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# Soil Microbial Community Dynamics during the Decomposition Period of Winter Cover Crops

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# SOIL MICROBIAL COMMUNITY DYNAMICS DURING THE DECOMPOSITION PERIOD OF WINTER COVER CROPS

by

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# ABSTRACT

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Across the Midwest United States, there is an increasing number of agriculturalists incorporating cover crops and reduced tillage residue management into sustainable nutrient management plans. However, the impact of these treatments on the soil microbial community, and subsequently, on soil nutrient cycling and inorganic nitrogen (N) availability, is largely unknown. Therefore, this research had two objectives: i) to determine the impact of cover crop species on soil and litter enzyme activity and soil nitrogen supply during the cover crop decomposition period, and ii) to evaluate the impact of cover crops and reduced tillage on the soil microbial community diversity and composition during the cover crop decomposition period. Enzyme activity was measured through β-glucosidase and urease activity assessments on soil and cover crop litter. Soil microbial communities were characterized using the small subunit (16S) rRNA gene sequences determined using the Illumina MiSeq system and analyzed with the QIIME bioinformatics pipeline. Results indicate that cover crops and tillage treatment significantly influenced soil and litter enzyme activity and inorganic N availability during the decomposition period. Specifically, soil β-glucosidase activity peaked in all cover crop treatments in 2016 and 2017 within six decomposition degree-days (DCDs) following cover crop termination. Both seasons, peak inorganic N availability occurred within seven DCDs from peak β-glucosidase activity. In addition, this study revealed that the structure of the soil microbiome changed during the decomposition period. This was evident through changes in microbial community α- and β-diversity, and these

differences were greater as the decomposition period progressed, most notably after DCD 2.9 when a majority of the cover crop decomposition occurred. Residue management treatment was also a significant determinant of the soil microbial community structure, and the impact of these treatments was greatest at DCD 6.4. The variation of the soil microbiome structure at the beginning and end of the decomposition period was greater than the variation during peak cover crop decomposition. Overall, it was concluded that DCD, cover crop treatment, and residue management treatment were important variables in explaining the variance of the genus level observations. This study increases our understanding of how the soil microbial community responds to carbon inputs during the decomposition of winter cover crops, leading to the identification of bacteria that are highly responsive to management practices during the cash crop growing season and having the largest effect in differentiating cover crop species. Understanding the abundance patterns of the microbiota that are influenced by management practices has the potential to help optimize agriculture management practices to promote beneficial microorganisms as the adoption of management practices such as cover cropping and reduced tillage residue management treatments continues.

# CHAPTER 1. INTRODUCTION

## 1.1 Background

The contribution of nitrate from the Upper Mississippi River Basin to the Gulf of Mexico hypoxic zone continues to be an environmental issue for national water quality and a threat to the autonomy of row crop agriculture (Fageria et al., 2005; Sainju et al., 2002; Alexander et al., 2000). As a result, researchers have examined the effectiveness and economic feasibility of both in-field and edge-of-field practices to reduce the susceptibility of nitrogen (N) being lost through tile drainage. An example of an in-field management practice that has been considered for nutrient reduction is split N applications (Randall et al., 2003 and Randall and Vetsch, 2005). However, applying N in the fall is still common practice among Midwestern farmers (Smiciklas et al., 2008 and Lemke et al., 2011). Now, the assessment of multiple Midwestern states has concluded that cover crops, another in-field management practice, have the greatest efficacy to reduce nitrate loading to the Gulf of Mexico and the size of the hypoxic zone. Cover crops are vegetation most commonly grown between cash crop plantings to protect or improve the soil, water, or crop quality in an agroecosystem (Dabney, 1998). In Midwestern states, there has been a 212% increase in cover-cropped acres over a recent 5-year period, and there has been an increase in cover crop usage in conventional and organic agronomic production areas across the United States (Cover Crop Survey, 2017). The ability of cover crops to stabilize soil N and to scavenge and uptake mineralized and applied N from the soil are some of the main drivers of this adoption (Danso et al., 1991; Kasper and Singer, 2011; Fageria et al., 2005). However, to optimize N management using different cover crops, the cover crop knowledge base must be advanced beyond N scavenging ability of different cover crop species.

There is a lack of knowledge available to crop producers, and in the scientific literature, surrounding the timing of cover crop nutrient release from the cover crop residue during the decomposition period. The soil microbial community, as well as soil temperature, moisture, aeration and substrate quality (Stump and Binkley, 1992; Robertson and Paul, 2000; Vazquez et al., 2003) drive cover crop decomposition. These microbial-driven processes can be altered based on changes of in-field management practices such as tillage (Poffenbarger et al., 2015) or cover crop species adoption (Sievers and Cook, 2018). Recently, an Illinois study discovered that the decomposition rates of cover crops varied depending on the initial carbon (C): N ratio. Specifically, hairy vetch decomposed much faster than cereal rye after spring cover crop termination (Sievers and Cook, 2018). This illustrates that the soil C:N ratio is a critical variable in determining the rate of mineralization or conversion of residue N and C to plant available forms. The more wide or narrow the soil C:N ratio, the more rapid or slow the decomposition process, respectively, as illustrated with field scale plots by Sievers and Cook (2018). However, there is not a comprehensive understanding of how different cover crop species, with different C:N ratios, impact the soil microbial community functionality and diversity during the cover crop decomposition period, and how this influences the depolymerization of C and the bioavailability of N to subsequent cash crops. Thus, there is a critical need to focus on the response of the soil microbial community to cover crop residues, and how microbes facilitate C and N release from the cover crop residue.

