Value in the parenthesis is the standard error of the mean (n= 4). Different capital letters within each row for 2016 and 2017 indicate significant differences by depth according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments. Different lowercase letters for 2018 indicate significant differences by treatment and depth.

Table 2.7 Effects of cover crop treatments on GRSP (mg/kg) by soil depth and year.

	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	614.2 (±41.1)	551.5 (±37.4)	470.8 (±49.3)	311.5 (±50.9)	213.3 (±58.9)
Cereal rye	634.8 (±44.7)	547.2 (±57.7)	501.3 (±50.4)	271.5 (±79.5)	181.7 (±80.3)
Rye+Clover	600.4 (±45.4)	548.9 (±51.0)	460.7 (±29.8)	298.9 (±68.1)	195.1 (±58.7)
Rye+Radish	627.0 (±28.8)	567.2 (±16.3)	481.7 (±10.2)	260.3 (±11.0)	179.8 (±5.2)
Clover+Radish	606.2 (±102.2)	548.2 (±79.6)	425.0 (±69.7)	262.6 (±79.2)	144.3 (±40.0)
3 Way mix	606.9 (±48.2)	543.1 (±33.1)	448.1 (±47.0)	266.1 (±44.6)	174.7 (±44.4)
Mean	*614.9 (±5.5)A*	*551.0 (±3.4)A*	*464.6(±10.8)B*	*278.5 (±8.7)C*	*181.5 (±9.4)D*
2017					
Fallow	635.0 (±34.7)	533.2 (±37.2)	458.5 (±26.7)	304.0 (±42.6)	213.0 (±55.6)
Cereal rye	679.2 (±58.5)	564.2 (±55.6)	441.2 (±35.1)	281.9 (±62.4)	182.0 (±58.9)
Rye+Clover	649.7 (±56.2)	557.5 (±42.7)	477.2 (±35.1)	289.3 (±63.3)	186.0 (±41.0)
Rye+Radish	664.6 (±47.3)	536.5 (±29.4)	424.6 (±24.0)	241.2 (±62.6)	167.0 (±45.7)
Clover+Radish	641.4 (±77.7)	553.6 (±78.7)	439.4 (±70.5)	297.6 (±85.2)	169.6 (±28.4
3 Way mix	644.2 (±55.7)	545.2 (±43.1)	423.0 (±43.2)	254.9 (±42.6)	163.3 (±29.2)
Mean	*652.3 (±6.8)A*	*548.4 (±5.0)B*	*444.0 (±8.5)C*	*278.1 (±10.2)D*	*180.2 (±7.5)E*
2018					
Fallow	572.9 (±9.4)	452.4 (±5.9)	415.3 (±17.8)	267.4 (±16.7)	205.8 (±19.9)
Cereal rye	724.0 (±45.5)	516.1 (±47.1)	440.3 (±43.5)	299.2 (±63.0)	209.9 (±37.8)
Rye+Clover	754.8 (±44.4)	593.3 (±49.4)	514.5 (±40.8)	382.6 (±60.7)	257.3 (±37.8)
Rye+Radish	708.5 (±41.4)	498.8 (±22.9)	432.5 (±35.3)	328.8 (±42.1)	238.2 (±46.3)
Clover+Radish	683.2 (±62.8)	550.8 (±66.6)	460.3 (±46.2)	268.6 (±35.4)	187.1 (±32.6)
3 Way mix	665.6 (±40.4)	506.6 (±44.3)	448.0 (±43.5)	248.1 (±32.1)	158.8 (±20.2)
Mean	*684.8 (±25.7)A*	*519.7 (±19.6)B*	*451.8 (±14.0)C*	*299.1 (±20.3)D*	*209.5 (±14.4)E*

Note: Value in the parenthesis is the standard error of the mean (n= 4). Different capital letters within each row indicate significant differences by depth according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments.

Table 2.8 Analysis of variance for soil health indicators and general soil properties for six cover crop treatments in 2016, 2017, and 2018.

	Source of variation		
Indicator	**Cover crop (CC)**	**Depth**	**CCxDepth**
2016			
SOC	ns	***	ns
TON	ns	***	ns
Bulk Density	*	***	ns
Active C	ns	***	ns
Soil Respiration	ns	***	ns
AMF	*	NA	NA
GRSP	ns	***	ns
2017			
SOC	ns	***	ns
TON	ns	***	ns
Bulk Density	ns	***	ns
Active C	ns	***	ns
MBC	ns	NA	NA
Soil Respiration	ns	***	ns
GRSP	ns	***	ns
2018			
Active C	***	***	***
MBC	**	NA	NA
Soil Respiration	***	***	ns
AMF	***	NA	NA
GRSP	***	***	ns

ns: not significant; *Significant at $P \leq 0.05$; **Significant at $P \leq 0.01$

***Significant at $P \leq 0.001$.

NA: one depth only

Table 2.9 Analysis of variance for ratios between biological soil health indicators and SOC and metabolic quotient (soil respiration/MBC) for six cover crop treatments in 2016, 2017, and 2018.

	Source of variation		
Ratio	**Cover crop (CC)**	**Depth**	**CCxDepth**
2016			
Active C/SOC	ns	**	ns
Soil respiration/SOC	ns	***	ns
GRSP/SOC	ns	***	ns
2017			
Active C/SOC	ns	***	ns
Soil respiration/SOC	ns	***	ns
GRSP/SOC	ns	***	ns
MBC/SOC	ns	NA	NA
Soil respiration/MBC	ns	NA	NA
2018			
Soil respiration/MBC	**	NA	NA

ns: not significant; *Significant at $P \leq 0.05$; **Significant at $P \leq 0.01$;

***Significant at $P \leq 0.001$.

NA: one depth only

Table 2.10 Pearson correlation coefficients for the soil health indicators measured in the study.

Variables	SOC	MBC	Active C	Soil respiration	GRSP	AMF
SOC	1					
MBC	0.16	1				
Active C	0.86***	0.10	1			
Soil respiration	0.75***	0.12	0.91***	1		
GRSP	0.93***	0.18	0.92***	0.83***	1	
AMF	-0.11	0.24	-0.02	-0.05	0.11	1

***Significant at $P \leq 0.001$

Table 2.11 Effects of cover crop treatments on the ratio between active C and SOC by depth and year.

	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	0.036 (±0.004)	0.037 (±0.005)	0.035 (±0.003)	0.025 (±0.004)	0.024 (±0.007)
Cereal rye	0.034 (±0.001)	0.035 (±0.003)	0.037 (±0.003)	0.026 (±0.003)	0.051 (±0.018)
Rye+Clover	0.039 (±0.003)	0.037 (±0.001)	0.035 (±0.002)	0.029 (±0.002)	0.028 (±0.005)
Rye+Radish	0.039 (±0.003)	0.042 (±0.005)	0.038 (±0.002)	0.036 (±0.009)	0.025 (±0.003)
Clover+Radish	0.038 (±0.005)	0.038 (±0.003)	0.034 (±0.003)	0.022 (±0.002)	0.031 (±0.014)
3 Way mix	0.039 (±0.003)	0.036 (± 0.003)	0.036 (±0.003)	0.028 (±0.005)	0.030 (±0.005)
2017					
Fallow	0.043 (±0.002)	0.042 (±0.002)	0.034 (±0.001)	0.016 (±0.002)	0.013 (±0.002)
Cereal rye	0.043 (±0.001)	0.039 (±0.004)	0.033 (±0.004)	0.020 (±0.003)	0.018 (±0.002)
Rye+Clover	0.046 (±0.002)	0.040 (±0.002)	0.038 (±0.003)	0.019 (±0.002)	0.030 (±0.006)
Rye+Radish	0.046 (±0.003)	0.048 (±0.002)	0.037 (±0.005)	0.024 (±0.004)	0.014 (±0.005)
Clover+Radish	0.046 (±0.004)	0.040 (±0.002)	0.036 (±0.004)	0.017 (±0.003)	0.019 (±0.010)
3 Way mix	0.049 (±0.001)	0.043 (±0.002)	0.034 (±0.002)	0.022 (±0.005)	0.024 (±0.007)

Note: Value in the parenthesis is the standard error of the mean (n=4).

Table 2.12 Effects of cover crop treatments on the ratio between MBC and SOC in 2017.

Treatment	**2017**
Fallow	0.018 (±0.002)
Cereal rye	0.017 (±0.002)
Rye+Clover	0.022 (±0.004)
Rye+Radish	0.022 (±0.003)
Clover+Radish	0.018 (±0.004)
3 Way mix	0.017 (±0.002)

Note: Value in the parenthesis is the standard error of the mean (n=4).

Table 2.13 Effects of cover crop treatments on the ratio between soil respiration and SOC by depth and year.

	Depth (cm)		
Treatment	**0–5**	**5–10**	**10–15**
2016			
Fallow	0.004 (±0.0006)	0.003 (±0.0005)	0.002 (±0.0006)
Cereal rye	0.004 (±0.0005)	0.003 (±0.0005)	0.002 (±0.0002)
Rye+Clover	0.004 (±0.0005)	0.002 (±0.0007)	0.002 (±0.0004)
Rye+Radish	0.004 (±0.0006)	0.004 (±0.0003)	0.003 (±0.0004)
Clover+Radish	0.005 (±0.0006)	0.004 (±0.0005)	0.002 (±0.0002)
3 Way mix	0.004 (±0.0004)	0.003 (± 0.0005)	0.003 (±0.0002)
2017			
Fallow	0.008 (±0.0005)	0.005 (±0.0009)	0.004 (±0.0005)
Cereal rye	0.008 (±0.0004)	0.006 (±0.0007)	0.005 (±0.0006)
Rye+Clover	0.008 (±0.0007)	0.005 (±0.0001)	0.005 (±0.0005)
Rye+Radish	0.008 (±0.0005)	0.005 (±0.0003)	0.005 (±0.0006)
Clover+Radish	0.008 (±0.0005)	0.005 (±0.0005)	0.005 (±0.0012)
3 Way mix	0.008 (±0.0005)	0.005 (±0.0003)	0.004 (±0.0004)

Note: Value in the parenthesis is the standard error of the mean (n=4).

Table 2.14 Effects of cover crop treatments on metabolic quotient (qCO_2) in 2017 and 2018.

Treatment	**2017**	**2018**
Fallow	0.25 (±0.04)	0.48 (±0.04)
Cereal rye	0.24 (±0.04)	0.53 (±0.02)
Rye+Clover	0.24 (±0.04)	0.51 (±0.01)
Rye+Radish	0.23 (±0.06)	0.53 (±0.02)
Clover+Radish	0.25 (±0.06)	0.54 (±0.03)
3 Way mix	0.24 (±0.06)	0.53 (±0.04)

Note: Value in the parenthesis is the standard error of the mean (n=4).

Table 2.15 Effects of cover crop treatments on the ratio between GRSP and SOC by soil depth and year.

	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	0.081 (±0.008)	0.085 (±0.007)	0.078 (±0.003)	0.021 (±0.001)	0.020 (±0.001)
Cereal rye	0.075 (±0.003)	0.082 (±0.006)	0.078 (±0.003)	0.020 (±0.001)	0.020 (±0.001)
Rye+Clover	0.079 (±0.010)	0.080 (±0.006)	0.072 (±0.005)	0.022 (±0.001)	0.020 (±0.000)
Rye+Radish	0.081 (±0.005)	0.083 (±0.007)	0.074 (±0.006)	0.022 (±0.002)	0.019 (±0.001)
Clover+Radish	0.068 (±0.006)	0.079 (±0.007)	0.067 (±0.006)	0.018 (±0.002)	0.015 (±0.004)
3 Way mix	0.078 (±0.005)	0.080 (± 0.008)	0.075 (±0.008)	0.021 (±0.000)	0.020 (±0.001)
2017					
Fallow	0.089 (±0.003)	0.089 (±0.006)	0.073 (±0.004)	0.022 (±0.001)	0.020 (±0.002)
Cereal rye	0.081 (±0.004)	0.086 (±0.003)	0.081 (±0.007)	0.022 (±0.002)	0.021 (±0.002)
Rye+Clover	0.086 (±0.004)	0.079 (±0.005)	0.080 (±0.005)	0.021 (±0.001)	0.019 (±0.001)
Rye+Radish	0.087 (±0.008)	0.084 (±0.004)	0.079 (±0.005)	0.021 (±0.003)	0.021 (±0.002)
Clover+Radish	0.087 (±0.002)	0.083 (±0.002)	0.074 (±0.001)	0.020 (±0.000)	0.022 (±0.002)
3 Way mix	0.086 (±0.002)	0.081 (±0.004)	0.073 (±0.005)	0.023 (±0.003)	0.023 (±0.002)

Note: Value in the parenthesis is the standard error of the mean (n=4).