## 1.2 Rationale for Cover Crops

### 1.2.1 Haber-Bosch Process

Nitrogen is essential for the synthesis of proteins and nucleic acids (Canfield et al., 2010), and therefore is essential in agriculture production systems. Prior to the 20$^{th}$ century, the marriage

of crop and animal production systems to form agroecosystems dominated the agriculture realm worldwide (Smil, 2001). Plants grown in cropping systems sustained livestock, while livestock returned nutrient rich manure to the soil, which supported the crops. Over time, the global N and phosphorus cycles have been altered to better accommodate for the advancement of societal practices such as the fertilization of agriculture fields, the generation of wastewater, and nutrient atmospheric deposition from fossil fuel combustion (Peierls et al. 1991, Howarth et al. 1996). An additional alteration was the introduction of the Haber-Bosch process, patented in 1908 by Fritz Haber. Fritz Haber and Carl Bosch discovered that ammonia could be synthesized from its elements N and hydrogen (H) by reacting atmospheric $N_2$ with H. This process was termed the Haber-Bosch process. (Erisman et al., 2008). The agriculture industry has since utilized the Haber-Bosch process to create ammonia for N fertilization, and subsequently the agriculture industry has relied less on nutrients from animal production.

### 1.2.2 Tile drainage

Approximately 25% of the cropland in the United States uses tile drainage (Pavelis, 1987; Skaggs et al., 2012). Tile draining farmland leads to increases in crop productivity and yield, but with the potential consequence of increased N fertilizer leaching through the drainage systems (Smiciklas et al., 2008). The tile drains act as a sink, removing the excess N not utilized by the crop. Tile drainage in the Midwest United States Corn Belt Region delivers the N rich water to coastal areas in the Gulf of Mexico, leading to the creation of hypoxic zones and eutrophication. Linear relationships have been found between tile drained, row crop agriculture and N concentrations in local surface water (Schilling and Libra, 2000), and it has been concluded that tile drainage in agriculture fields enhances nitrate flows to surface waters (Dinnes et al., 2002).

### 1.2.3 Eutrophication

Eutrophication of the Gulf of Mexico has created an oxygen deficient 'dead zone' spreading over a 20,000 square mile radius, which is of global environmental concern. The vast drainage basin of the Mississippi river, covering nearly 3,028,700 $km^2$ of the United States, is the source of the N loading into the Gulf of Mexico (David et al., 2010). The Mississippi river basin, the third largest river basin in the world, drains approximately 41% of the continental United States stretching from New York to the Rocky Mountains and from southern Canada to the Gulf of Mexico. Approximately 58% of the basin is cropland (Goolsby et al., 2001). On average, this land area contributes 1.6 million tons of N annually to the Mississippi river, and subsequently, into the Gulf of Mexico (Rabalais et al., 2002b).

## 1.3 Cover Crop Benefits and Barriers to Adoption

Scientists, policy makers, and community groups have advanced the idea of multifunctional agroecosystems (MFA). MFAs are the combination of production agriculture commodities and ecological services. Examples of services that are included in MFAs vary from beneficial effects on pest and nutrient management, water quality, and ecosystem biodiversity (Wilson, 2007). Despite the benefits of these systems, MFAs have not been widely adopted in the United States due to interrelated sociopolitical, economical, and ecological factors, but exceptions to this adoption are agriculture systems that include cover crops and reduced tillage residue management (Jordan and Warner, 2010). Cover crops are an example of a tool that can be used to create MFAs through the ability to increase water quality. Since 2010, farmland in the United States with cover crops has increased from 48,000 hectares to over 150,000 hectares, as recorded in 2015 (Cover Crop Survey, 2015).

### 1.3.1 Benefits of adoption

One way to reduce the amount of N that leaches through tile-drained fields is incorporating cover crops into a nutrient management plan (Dean and Weil, 2009). Beyond N scavenging, cover crops positively impact hydrology and soil ecosystems through several mechanisms. Cover crops increase water infiltration rates and the hydrological resistance of the soil surface, leading to decreased surface runoff. (Dabney, 1998). Additionally, soil erodibility decreases with cover crop adoption as the cover crop roots and the associated fungal hyphae aid with the binding of soil particles. Aboveground, the cover crops act as a barrier between raindrops and bare soil, lessening the impact of splash erosion (Hartwig et al., 2002). Overall, the ability of cover crops to reduce soil erosion prevents the removal of fertile topsoil from an agroecosystem. In addition to decreased soil erosion, cover crops are being adopted by farmers because of their potential to reduce the pressure of weeds, increase water holding capacity, improve soil structure, increase soil organic carbon (Danso et al., 1991; Hartwig et al., 2002), enhance crop productivity (Hobbs et al., 2008), and improve soil heath and enhance ecosystem services (Banclo-Canqui, 2015; Bender, 2016).

### 1.3.2 Barriers to adoption

There are potential risks and barriers with cover crop adoption. Two primary barriers include expenses associated with purchasing the cover crop seed and time required for soils to dry in the spring following winter cover crops prior to planting the cash crop (Teasdale, 1993; Roth et al., 2018). Additional costs such as cover crop seed, as well as the potential for decreased grain yield (Ismail et al., 1994), have led to increased interest in cost-benefit analyses to examine the economic impacts of cover cropping (Roth et al., 2018). Another barrier to adoption is the lack of confidence among growers when incorporating the nutrient credit from cover crops into their nutrient management plan (Roth, 2017). Currently, it is unknown when the N that the crop has

scavenged is converted from its organic to inorganic form and made available to the cash crop. Because of this, it has been suggested that different timings of applications and rates of N and different combinations of cover crop species may be best depending on the individual nutrient management plan and the long and short-term goals of that plan (Snapp et al., 2005). However, each of these additional management practices add expenses to the nutrient management planning.