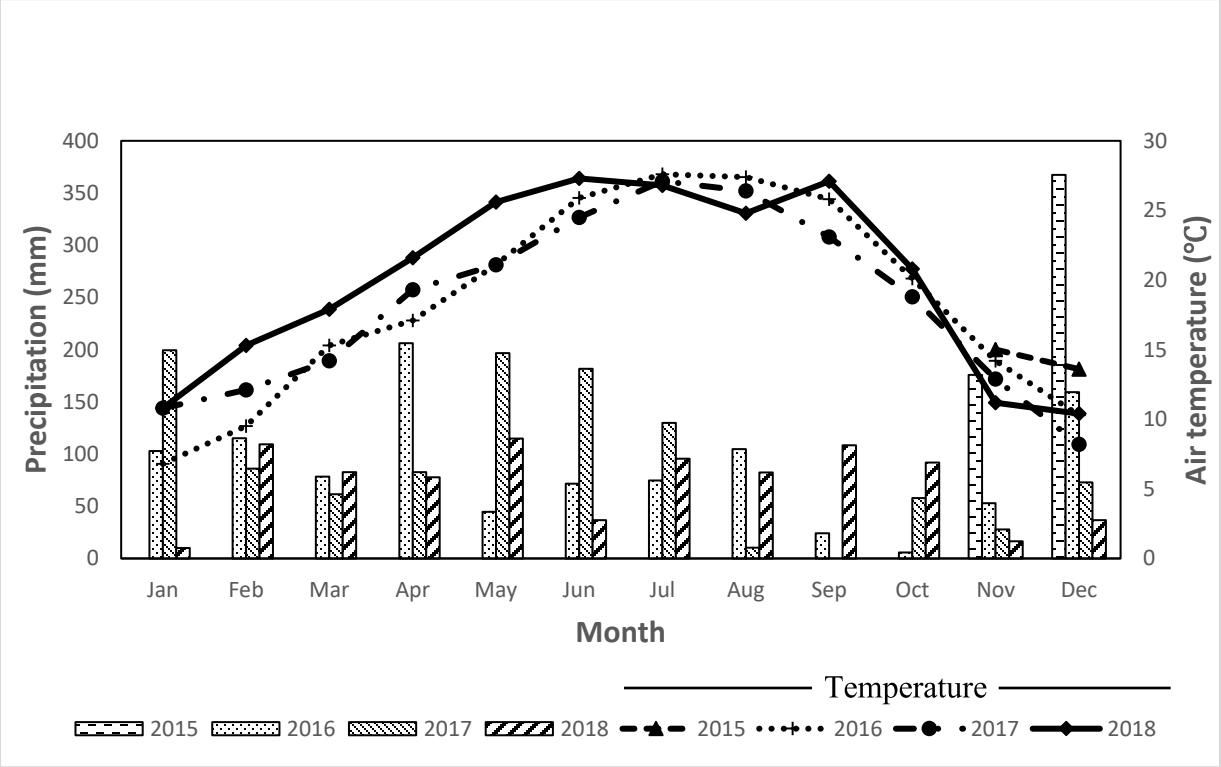

Figure 2.1 Mean monthly air temperature and precipitation during the study period in Shorter, AL.

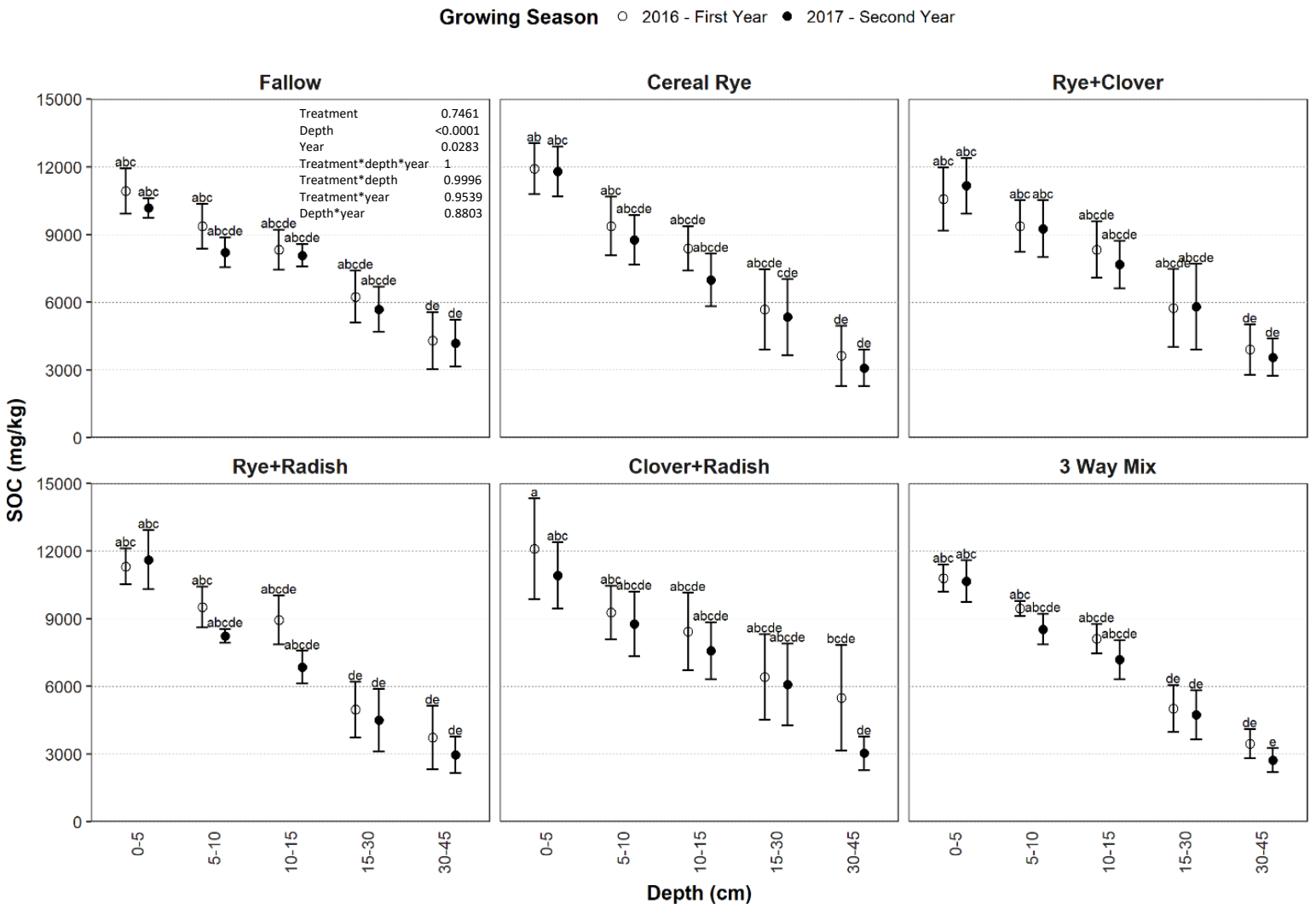

Figure 2.2 SOC by cover crop treatment, depth, and year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for SOC with main effects (cover crop treatment, depth, and year) and their interactions, as well as significant differences from Tukey's HSD at $\alpha = 0.05$ shown as different letters.

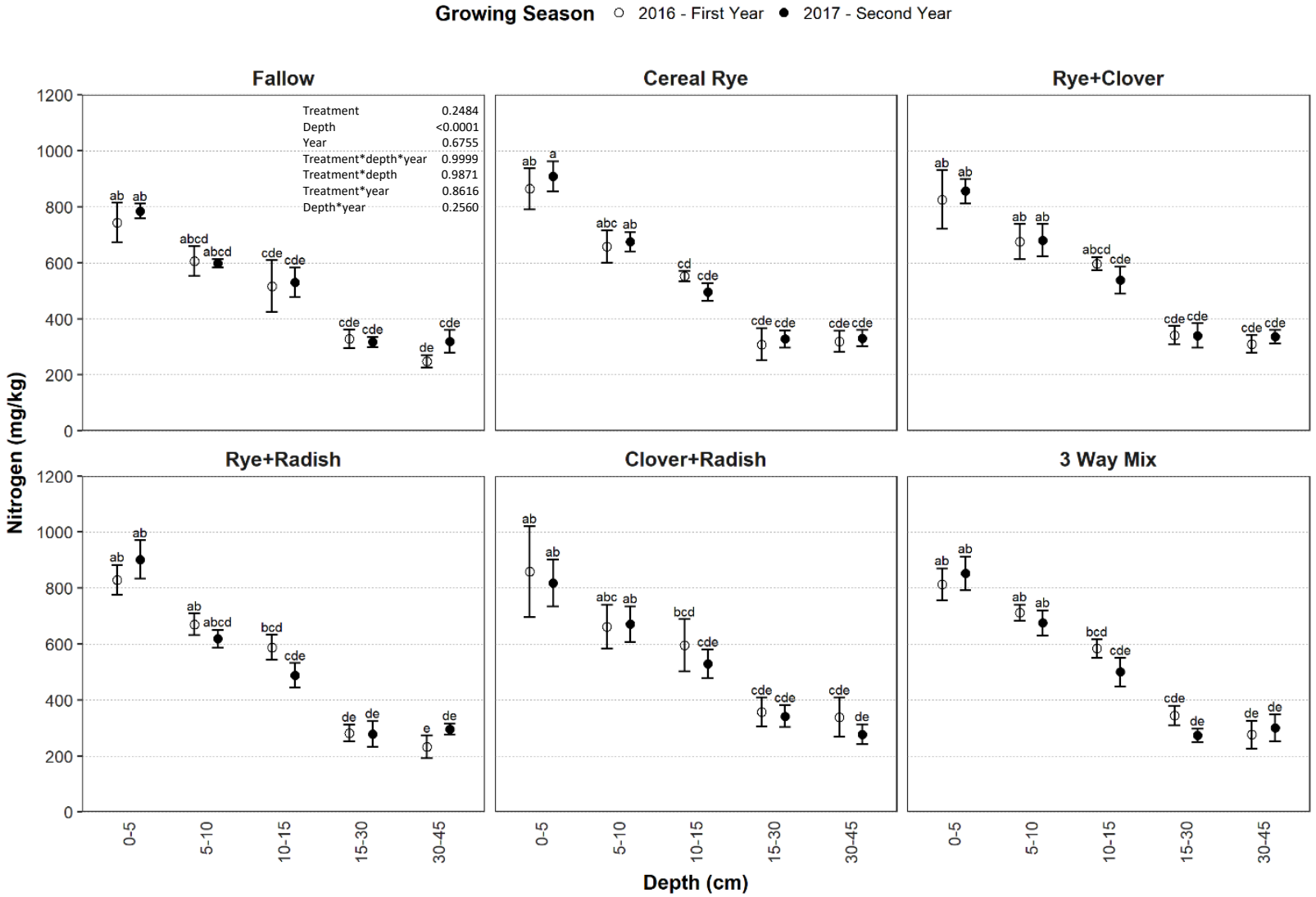

Figure 2.3 Nitrogen by cover crop treatment, depth, and year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for nitrogen with main effects (cover crop treatment, depth, and year) and their interactions, as well as significant differences from Tukey's HSD at α= 0.05 shown as different letters.