Another potential barrier is the difficulty encountered when incorporating cover crops into the soil after termination. The cover crop biomass buildup after termination can lead to decreased N mineralization early in the decomposition period if cover crops are not incorporated with tillage, such as the case with no-tillage farming systems. To overcome this barrier, it has been hypothesized that combining a legume cover crop, which biologically fixes atmospheric N and has a narrow C:N ratio, with a N scavenging grass cover crop with a wide C:N ratio could increase the N mineralization rates. From another perspective, studies have examined the feasibility of applying N in the fall into a living cover crop stand instead of in the spring, and the potential of this practice to decrease N losses while sustaining cash crop yield (Lacey and Armstrong, 2015). Results indicate that applying N in the fall into a living cover crop stand leads to the stabilization of a minimum of 60 and 80% of that N in cereal rye and tillage radish plots, respectively (Lacey and Armstrong, 2015). With that being said, Randall and Vetsch (2005) found that fall applied N is more susceptible to leaching than spring applied N, but this study lacked organic matter amendments.

## 1.4 Carbon Cycling Transformations

Carbon cycling transformations in the soil drives plant material decomposition. Microbes in nature, including soil ecosystems, are limited by energy sources such as glucose derived from cellulose (Hobbie and Hobbie, 2013), which is a member of a group of compounds (cellulose,

hemicellulose, and pectins) that are the most abundant biosynthesized polymers on Earth (Horwath, 2015). The cellulose content in leaf tissues of agriculture crops range from 13-42% of the plant biomass (Whalen and Sampedro, 2010). Cellulose is insoluble and therefore too large to enter a microbial cell wall; it must be broken down for microorganisms to obtain energy from cellulose material. Several enzymes are responsible for the breakdown of cellulose to glucose, and these enzymes are termed cellulases. First, endoglucanase enzymes act on soluble and insoluble glucose chains by cleaving the β-1, 4 linkages. This results in glucose and cellooligosaccharides. These products are then further degraded by exoglucanases, including the enzyme glucanhydrolase (Horwath, 2015). Exoglucanases act on the non-reducing ends of cellulose chains, and this creates glucose, cellobiose and cellotriose, which are cellulose chain fragments. The cellobiose and cellotriose molecules liberated by these enzymes from cellulose are small enough to enter the outer cell membrane of the microorganism (Whalen and Sampedro, 2010). Once inside the cell, these molecules are further hydrolyzed to glucose by β-1, 4 glucosidase. This resulting glucose is a vital energy source in microbial metabolism (Eivazi and Tabatabai. 1988). Virtually all soil microorganisms produce β-1, 4 glucosidase and can obtain glucose from cellobiose and cellotriose, but not all organisms produce sufficient amounts of all cellulase enzymes (Horwath, 2015). Soil organisms that are known to depolymerize cellulose include the common aerobic bacteria *Cellulomonas* (Actinobacteria), *Pseudomonas* (Proteobacteria), and *Bacillus* (Firmicutes) and common anaerobic bacteria *Acetobacter* (Proteobacteria), *Bacteroides* (Bacteroidetes), *Clostridium* (Firmicutes), *Fibrobacter* (Fibrobacteres), and *Ruminococcus* (Firmicutes) (Horwath, 2007; Coughlan and Mayer, 1992; Iakiviak et al., 2016). As high-throughput DNA sequencing techniques such as DNA-stable isotope probing rapidly become cost and time efficient, our

understanding of the global C cycle, including the microbes that drive it, will continue to increase (Pepe-Ranney et al., 2016).

## 1.5 Nitrogen Cycle Transformations

The N cycle is complex, and becoming more so with discoveries occurring regularly. The core of the N cycle is the transformations that occur between organic and inorganic N (Schimel and Bennett, 2004). The conversion of N from the organic to the inorganic forms of either ammonium ($NH_4^+$), nitrite ($NO_2^-$) or nitrate ($NO_3^-$) is broadly known as mineralization. Ammonification is the conversion of organic N to ammonium, and nitrification is the oxidation of reduced N in the form of either ammonium or organic N to nitrite or nitrate. Immobilization is the assimilation of inorganic N to organic N. Microbial communities mediate these N cycling transformations (Robertson and Groffman, 2007).

When converting organic N to inorganic N, the large polymers that contain the bulk of the N in the organic substances must be broken down through depolymerization. This is carried out through enzymes that originate primarily from microorganisms. These N cycling extracellular enzymes include proteinases, chitinases, kinases, amidases, and amidohydrolases (Ladd and Jackson, 1982). The substrates that undo depolymerization range from proteins, microbial cell wall constituents such as amino sugars and their polymers, chitin, and peptidoglycan, and nucleic acids. These extracellular enzymes break down complex organic N molecules, and facilitate the further decomposition of these substances (Kandeler and Gerber, 1988). This step of decomposition is known to be the rate limiting step in N mineralization (Wallenstein and Weintraub, 2008; Jones et al., 2009).

## 1.6 Enzyme Activity

The soil microbial community plays an important role in plant productivity and ecosystem functioning. Claire Horner-Devine et al. (2003) estimates that a gram of soil can contain up to $10^{10}$-$10^{11}$ bacteria, and this gram of soil can contain up to 6,000-50,000 bacteria species (Curtis et al. 2002). Upwards of 80–90% of the processes in soil are reactions mediated by microbes (Coleman and Crossley, 1996; Nannipieri and Badalucco, 2003), and therefore, the microbial community plays a major role in nutrient cycling in agroecosystems, specifically, the decomposition of cover crops. Groups and successions of enzymes drive the transformation of cellulose in leaf tissues to glucose, an energy source for microorganisms, and the conversion of N from the organic to the plant-available inorganic forms. Soil enzymology research examining the short and long-term dynamics of C and N cycling enzyme activity in agroecosystems has shown that different field management practices, including cover cropping and reduced tillage residue management, impact enzyme activity (Bandick and Dick, 1999; Dilly et al, 2007).

### 1.6.1 Soil and litter enzyme activity

Recent research has aimed to better understand the relationships between nutrient availability and soil enzyme activity in soil ecosystems. Dilly et al. (2007) examined the dynamics of β-glucosidase and urease enzyme activity on litter and soil in a cover crop litter bag decomposition experiment. They discovered that certain enzyme responses, such as the stimulation of N cycling enzymes, were correlated with the rapid decomposition of litter. Likewise, studies have discovered that N cycling enzyme activity increased with C availability in organic agriculture systems with manure and legume cover crops, and C cycling enzyme activities increased with inorganic N availability (Bowles et al., 2015). Kotroczó et al. (2014) measured litter decomposition in a forest ecosystem simultaneously with soil enzyme activity. In this study, aboveground litter

was not a significant source of labile C to microorganisms as the C did not stimulate soil enzyme activity, unlike other studies (Bowles et al., 2015). However, it was discovered that removing the detritus from the soil ecosystem significantly reduced soil enzyme activities. Chavarria et al. (2016) discovered that soil enzyme activities in cover cropped fields were related to bacterial PLFA analyses results, especially gram-positive bacteria, as well as the availability of N, phosphorus, and potassium. Enzyme activity has also been measured on decomposing plant residue or litter. Litter decomposition is crucial in agriculture systems because this residue contains valuable nutrients for ecosystem functions. Dilly et al. (2007) measured litter enzyme activity on decomposing rye grass and wheat straw litter at five sampling dates from February to August in a single year in North, Central, and Southern Germany. The litter β-glucosidase activity increased until day 58 of the experiment, and then declined at one of the research sites. At another site, the β-glucosidase activity continuously decreased. The litter urease activity increased until day 118 and then decreased in all litter types. Overall, research as shown that enzyme activities in soil and litter ecosystems are dependent on the biochemical characteristics of the amendment being incorporated and the environmental factors such as temperature and precipitation. With that being said, there is a lack of knowledge surrounding how cover crops impact these activities during the decomposition period of these organic amendments in the Midwest Corn Belt.

## 1.7 Understanding the Microbiome

Recently, the breakthrough of high-throughput sequencing technologies has allowed scientists to gain a deeper understanding of microbial communities in an array of ecosystems, ranging from the human gut (Yatsunenko et al., 2012), the insect gut (Wang et al., 2011), air in hospitals (Kembel et al., 2012), a United States Space Station (Lang et al., 2017) and the soil of the Earth (Gilbert et al., 2010). This research has created a massive advancement of knowledge

regarding the microbiome of our Earth. However, soil is one of the most microbial dynamic and diverse biospheres on Earth and using this technology to generate and accurately comprehend gigabytes of sequencing data proves to be a complex but intellectually rewarding task.

### 1.7.1 Microbial diversity and ecosystem functioning

It is currently unclear whether soil microbial diversity is correlated with plant diversity and/or ecosystem functioning (Van der Heijden et al., 2007), however, it is known that ecosystem productivity commonly increases with plant species diversity (Schnitzer et al., 2011; Luo et al., 2017). Now, researchers seek to understand what drives this phenomenon. Understanding the mechanisms behind the link between ecosystem productivity and species diversity could aid with the prediction of the future productivity of many of Earth's ecosystems as they continue to undergo species loss, with some losses caused by an increase in perturbations associated with climate change (Schnitzer et al., 2011). Now, researchers predict that the soil microbiome could play a role in the link between ecosystem productivity and species diversity (Schnitzer et al., 2011; Schnitzer and Klironomos, 2011; van der Heijden et al., 2008; Wagg et al., 2011). Overall, these connections between soil microbial diversity and ecosystem productivity provide incentive to further investigate these relationships, specifically, in intensively managed agroecosystems that are expected to have high levels of productivity, despite consistent perturbations.

### 1.7.2 The temporal dynamics of the litter and soil microbiome

Studies have focused on understanding soil microbial diversity during organic matter amendment decomposition and on the relationship between soil microbial diversity and ecosystem function and productivity. Van der Heijden et al. (1988) found that increased fungal diversity led to increased nutrient acquisition and plant productivity, but another study found no effects when fungal diversity was increased (van der Heijden et al., 2006). Similarly, Finney and Kaye (2017)

found little impact when increasing the species richness of over wintering cover crops in agriculture landscapes, but did determine that increasing cover crop species functional diversity would lead to stronger multifunctionality of the agroecosystem.

Dilly et al. (2004) completed a decomposition experiment, and bacterial diversity in soil and litter was determined via denaturing gel electrophoresis (DGGE) of amplified fragments of genes coding for the 16S rRNA gene region. It was discovered that the bacteria community structure and diversity changed significantly over the decomposition period of 180 days in both rye and wheat litter. Once the litter bags were added to the soil the diversity increased, especially in the rye litter. The rye litter had a more narrow C:N ratio than the wheat material, leading to more rapid decomposition. Additionally, the wheat material had low nutritional quality. This was evident by the low count of bacterial DNA discovered during denaturing gradient gel electrophoresis (DGGE). The rapidly decomposing rye grass with higher nutritional quality had a spike in bacterial DNA. With that being said, the low number of DNA bands and lower diversity indicated that the rye was dominated by few organisms. The wheat promoted the development of microbial communities with greater diversity compared to the rye. In conclusion, the number of DNA bands increased as the decomposition proceeded over the 180 days, but the microbial biomass and activity decreased. Purahong et al. (2016) took a similar approach to analyze the microbial community succession in leaf litter but utilized pyrotag sequencing of the bacterial 16S rRNA genes. When analyzing decomposing leaf litter of temperate beach forest through a litter bag method, they determined that the bacteria community underwent rapid changes. Additionally, they found that Bacteria richness was positively correlated with three hydrolytic enzymes representing the N, phosphorus, and C macronutrients. Tláskal et al. (2016) found similar results with bacteria succession in leaf litter decomposition. There were distinct successional changes

over the 24-month decomposition period. Interestingly, they determined that cellulose-degrading bacteria were less frequent and their abundance ranged from 4-15% during the initial two months of decomposition.