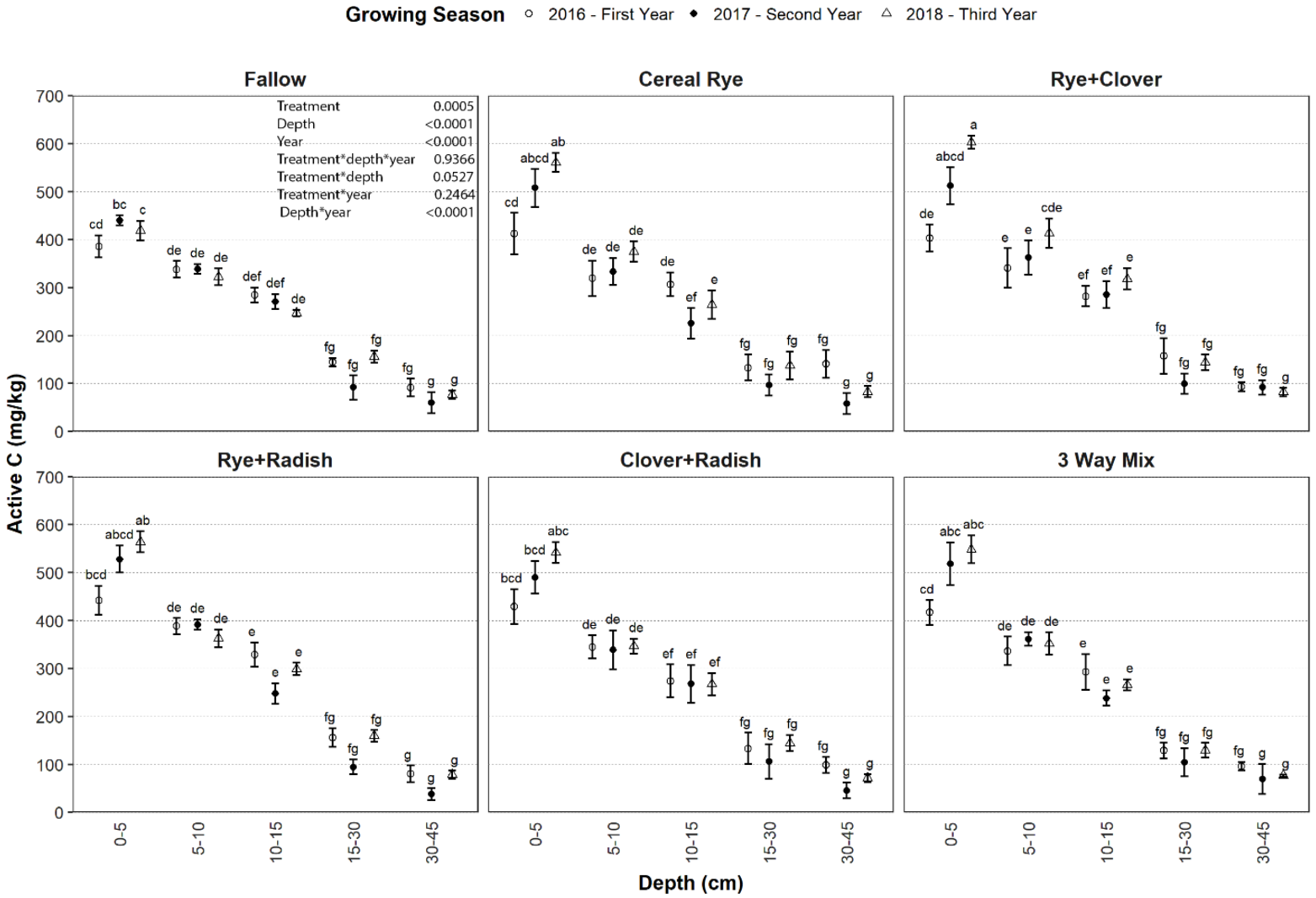

Figure 2.4 Active C by cover crop treatment, depth, and year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for active C with main effects (cover crop treatment, depth, and year) and their interactions, as well as significant differences from Tukey's HSD at $\alpha = 0.05$ shown as different letters.

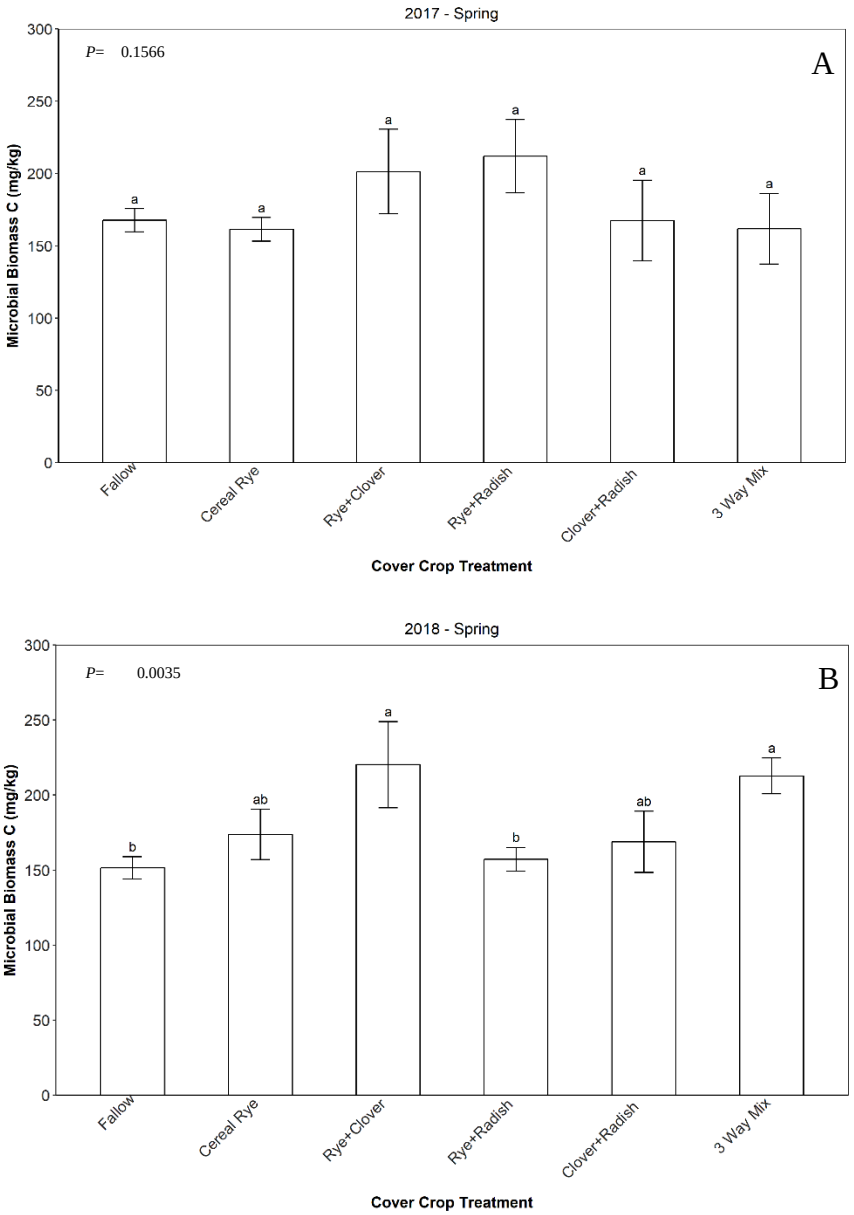

Figure 2.5 Effects of cover crop treatments on microbial biomass C (MBC) in 2017 (A) and 2018 (B). Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for MBC with the main effect (cover crop treatment), as well as significant differences from Tukey's HSD at $\alpha = 0.05$ shown as different letters.

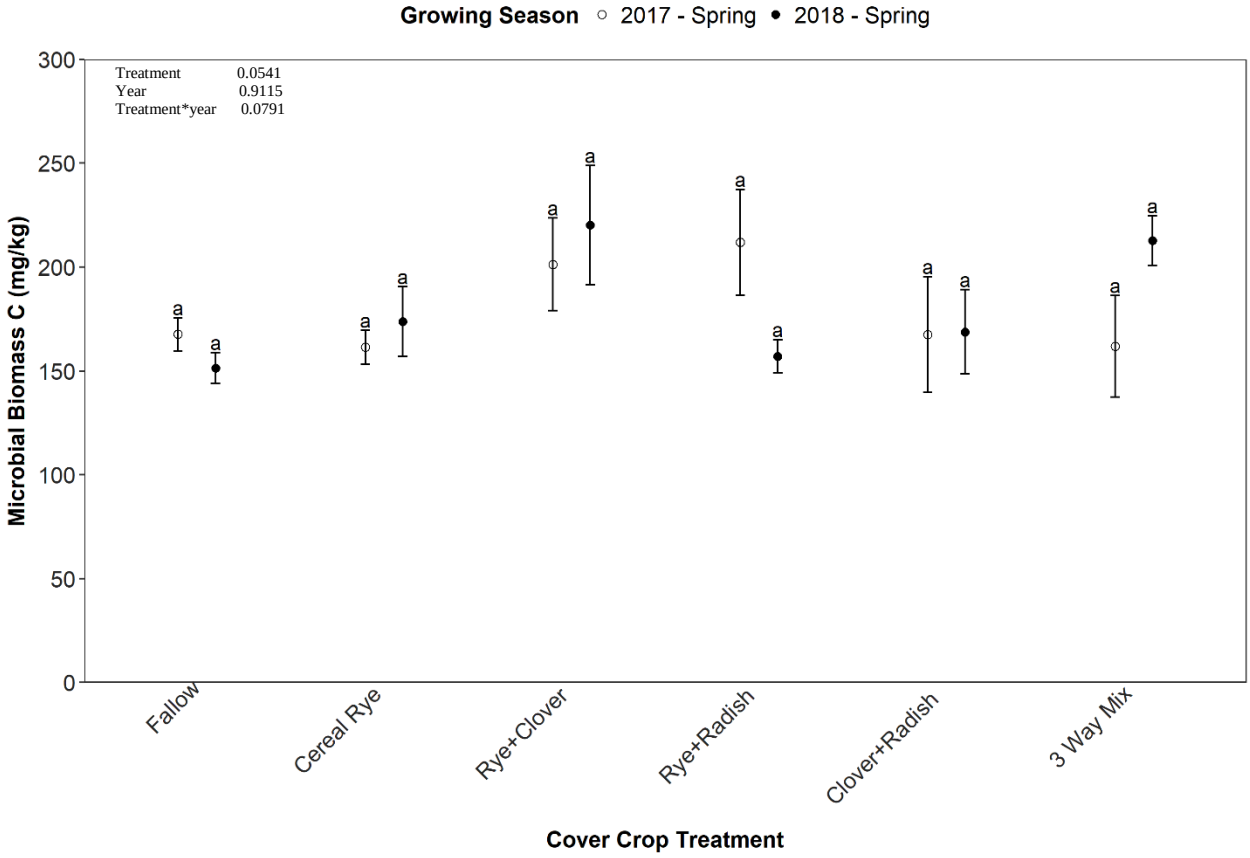

Figure 2.6 Effects of cover crop treatments on microbial biomass C (MBC) by year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for MBC with main effects (cover crop treatment and year) and the interaction, as well as significant differences from Tukey's HSD at $\alpha = 0.05$ shown as different letters.

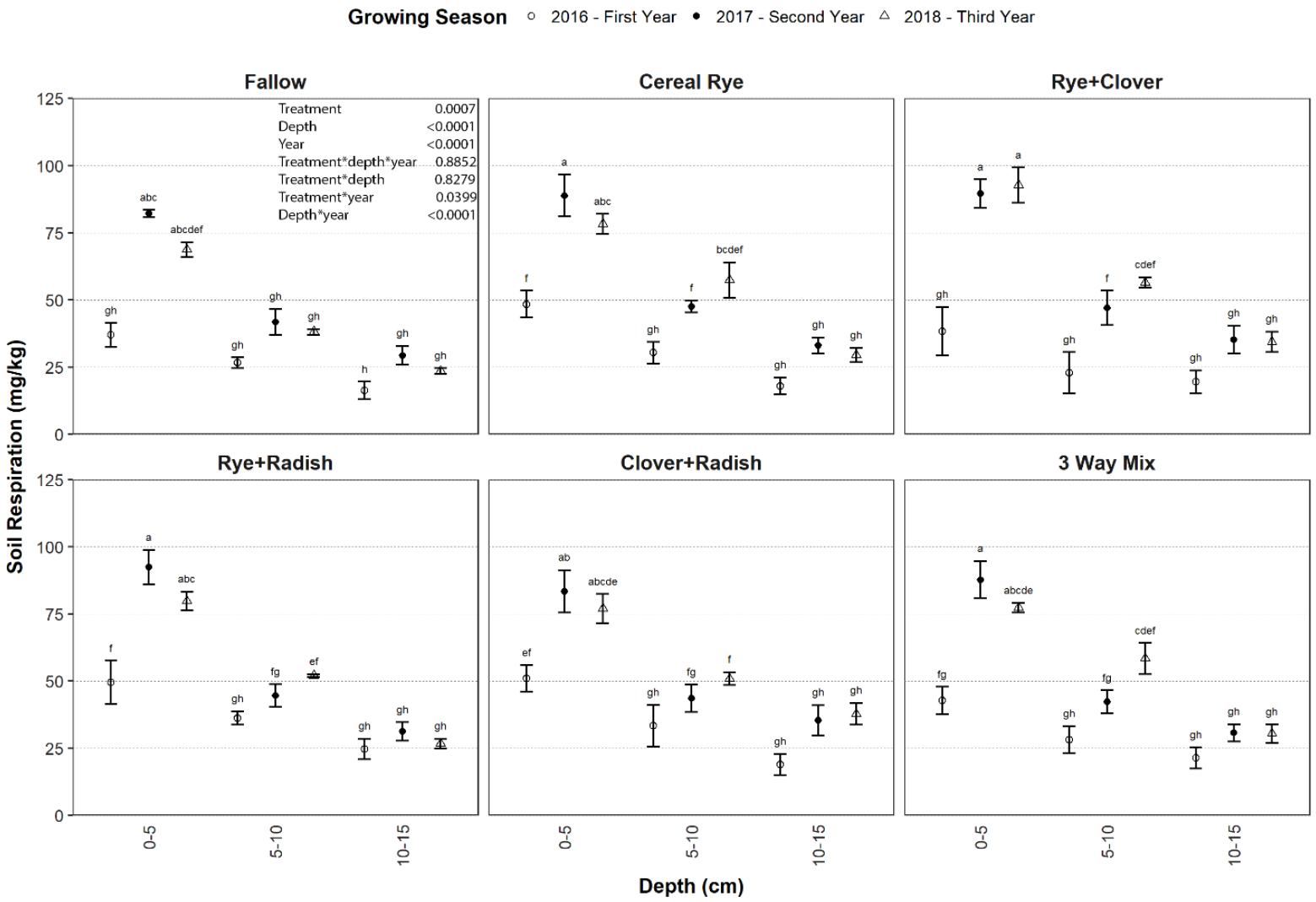

Figure 2.7 Soil Respiration by cover crop treatment, depth, and year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for soil respiration with main effects (cover crop treatment, depth, and year) and their interactions, as well as significant differences from Tukey's HSD at α= 0.05 shown as different letters.