A recent study by Štursová et al. (2016) examined the enzyme activity, microbial abundance and composition, among other biological characteristics, in the bulk topsoil and the fallen leaf litter of spatially heterogeneous temperate natural forest ecosystems to determine the relationships between the biological variables. They found that the effect of the litter vegetation type was not as robust as the chemical composition. The chemical composition of the soil effected the microbial abundance and the rate of decomposition of the litter. Additionally, Burns et al. (2015) examined the Bacteria and Archaea diversity of different management practices including cover cropping, compost additions, tillage, and system design (conventional, organic, or bioproduction) in vineyard soils in Napa Valley California using the Illumina MiSeq system. They found that management practices in vineyards directly affected the structure of the soil microbial community. Using similar approaches, the use of synthetic fertilizers and cover crops in agriculture systems was studied to determine how these two management practices influence soil bacterial diversity, as well as a number of other soil properties (Verzeaux et al., 2016). The results showed that Bacteria communities are modified when a synthetic N fertilizer application is applied to the soil, but the effects are dampened and bacteria community composition and diversity remains similar under legume and non-legume cover crop treatments. Possibly most related to the proposed research in this thesis, was a study completed by Fernandez et al. (2016) that examined the structure of Bacteria communities under different cover crop and fertilizer treatments in bulk and rhizosphere soil in three organic agriculture systems. There were four cover crop treatments in the experimental design: hairy vetch (*V. villosa*), winter rye (*S. cereale*), oilseed radish (*R. sativus*),

and buckwheat (*F. esculentum*). Soil samples were collected at three sampling times: once in August following cover crop planting, again in May before corn planting, and lastly in July when the corn plants were at the V13 vegetative stage. After Illumina sequencing of the 16S rRNA gene, they determined that community structure was most effected by sampling site. Additionally there was a trend that some cover crop treatments decreased and increase α-diversity in the earlier and later season soil samples compared to the no amendment control, respectively.

Proposed linkages between soil microbiome diversity and ecosystem function and productivity have led to an increase in research investigating the microbiomes of soil ecosystems. The aforementioned studies recognized the temporal dynamics of soil microbiomes, some during organic amendment decomposition, and these findings indicate that cover crops may influence the soil microbial community function, diversity and composition. However, there is not a comprehensive understanding of the impact of cover crops and reduced tillage systems on the soil microbial community, and subsequently, on soil nutrient cycling and inorganic N availability. The research presented in this thesis aimed to increase our understanding of cover crop decomposition period soil microbial community dynamics by: i) determining the impact of cover crop species on soil and litter enzyme activity and soil nitrogen supply during the cover crop decomposition period, and ii) by evaluating the impact of cover crops and reduced tillage on the soil microbial community diversity and composition during the cover crop decomposition period. As cover crop use as organic amendments in agroecosystems increases in the Midwest United States, investigating the temporal dynamics of the soil microbiome will yield valuable information about the connection between soil microbiome diversity, organic amendment decomposition, and nutrient bioavailability.

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# CHAPTER 2. THE IMPACT OF COVER CROPS AND REDUCED TILLAGE SYSTEMS ON ENZYME ACTIVITY AND NITROGEN SUPPLY DURING THE COVER CROP DECOMPOSITION PERIOD

## 2.1 Abstract

Cover crop adoption in the Midwest Corn Belt region is a rapidly emerging adaptive management practice being used as a tool to reduce nitrate loading to tile-drainage systems. However, little is known about the inclusion of cover crops on the soil microbial community and their efficiency in facilitating nitrogen (N) release from cover crop litter. Therefore, the goal of this study was to determine if N release from cover crop litter in no-tillage or spring tillage corn (*Zea mays L.*) production systems is related to soil and litter enzyme activity (EA), and to understand the timing of cover crop N release as it relates to the growth of the corn cash crop. Specifically, the objectives were: i) to determine how soil and cover crop litter EA (β-glucosidase and urease) is influenced by different cover crops and residue management during the cover crop decomposition period in the first-year transition to a cover cropped and reduced tillage agroecosystem and; ii) to determine if there is a relationship between EA, soil inorganic N availability, and cover crop C decomposition during the cover crop decomposition period. Specifically, cover crop litter β-glucosidase and urease activity was quantified on decomposing cover crop litter at five sampling dates from May-October during the 2016 and 2017 cover crop decomposition periods. Soil β-glucosidase and urease activity was quantified at seven sampling dates from April-October in the same seasons. Sampling dates corresponded to a decomposition degree day (DCD), which was calculated with daily precipitation and temperatures values. Cover crops used in this study included cereal rye (*Secale cereal* L.), hairy vetch (*Vicia villosa Roth*), a cereal rye/hairy vetch mixture, and a no cover crop control. Residue management treatments