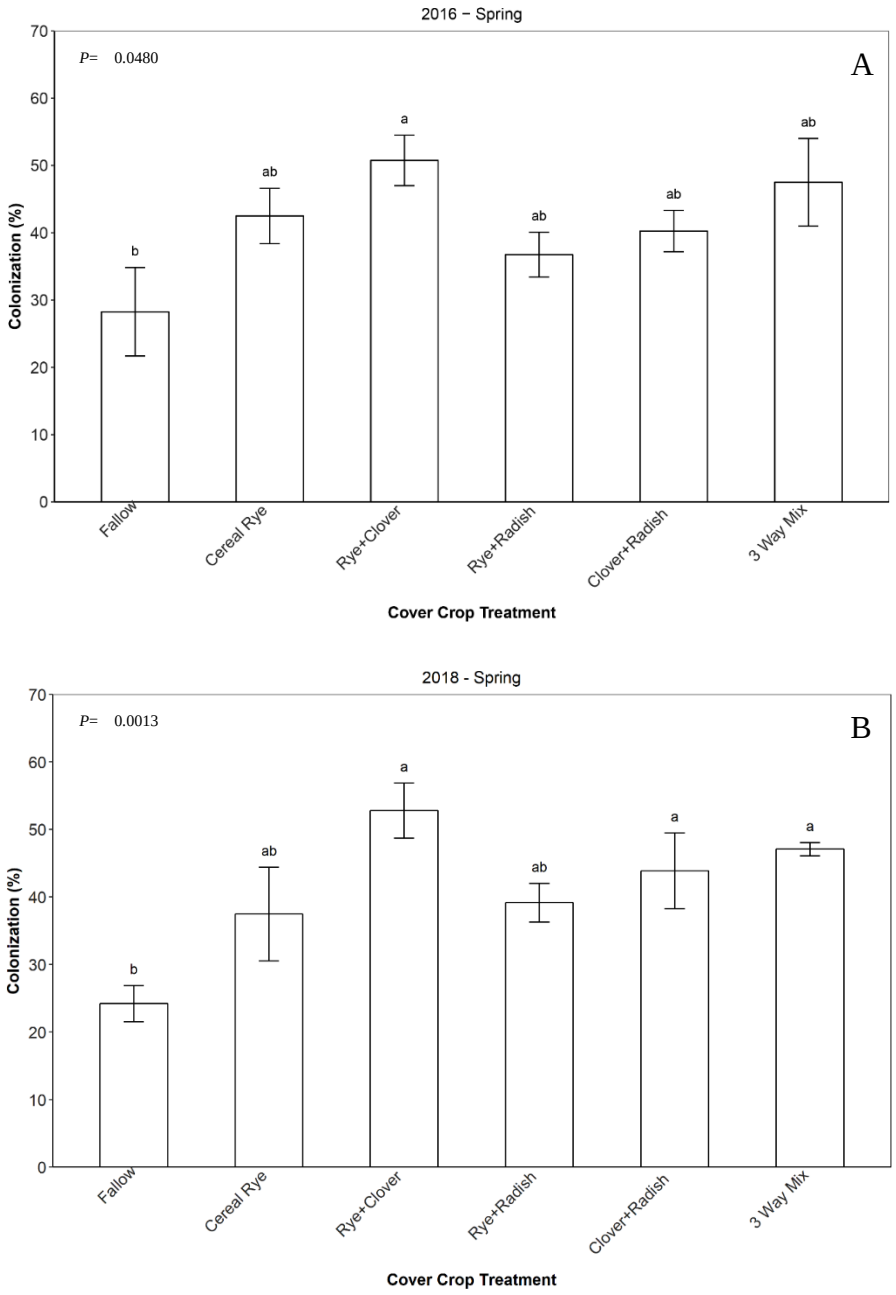

Figure 2.8 Effects of cover crop treatments on arbuscular mycorrhizal fungi colonization rate of cotton roots (AMF) in 2016 (A) and 2018 (B). Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for AMF with the main effect (cover crop treatment), as well as significant differences from Tukey's HSD at α= 0.05 shown as different letters.

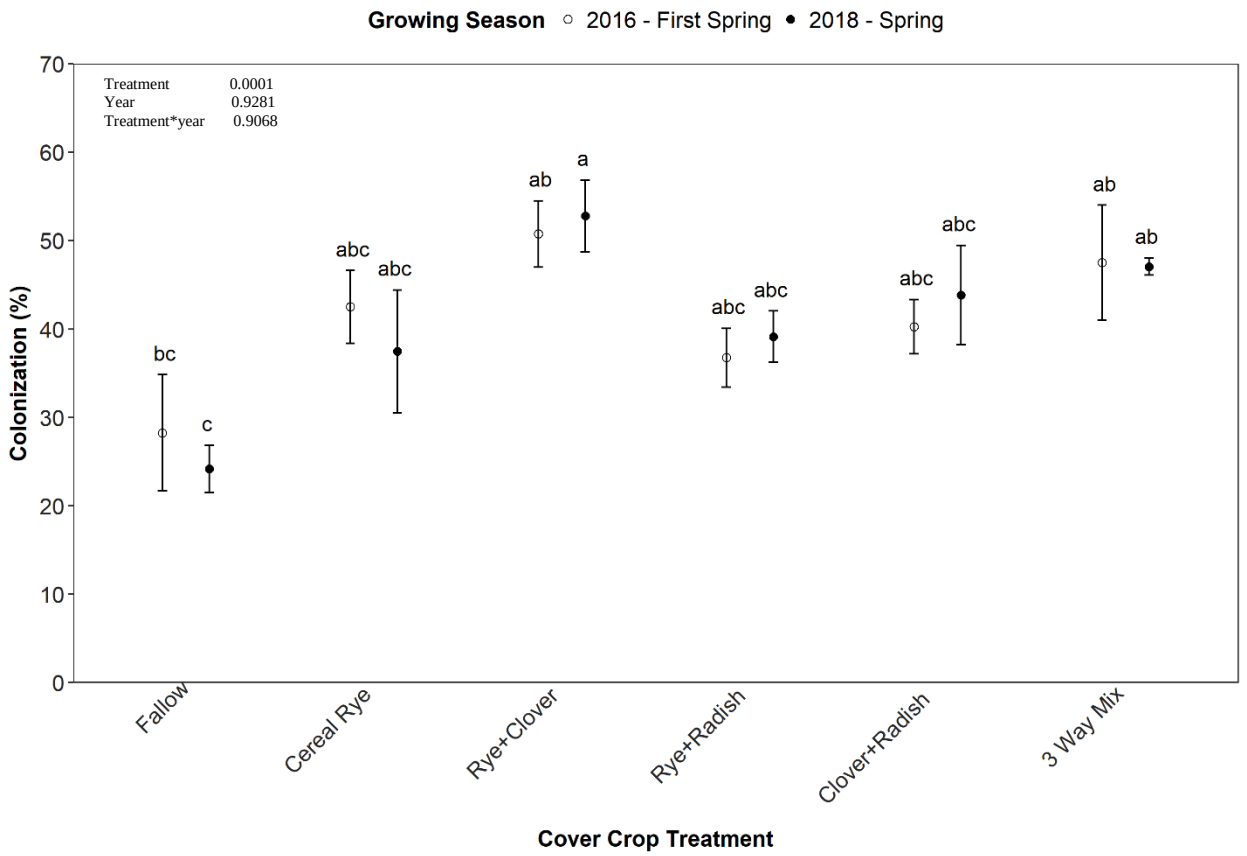

Figure 2.9 Effects of cover crop treatments on arbuscular mycorrhizal fungi colonization rate of cotton roots by year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for AMF with main effects (cover crop treatment and year) and the interaction, as well as significant differences from Tukey's HSD at α= 0.05 shown as different letters.

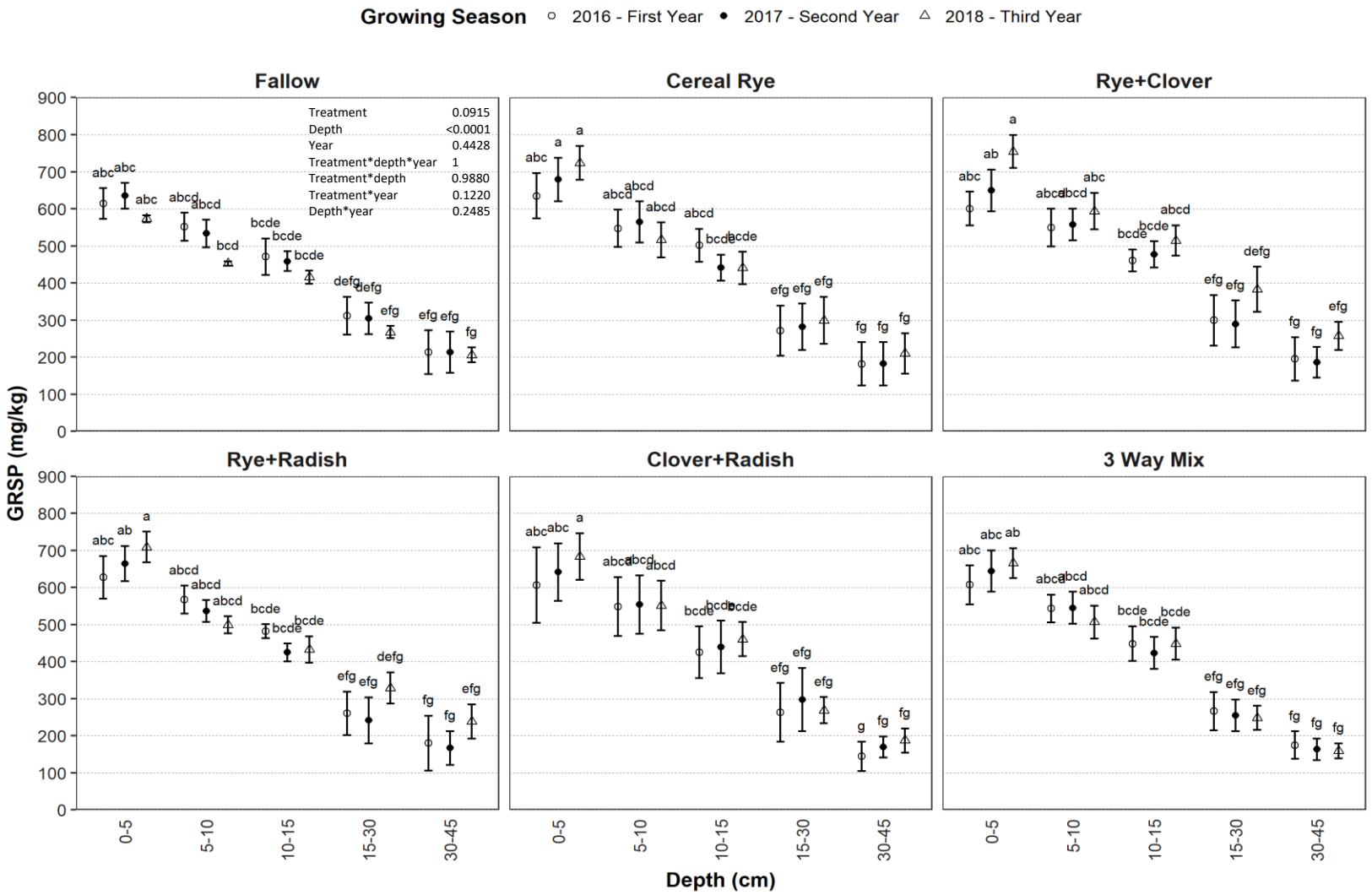

Figure 2.10 Glomalin-related soil protein (GRSP) by cover crop treatment, depth, and year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for GRSP with main effects (cover crop treatment, depth, and year) and their interactions, as well as significant differences from Tukey's HSD at $\alpha = 0.05$ shown as different letters.