included 15 cm spring tillage and no-tillage. Results indicate that cover crop litter β-glucosidase activity decreased in all treatments during each of the decomposition periods. Similarly, cover crop litter urease activity decreased in both residue management systems across the 2017 decomposition period. Litter urease activity in 2016 increased in cereal rye treatments on August 1 (DCD 27.9), while the activity in hairy vetch treatments remained more constant over the decomposition period. Soil β-glucosidase activity was related to increasing soil temperatures leading up to June 6 (DCD 10.5) and July 31 (DCD 29.8) in 2016 and 2017, respectively. These elevated soil β-glucosidase activities were related to an increased in soil inorganic N in both decomposition periods. The peak inorganic N availability for all treatments occurred one sampling date after the peak soil β-glucosidase activity. Soil β-glucosidase activity was highest between 4.5 and 9.9 DCDs after cover crop termination, and the most soil inorganic N was available between 9.9 and 19.8 DCDs following cover crop termination. In 2016, the most soil inorganic N was available when the corn was at the VT growth stage and in 2017, the most soil inorganic N was available when the corn was at VE and V6. Lastly, soil urease activity in all treatments in 2016 steadily increased from cover crop termination in April (DCD 0.0) until corn physiological maturity in September (DCD 40.8). In 2017, soil urease activity was more constant during the decomposition period in the control and hairy vetch treatments, but gradually decreased in the cereal rye and mixture plots leading up to corn physiological maturity (DCD 42.5). Data generated from this study increases our understanding of how cover crops and reduced tillage management impacts EA, and the timing of N release from cover crop residue. Understanding the timing of N release from cover crop residue could lead to the optimization of agriculture management practices to promote inorganic N availability at corn critical growth stages. These results indicate that the most soil inorganic N is available within seven DCDs following peak soil β-glucosidase activity.

**Abbreviations:** N, nitrogen; C, carbon; CR, cereal rye; HV/CR, hairy vetch/cereal rye; HV, hairy vetch; EA, enzyme activity

## 2.2 Introduction

The use of cover crops and reduced tillage residue management systems in sustainable agriculture management plans of row crop farmers is a growing trend in the U.S. Corn Belt region (Cover Crop Survey, 2017). Cover crops are being adopted to absorb N from the soil profile and improve water quality (Ditsch et al., 1993; Hartwig and Ammon, 2002; Kaspar and Singer, 2011). Additionally, cover crops and reduced tillage systems are being incorporated in sustainable nutrient management plans by growers to increase soil organic C (Liu et al., 2005), reduce soil and wind erosion (Langdale et al., 1991; Decker et al., 1994; Dabney, 1998; Delgado et al., 1998), and enhance crop productivity (Hobbs et al., 2008). One important aspect of soil quality and health is the status of the soil biological properties, such as enzyme activity (EA), which drive biogeochemical cycling, and therefore influence the ability of the soil to function and support cash crop production. Developing a better understanding of the availability of N from cover crop residue during the cash crop growing season could lead to increased adoption of these practices among farmers who are currently hesitant to incorporate these practices in agroecosystems because of the potential for N tie-up at cash crop critical growth stages.

While studies have investigated the impact of reduced tillage treatments and cover cropping on changes in soil microbial community structure (Fernandez et al., 2016; Degrune et al., 2017), phospholipid fatty acid profiles (Finney et al., 2017), and biochemical properties (Linn and Doran, 1984; Beare et al., 1992; Spedding et al., 2004; Simmons and Coleman, 2008; Acosta-Martinez et al., 2011), there is still a lack of consensus surrounding the effects of these

management practices on EA, soil N dynamics, and the relationship between the two during cover crop decomposition and corn growth. Only recently has a link between C and N cycling enzyme activity and inorganic N been confirmed in the context of agroecosystem management practices (Bowles et al., 2014), but this link was only investigated with relation to a single sampling point in time in June 2011. It is common for cover crop studies to examine the impacts of organic amendments by soil sampling prior to cover crop termination or planting (Bandick and Dick, 1999; Fernandez et al., 2016). However, there is growing evidence that EA has in-season variability (Debosz et al., 1999; Bell et al., 2009), and this in-season variability could be impacted by the addition of organic amendments, which are an energy source for the microbial community as C and N are released from the amendment.

Enzyme activity assessments have been used as compliments to other soil nutrient analyses because EA is the driver of organic matter decomposition through chemical reactions (Allison et al., 2007). Specifically, the enzyme β-glucosidase depolymerizes cellulose, yielding glucose, and the enzyme urease hydrolyzes pools of organic N such as proteins (Sinsabaugh et al., 2008). The EA of β-glucosidase and urease are indicators for the potential degradation of C polymers and the release of ammonia, respectively (Dilly et al., 2003). Both β-glucosidase and urease activity has been found to be highly responsive to nutritional conditions (Dilly and Nannipieri, 2001; Dilly et al., 2007), but this response has not been observed during the decomposition period of winter cover crops at corn critical growth stages. To fully understand the in-season dynamics of N availability in cover cropped agroecosystems, research should focus on the in-season dynamics of EA following cover crop termination. Therefore, the primary goal of this research is to explore the dynamics of soil and litter EA during the cover crop decomposition period. Specifically, there are two objectives: i) to determine how soil and litter EA (β-glucosidase and urease) is influenced by

different cover crops and residue management during the cover crop decomposition period in the first-year transition to a cover cropped and reduced tillage agroecosystem and; ii) to determine if there is a relationship between EA, soil inorganic N availability, and cover crop C decomposition during the cover crop decomposition period. Understanding the dynamics of EA and inorganic N availability during the cover crop decomposition period will aid with the production of tools capable of detecting short-term improvements and dynamics in soil health to help growers with their nutrient management strategies.

## 2.3 Materials and Methods

### 2.3.1 Research site and field activities

The research site was located at the Purdue University Agriculture Center for Research and Education (ACRE) in Tippecanoe County Indiana. Precipitation and bare soil temperature measurements were collected from the Indiana State Climate Office (https://iclimate.org). The winter of 2015/2016 was the first year the cover crop and tillage residue management treatments were implemented. Prior to these cover crop and tillage regimes, the site was a corn-soybean annual rotation with spring tillage prior to corn planting. The experiment had a split-plot design with three field replications. There were six treatments used: three cover crops, no cover crop controls, and two tillage practices. Each plot was 12 rows wide and approximately 80 m in length. Cover crop treatments included hairy vetch (HV) (*Vicia villosa*), cereal rye (CR) (*Secale cereale*), and a HV/CR mixture (mixture). Residue management treatments included a 15 cm spring disk tillage and a no-tillage. Cover crops were planted on September 28-30, 2015 and September 19, 2016 during the first and second years of the study, respectively. In September 2015, the HV and CR seeding rates were 34 and 56 kg $ha^{-1}$ drilled, respectively. The HV/CR mixture seeding rate was 17/45 kg $ha^{-1}$ drilled. In October 2016, the HV and CR seeding rates were 67 and 112 kg $ha^{-1}$

drilled, respectively. The HV/CR mixture seeding rate was 28/84 kg $ha^{-1}$ aerial seeded. Cover crops matured over the winter and were chemically terminated on April 14 for each sampling season. Following termination each year, anhydrous ammonia was applied at a rate of 78 kg N $ha^{-1}$. On April 25, 2016, the spring tillage plots were disk tilled twice to a depth of approximately 15 cm. On April 26, 2016, the plots were cultivated to prepare the seedbed and maize (*Zea mays*) was planted. On May 18, 2017, the spring tillage plots were disk tilled twice to a depth of approximately 15 cm. On May 30, 2017, the plots were cultivated to prepare the seedbed and maize was planted.

### 2.3.2 Soil sampling

Soil samples were collected at seven dates over the 2016 and 2017 corn-growing season, with each soil sampling date corresponding with a decomposition degree day (DCD) and corn growth stage. Decomposition degree days (DCD) were calculated according to Ruffo and Bollero (2003). During each sampling, 15 randomly distributed soil cores were removed per plot at a depth of 4 cm using a 4 cm diameter soil probe. The cores were homogenized during sampling to create a composite bulk sample for each plot. The bulk samples were transported from the field to lab on ice in a cooler. Approximately 100 g of each bulk sample was stored at 4°C for enzyme assays, and the assays were completed within 48 hours of sampling. An additional subsample of the bulk soil was air dried and used for physiochemical analyses.

### 2.3.3 Soil physiochemical analysis

Soil moisture was determined by oven drying a subsample of the fresh, 2 mm sieved soil at 105°C for 48 hours. Nitrate ($NO_3^-$-N) and ammonium ($NH_4^+$-N) were extracted from soil by shaking 2 g of each soil sample with 20 mL of 1 M KCl for 1 hr on a rotary shaker. The soil extracts were centrifuged for 2 min and filtered. The supernatants were analyzed on a SEAL AQ2 analyzer using USEPA methods 132-A Rev.1 and 103-A Rev. 4 (USEPA, 1993).

### 2.3.4 Litter bags for litter enzyme assessment

Cover crop biomass was collected prior to termination in the spring of 2016 and 2017. Cover crop residue was collected from 1 $m^2$ quadrants placed randomly in each plot. Biomass was oven dried at 105°C to remove water content and determine winter cover crop biomass accumulation.

Additional cover crop biomass samples were collected the day after termination and air-dried to be used in the litter bag study. Nylon mesh (1 mm) litter bags were created to house cover crop residue, as done previously (Organization for Economic Cooperation and Development; Melkonian et al., 2017; Jahanzad et al., 2016; Varela et al., 2017). The HV, CR, and mixture litter bags were filled with 10 g, 20 g, and 22 g of biomass, respectively. The 22 g of biomass in the mixture bag contained 20 g of CR and 2 g of HV, therefore it was predominately a cereal rye-based treatment. To simulate the 15 cm tillage treatment, litter bags were buried approximately 15 cm deep in plots that underwent spring tillage. Litter bags were applied to the soil surface between corn rows in the no-tillage field treatments and were held in place with lawn staples. Bags were retrieved at five sampling dates during the decomposition period. A subsample of the fresh litter was air-dried to determine moisture content. Another fresh subsample of the litter was stored at 4°C for enzyme assays, and the assays were completed within 72 hours of sampling.

### 2.3.5 β-glucosidase enzyme activity

β-glucosidase activity (μmoles *para*-nitrophenol released g dry $soil^{-1}$ $hr^{-1}$) was quantified according to Eivza and Tabatabai (1988) on soil and cover crop residue from collected litter bags. After transporting from the field to the lab on ice in a cooler, soil was sieved (< 2 mm) and 1 g was weighed into Erlenmeyer flasks. Triplicates were performed for each sample as well as a negative control and substrate control. Each of the Erlenmeyer flasks were treated with 0.2 mL of