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Appendix A. SOC data

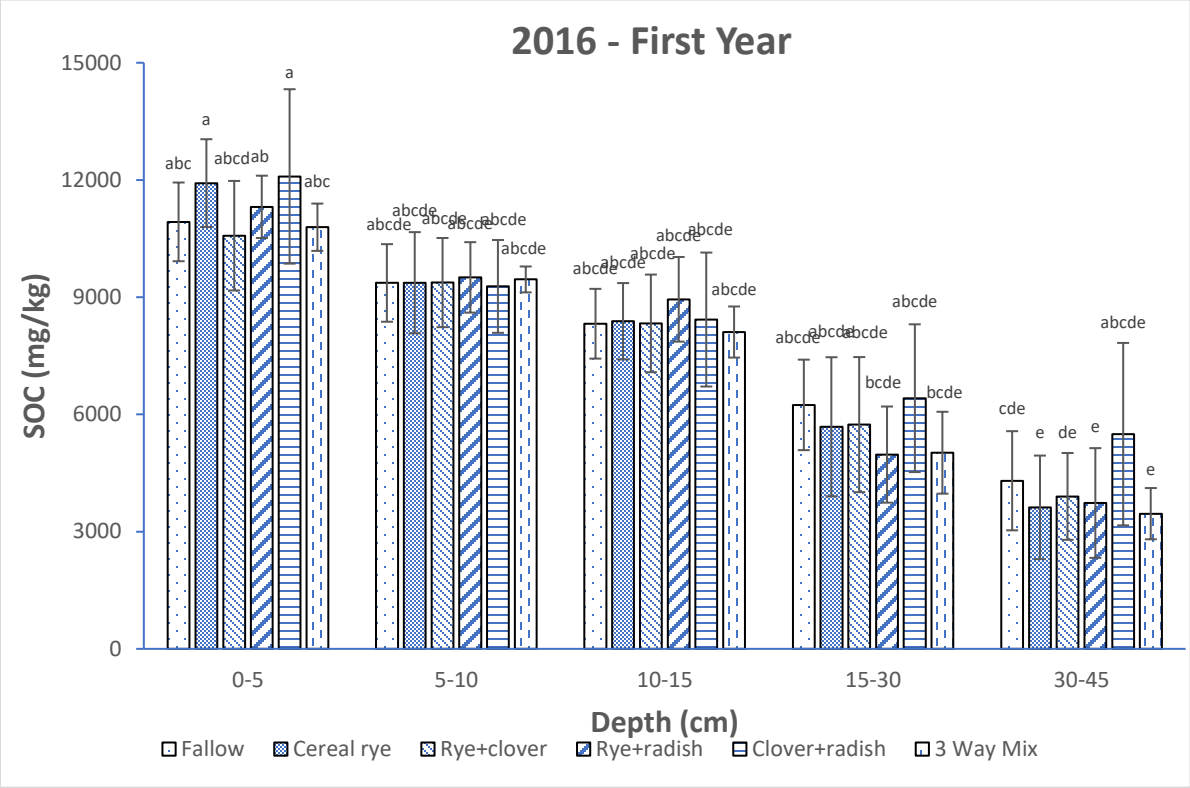


Figure A.1 SOC by cover crop treatment and soil depth in 2016. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

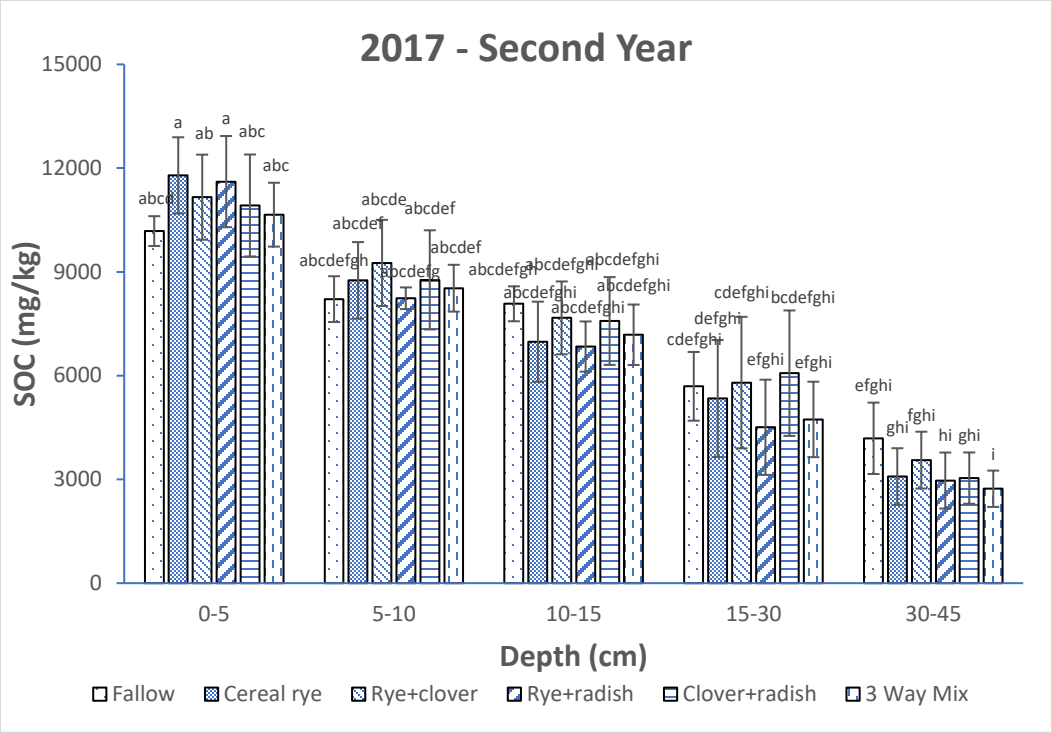


Figure A.2 SOC by cover crop treatment and soil depth in 2017. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

Appendix B. TON data

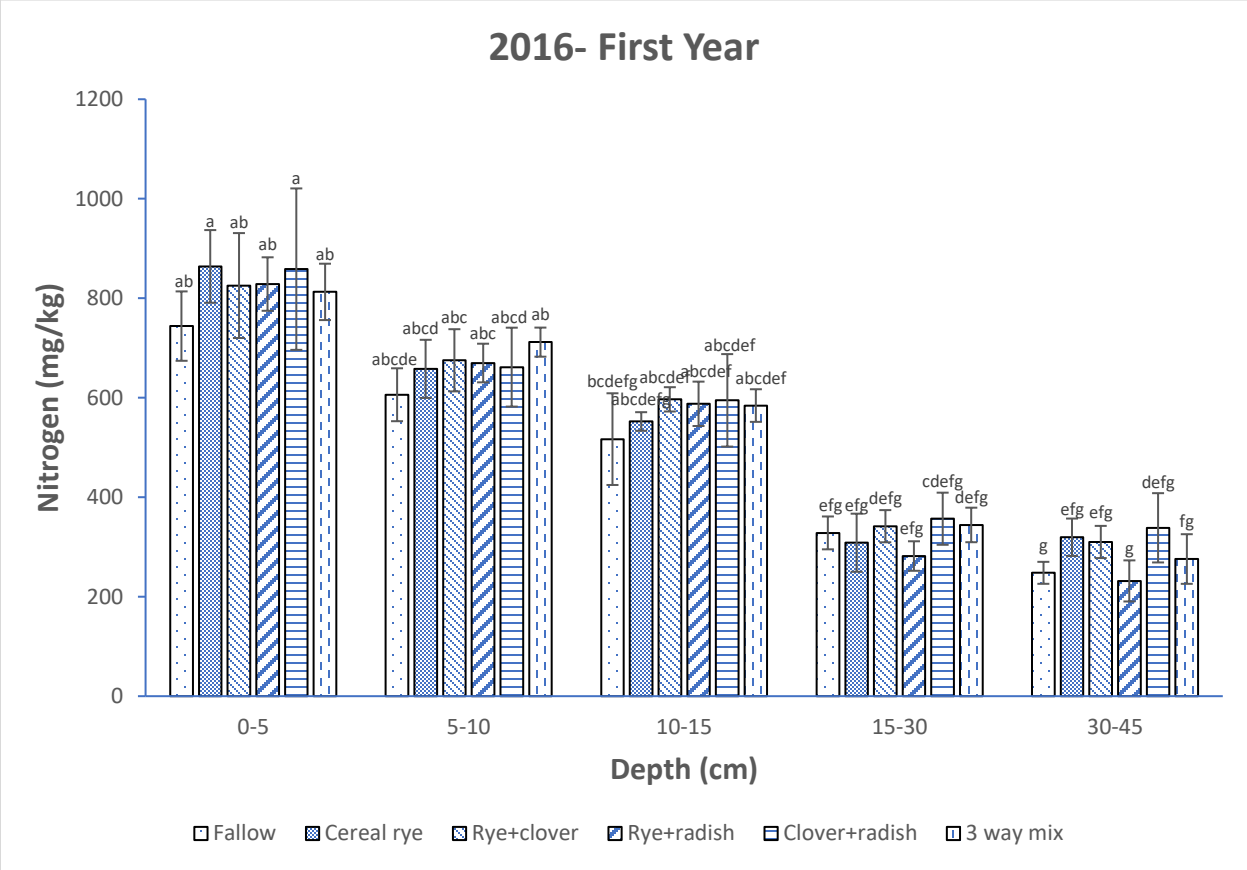


Figure B.1 Total organic nitrogen by cover crop treatment and soil depth in 2016. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

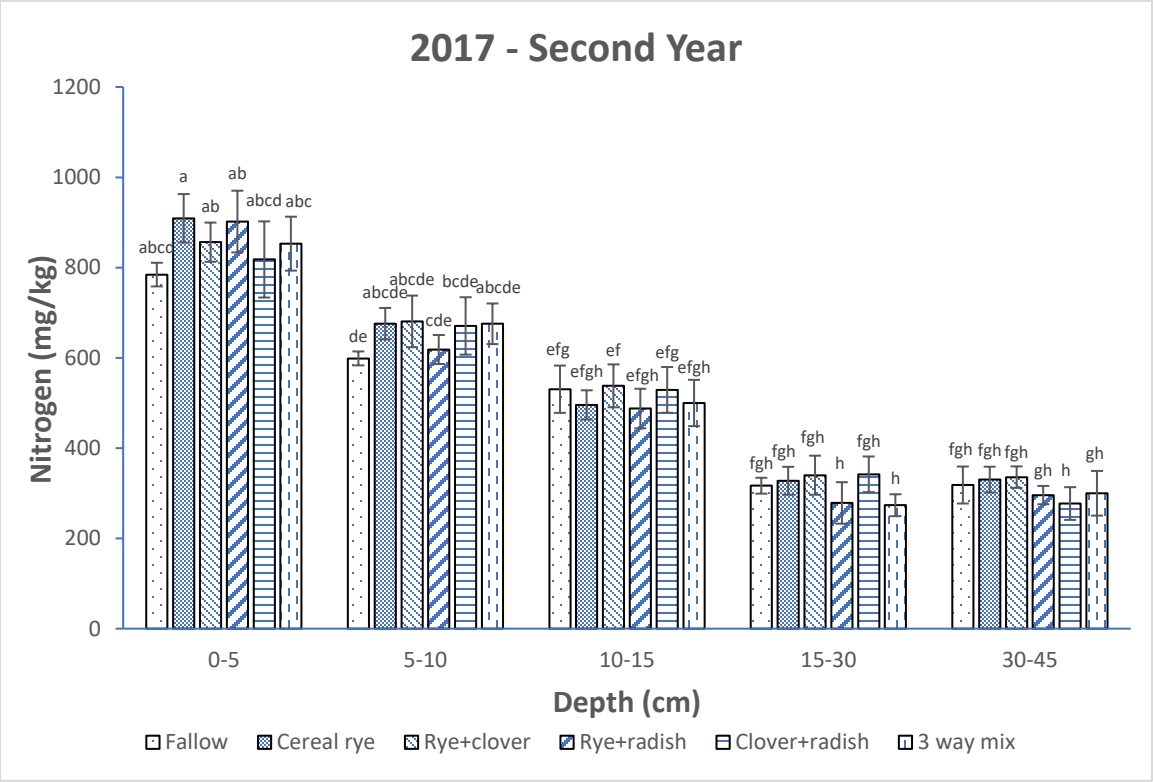


Figure B.2 Total organic nitrogen by cover crop treatment and soil depth in 2017. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at $\alpha = 0.05$.

Appendix C. Bulk density data

Table C.1 Effects of cover crop treatments on bulk density by soil depth and year.

	Bulk density (g/cm^3)				
	Depth (cm)				
Treatment	**0–5**	**5–10**	**10–15**	**15–30**	**30–45**
2016					
Fallow	1.43 (±0.11)	1.41 (±0.01)	1.45 (±0.04)	1.61 (±0.04)	1.68 (±0.06)
Cereal rye	1.42 (±0.06)	1.47 (±0.04)	1.56 (±0.04)	1.67 (±0.06)	1.65 (±0.03)
Rye+Clover	1.50 (±0.04)	1.49 (±0.05)	1.60 (±0.06)	1.69 (±0.06)	1.67 (±0.01)
Rye+Radish	1.38 (±0.06)	1.47 (±0.06)	1.51 (±0.03)	1.65 (±0.06)	1.69 (±0.06)
Clover+Radish	1.53 (±0.04)	1.52 (±0.03)	1.59 (±0.03)	1.64 (±0.04)	1.65 (±0.03)
3 Way mix	1.44 (±0.05)	1.46 (±0.05)	1.50 (±0.06)	1.70 (±0.05)	1.67 (±0.06)
Mean	*1.45 (±0.02)C*	*1.47 (±0.01)BC*	*1.53 (±0.02)B*	*1.66 (±0.01)A*	*1.67 (±0.01)A*
2017					
Fallow	1.40 (±0.03)	1.48 (±0.07)	1.57 (±0.02)	1.65 (±0.04)	1.76 (±0.06)
Cereal rye	1.43 (±0.03)	1.51 (±0.02)	1.63 (±0.02)	1.74 (±0.02)	1.80 (±0.03)
Rye+Clover	1.36 (±0.02)	1.57 (±0.04)	1.59 (±0.02)	1.73 (±0.05)	1.81 (±0.05)
Rye+Radish	1.35 (±0.06)	1.55 (±0.04)	1.59 (±0.04)	1.76 (±0.04)	1.81 (±0.04)
Clover+Radish	1.36 (±0.01)	1.53 (±0.04)	1.57 (±0.03)	1.69 (±0.03)	1.80 (±0.02)
3 Way mix	1.41 (±0.03)	1.58 (±0.05)	1.62 (±0.0)	1.70 (±0.04)	1.80 (±0.03)
Mean	*1.39 (±0.01)E*	*1.54 (±0.02)D*	*1.60 (±0.01)C*	*1.71 (±0.02)B*	*1.80 (±0.01)A*

Note: Value in the parenthesis is the standard error of the mean (n= 4). Different capital letters within each row indicate significant differences by depth according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments.

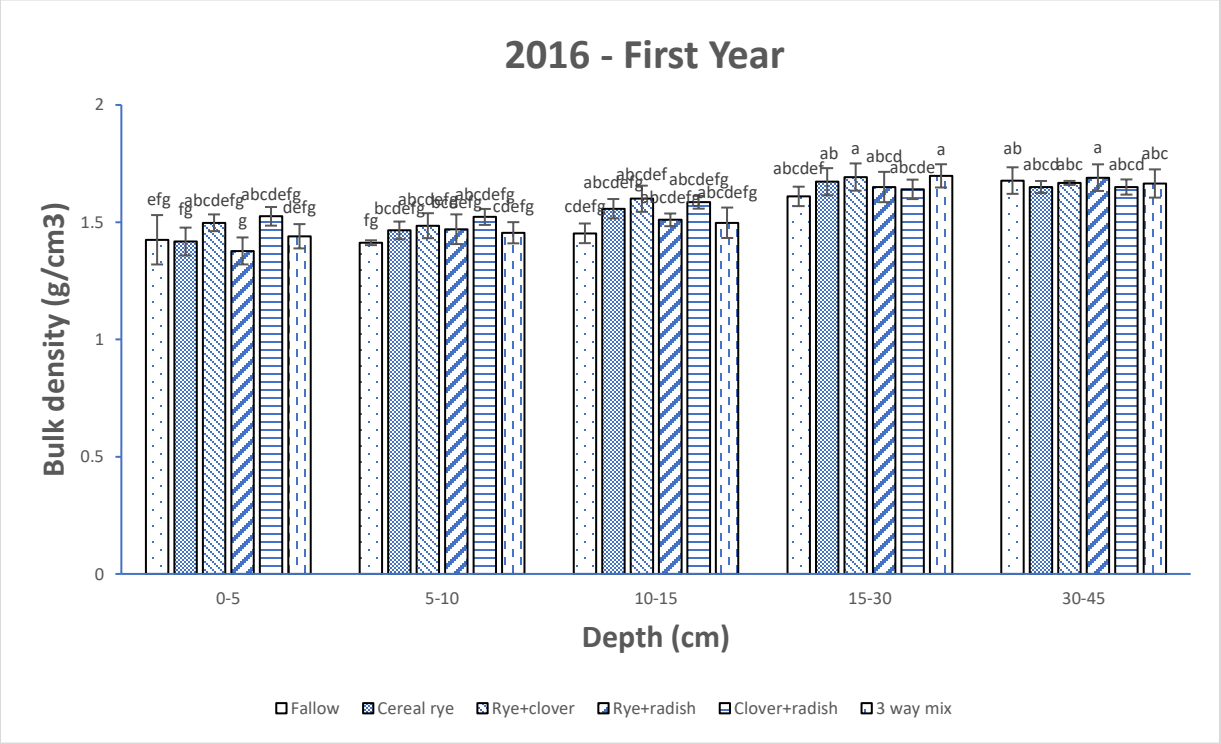


Figure C.2 Bulk density by cover crop treatment and soil depth in 2016. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

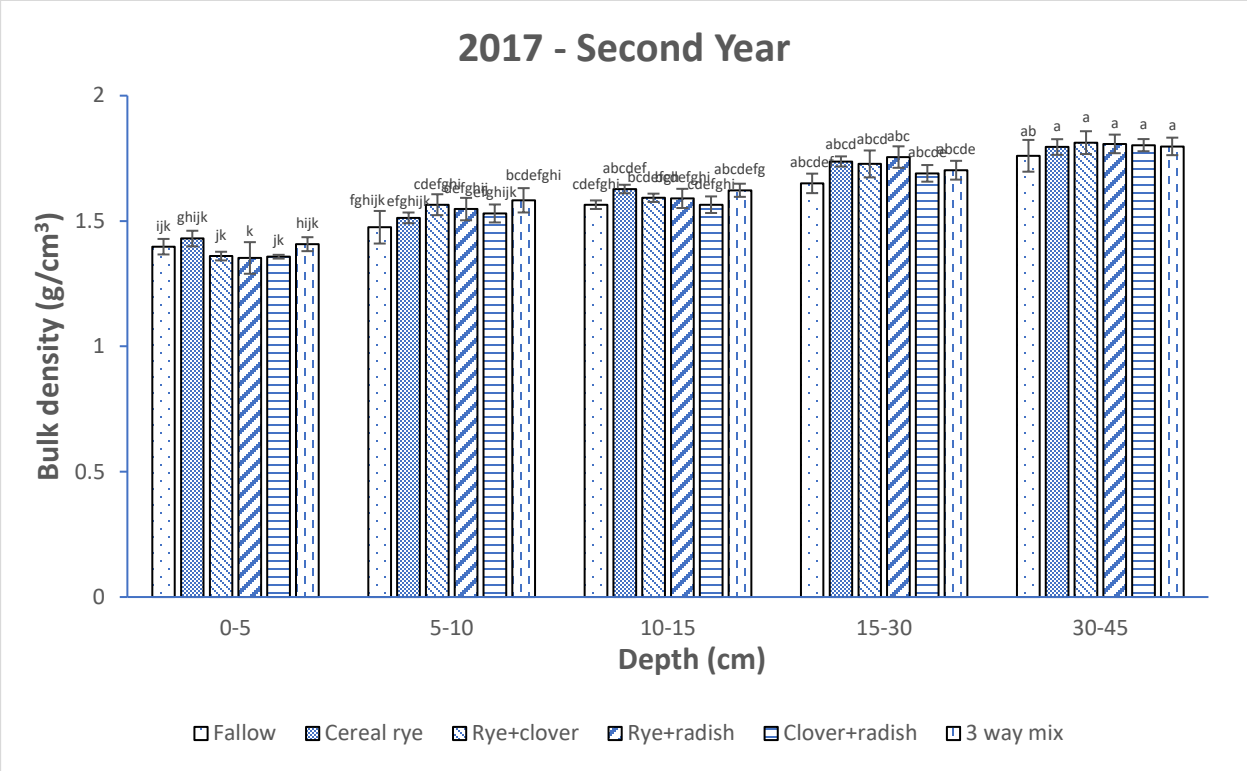


Figure C.3 Bulk density by cover crop treatment and soil depth in 2017. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

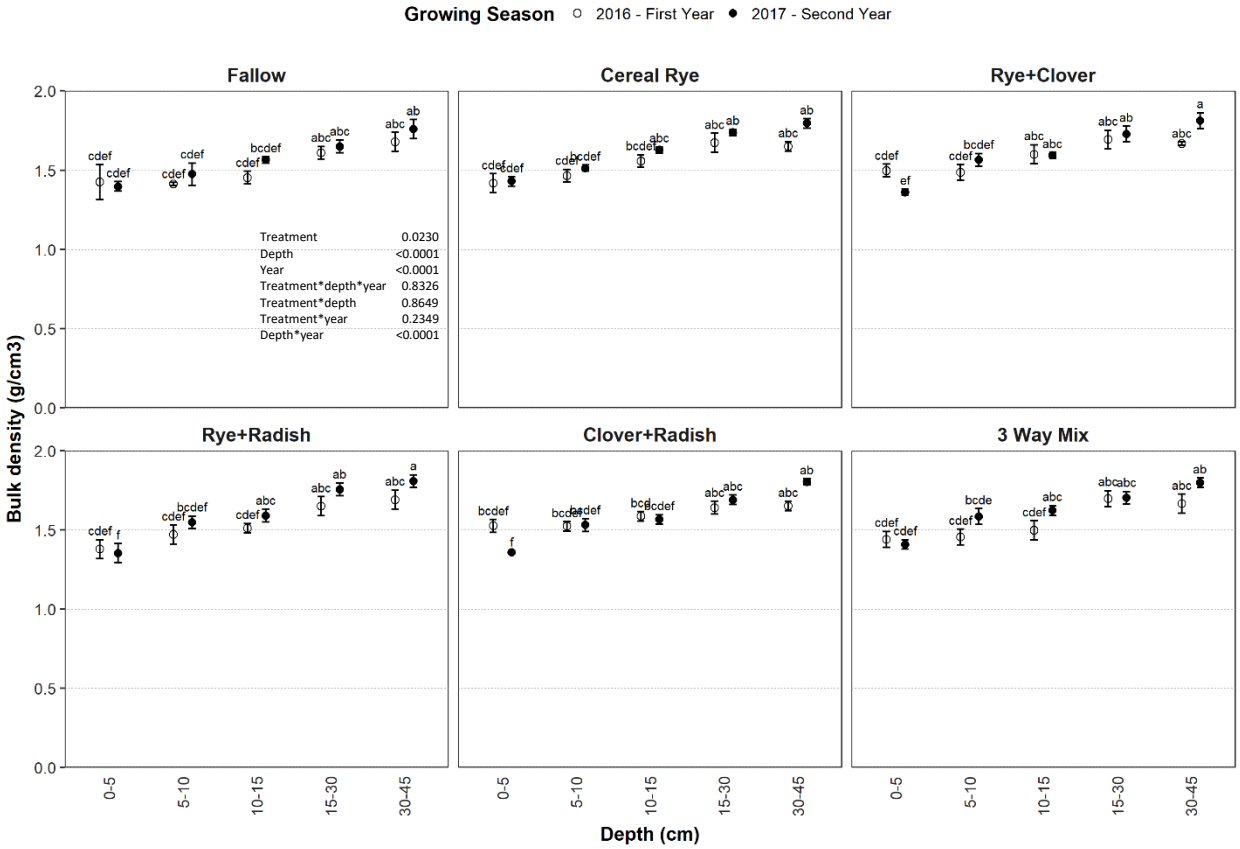


Figure C.4 Bulk density by cover crop treatment, depth, and year. Means (n= 4) are shown, and error bars are standard errors. P values from ANOVA results are shown for bulk density with main effects (cover crop treatment, depth, and year) and their interactions, as well as significant differences from Tukey's HSD at α= 0.05 shown as different lowercase letters.

Appendix D. Active C data

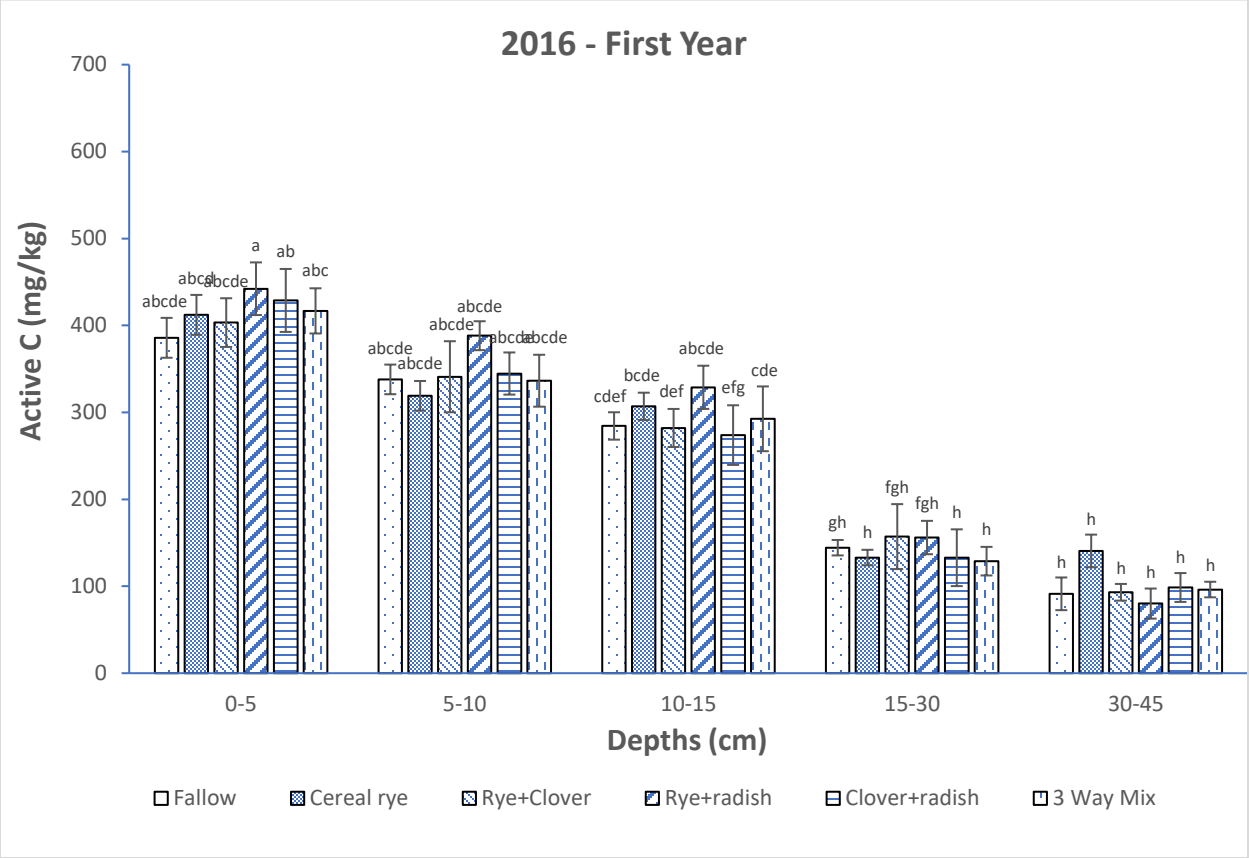


Figure D.1 Active C by cover crop treatment and soil depth in 2016. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

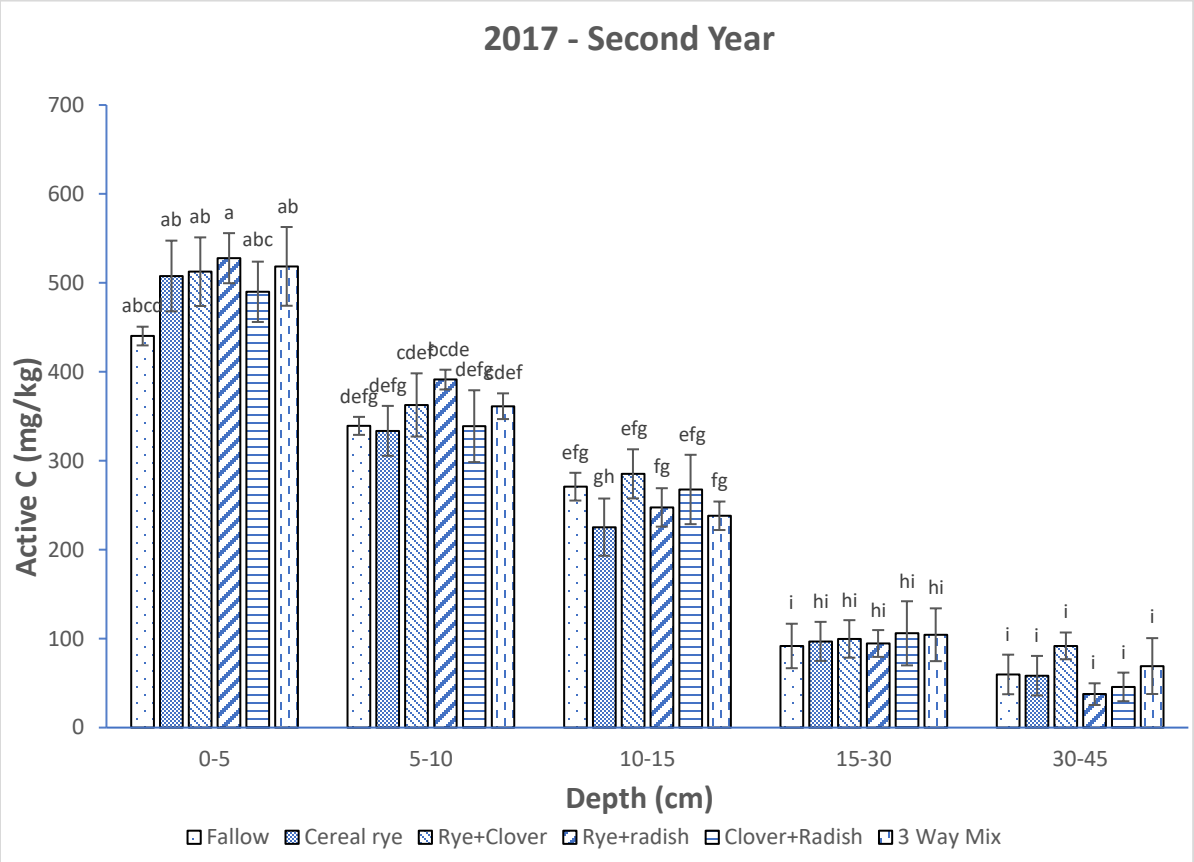


Figure D.2 Active C by cover crop treatment and soil depth in 2017. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at $\alpha = 0.05$.

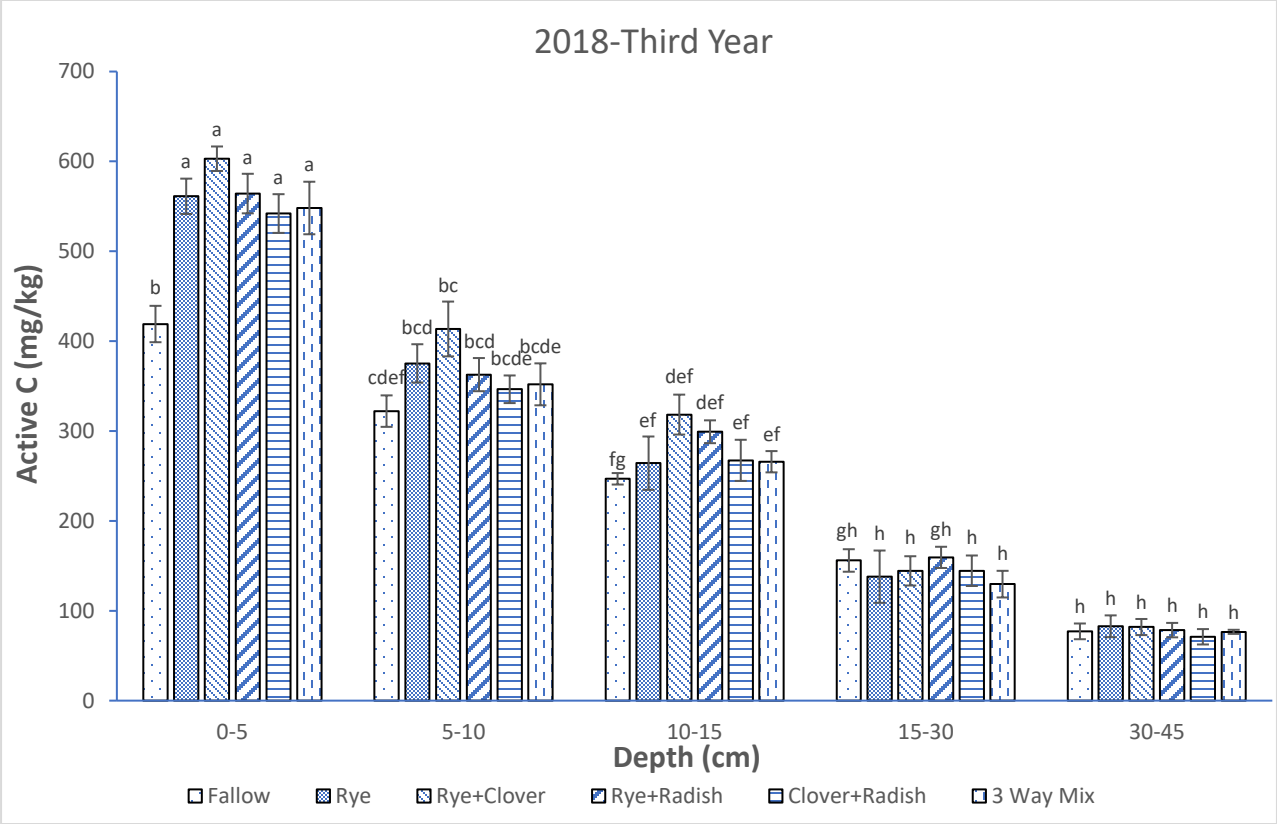


Figure D.3 Active C by cover crop treatment and soil depth in 2018. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

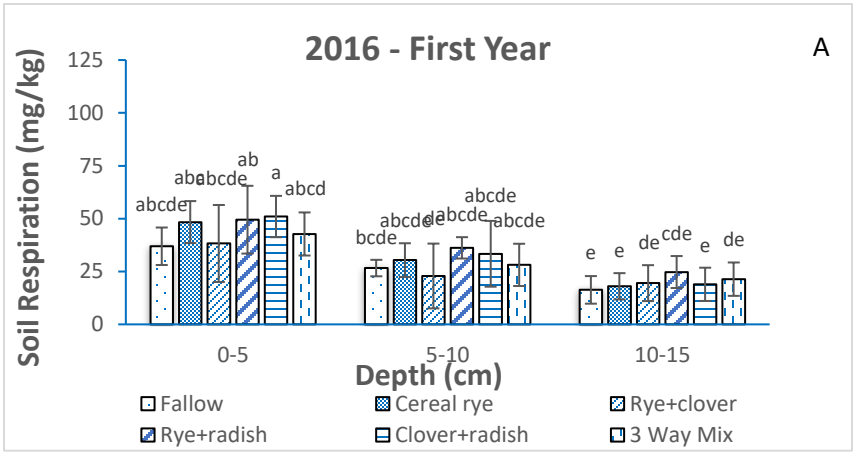
Appendix E. Microbial biomass C data

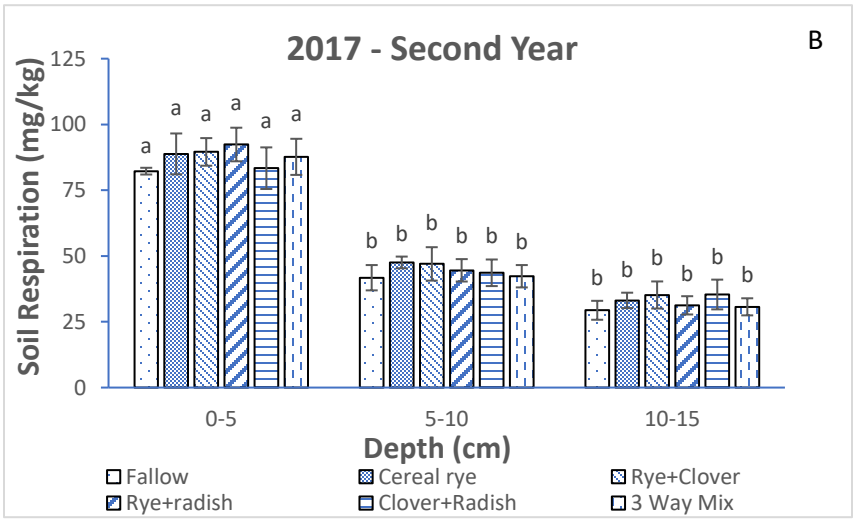
Table E.1 Effects of cover crop treatments on microbial biomass carbon (MBC) (mg/kg) in 2017 and 2018.

Treatment	**2017**	**2018**
Fallow	167.6 (±8.0)	151.4 (±7.4)b
Cereal rye	161.4 (±8.2)	173.7 (±16.7)ab
Rye+Clover	201.3 (±29.3)	220.2 (±28.7)a
Rye+Radish	211.9 (±25.4)	157.1 (±8.0)b
Clover+Radish	167.4 (±27.9)	168.8 (±20.4)ab
3 Way mix	161.8 (±24.4)	212.7 (±11.9)a

Note: Value in the parenthesis is the standard error of the mean (n=4). Different letters within each column indicate significant differences according to Tukey's HSD at α=0.05. Absence of letters indicates no significant differences among treatments.

Appendix F. Soil respiration data





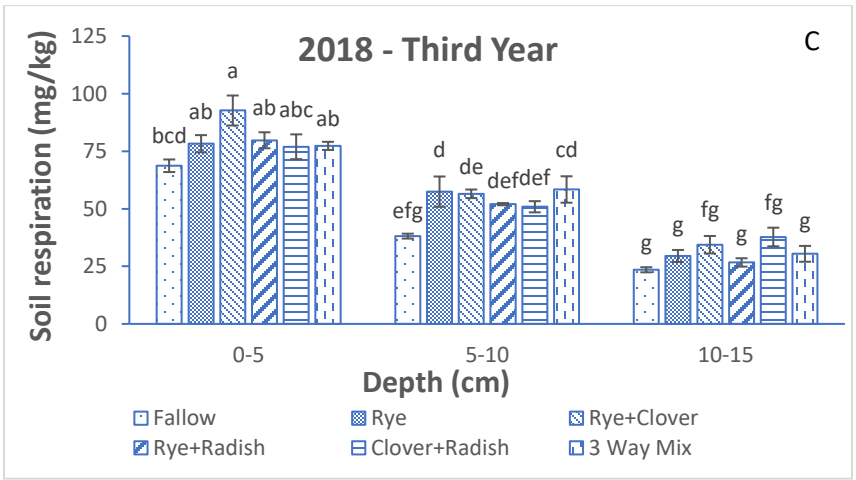


Figure F.1 Soil respiration by cover crop treatment and soil depth in different years: (A) 2016, (B) 2017, and (C) 2018. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

Appendix G. Arbuscular mycorrhizal colonization data

Table G.1 Effects of cover crop treatments on AMF colonization rates (%) in 2016 and 2018.

Treatment	**2016**	**2018**
Fallow	28.3 (±6.6)b	24.2 (±2.7)b
Cereal rye	42.5 (±4.1)ab	37.5 (±6.9)ab
Rye+Clover	50.8 (±3.8)a	52.8 (±4.1)a
Rye+Radish	36.8 (±3.3)ab	39.1 (±2.9)ab
Clover+Radish	40.3 (±3.1)ab	43.8 (±5.6)a
3 Way mix	47.5 (±6.5)ab	47.1 (±1.0)a

Note: Value in the parenthesis is the standard error of the mean (n=4). Different letters within each column indicate significant differences according to Tukey's HSD at α=0.05.

Appendix H. Glomalin-related soil protein data

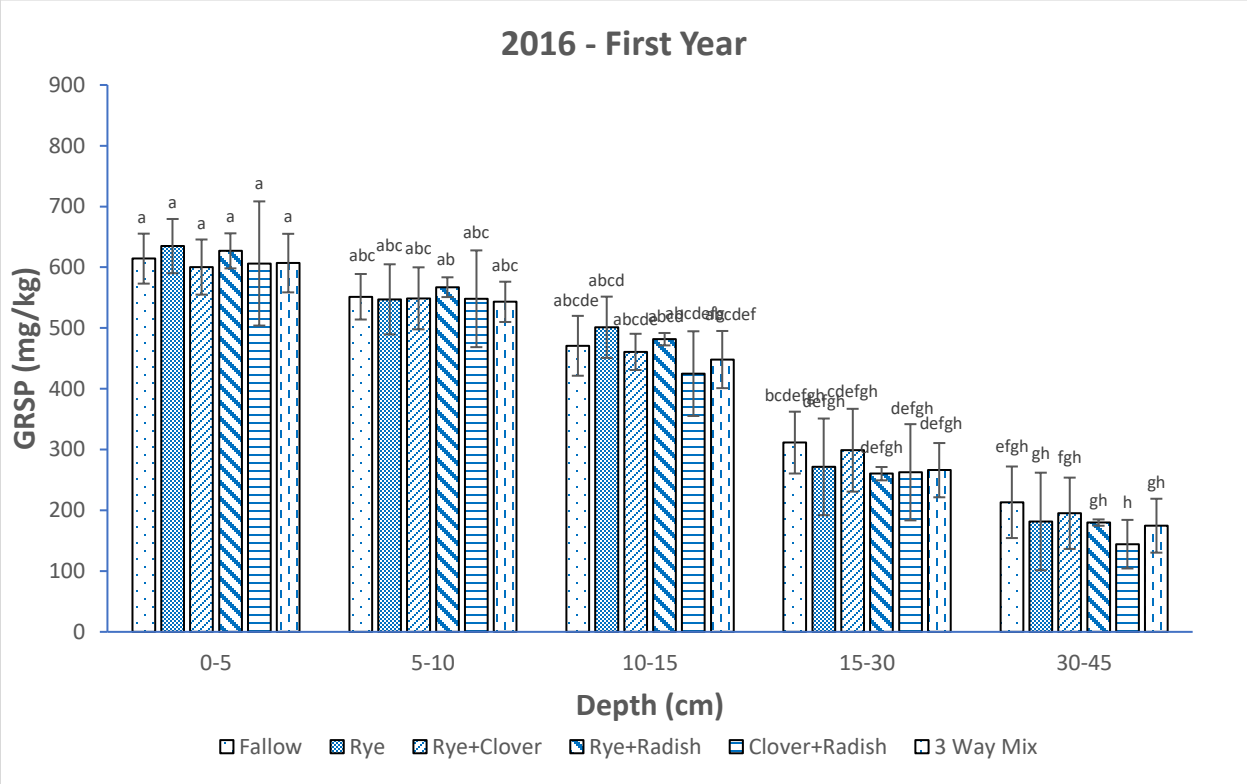


Figure H.1 Glomalin-related soil protein (GRSP) by cover crop treatment and soil depth in 2016. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

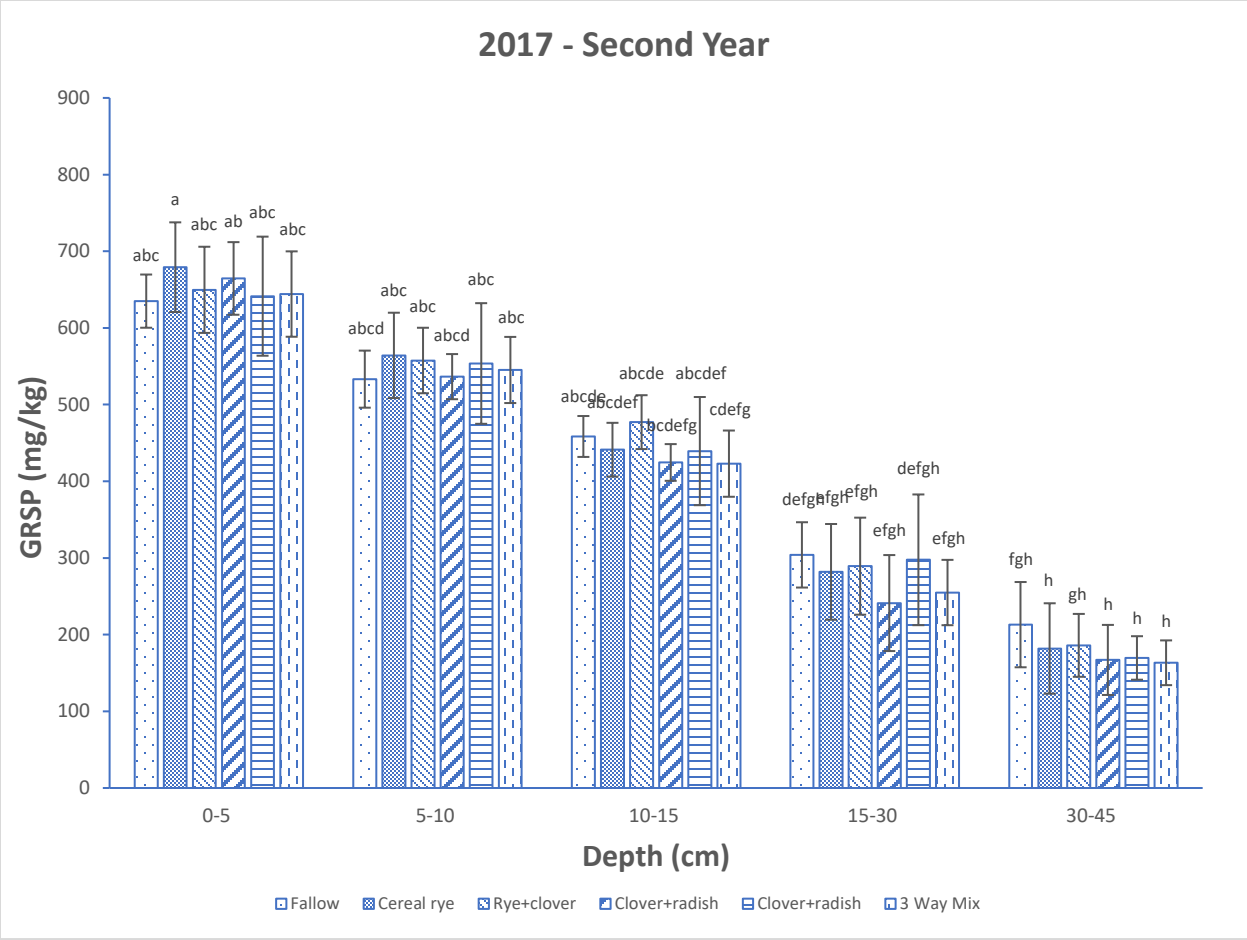


Figure H.2 Glomalin-related soil protein (GRSP) by cover crop treatment and soil depth in 2017. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at α= 0.05.

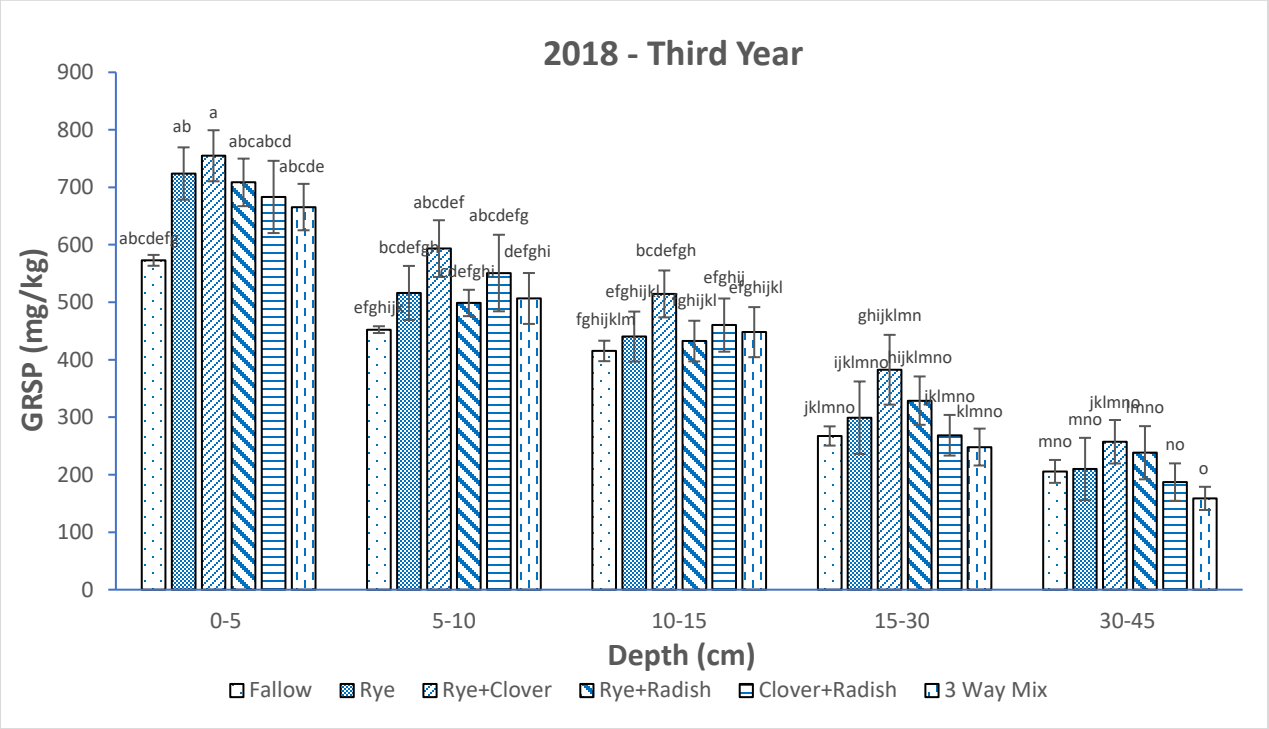


Figure H.3 Glomalin-related soil protein (GRSP) by cover crop treatment and soil depth in 2018. Means (n= 4) are shown, and error bars are standard errors. Significant differences shown as different lowercase letters based on Tukey's HSD at $\alpha = 0.05$.