toluene, the solution was mixed, and remained in the fume hood for 15 min. Flasks were then treated with 4 mL of a modified universal buffer (MUB) (pH 6.0) and 1 mL of *para*-nitrophenyl-β -D-glucoside (PNG) (50 mM). Substrate control flasks were not treated with PNG. The flasks were stoppered, mixed thoroughly, and incubated at 37°C for 1 hr. Due to generally higher enzyme activity in litter than in bulk soil (Kandeler et al, 1999b; Dilly et al., 2007), a smaller sample weight (e.g., 0.1 g instead of 1 g) and increased concentration of substrate solution was used in each litter assay to ensure that the enzyme substrate concentration was not a limiting factor in the assay (Dilly et al., 2007). After incubation, 1 mL of $CaCl_2$ (0.5 M) and 4 mL of tris(hydroxymethyl)aminomethane (THAM) buffer (100 mM, pH 12) was added, and then the product was filtered. The yellow intensity of the color reaction was measured on a spectrophotometer at 415 nm. Standards were created which contained 0, 100, 200, 300, 400, and 500 nanomoles of *para*-nitrophenol released in each flask. Standards were measured on the spectrophotometer to create a calibration curve. The curve was used to calculate the amount of *para*-nitrophenol released from each sample. If the filtrates had a color intensity exceeding the highest standard the filtrate was diluted with a 1:1 mixture of MUB (pH 6.0) and THAM (0.1 M, pH 12.0).

### 2.3.6 Urease enzyme activity

Urease activity (μg $NH_4$-N g dry $soil^{-1}$ $hr^{-1}$) in the soil and fresh litter subsampled from litter bags was quantified according to Kandeler and Gerber (1988). The same soil and litter sources were used for the urease assays as the β-glucosidase assays. Triplicates were performed for each sample as well as a negative control and substrate control. The flasks were treated with 2.5 mL of urea (720 mM) substrate solution and 20 mL of borate buffer (0.1 M, pH 10). The flasks were sealed and incubated at 37°C for 4 hours. After incubation, flasks were treated with 30 mL

of potassium chloride (2 M)-hydrochloric acid (0.01 M) solution. Due to generally higher enzyme activity in litter than in bulk soil (Kandeler et al, 1999b; Dilly et al., 2007), a smaller sample weight (e.g., 0.1 g instead of 1 g) and increased concentration of substrate solution was used in each litter assay to ensure that the enzyme substrate concentration was not a limiting factor (Dilly et al., 2007). Samples were placed on a rotary shaker for 30 min at 100 rpm, and following shaking the soil suspensions were filtered. The ammonium released during incubation was determined by combining a 1 mL subsample of the filtrate and 9 mL of Nanopure (Thermo Scientific, Barnstead Nanopure) water. A standard curve was prepared by mixing 1 mL of ammonium chloride standards containing 0, 2.5, 5, 10, 15, 20, 25, and 30 µg $NH_4$-N $mL^{-1}$ with 9 mL of Nanopure (Thermo Scientific, Barnstead Nanopure) water. For the color reaction to take place, 5 mL of sodium salicylate (1.06 M)-sodium hydroxide (0.3 M) solution and 2 mL of sodium dichloroisocyanurate (3.91 mM) solution were added to the extracts and the standards. The tubes were swirled for a few seconds for adequate mixing and the color developed for 30 min at room temperature. The absorbance of the extracts and standards were measured at 660 nm on a spectrophotometer.

### 2.3.7 Data analysis

Statistical analyses were performed using PROC MIXED (SAS Institute Inc., Cary, NC). Enzyme activity was completed in laboratory triplicates, and the mean of the triplicates was used for statistical analysis. A transformation was applied if there was a range of means divided by the grand mean that was greater than 0.5. If a transformation was necessary, a Box-Cox regression was used to determine which transformation was most appropriate. Log transformations were needed for the litter urease enzyme activity to restore linearity, and the Tukey-Kramer adjustment was used to consider all pairwise comparisons. All reported values were back-transformed if a transformation was applied. The error terms and the years were not pooled. Sampling date, cover

crop (main plot) and tillage (sub-plot) were each considered fixed effects and field replication (block) was treated as a random effect. Sampling date was modeled as a repeated measure using the ARH (heterogeneous first-order autoregressive structure) variance-covariance matrix. The effects of cover crop and tillage on EA within sampling date were determined with the SLICE statement. Figures for EA were created using the R project (http://www.R-project.org/) and figures for precipitation and temperature dynamics were created using Microsoft Excel 2010.

## 2.4 Results and Discussion

### 2.4.1 Weather conditions

Overall, the spring (March-May) of 2016 (Figure 2-1) and 2017 (Figure 2-2) were warmer than the 30 year air temperature average, and spring of 2016 (average of 11.70°C) was warmer than spring of 2017 (average of 11.45°C). Specifically, the months of March and May of 2016 were an average of 2.4°C and 0.6°C warmer than March and May of 2017, respectively. April had an average temperature that was 2.5°C warmer in 2017 than 2016. Additionally, temperatures peaked in 2016 in mid-August at > 31.5°C while in 2017 temperatures peaked on July 7 and the month of August was cooler compared to August 2016.

### 2.4.2 Cover crop biomass

In 2016, average cover crop biomass after winter growth was 1967, 1858, and 418 kg biomass $ha^{-1}$ in the mixture, CR, and HV treatments, respectively. In 2017, mean cover crop biomass was 915, 888, and 512 kg biomass $ha^{-1}$ in the mixture, CR, and HV treatments, respectively. Therefore, there was more than double the amount of biomass available for decomposition in 2016 compared to 2017 in the mixture and CR treatments. Contrary to this trend, in the HV treatment there was an additional 96 kg biomass $ha^{-1}$ available for decomposition in

2017 than 2016. In 2016, the average C:N ratios of the CR, HV, and mixture treatments were 16.6, 16.0, and 12.9, respectively. In 2017, the average C:N ratios of the CR, HV, and mixture treatments were 21.1, 11.9, and 20.5, respectively.

### 2.4.3 Litter enzyme activity

Similar trends of litter EA were observed in 2016 and 2017 for both urease and β-glucosidase. There was not an interaction effect of date by cover crop or tillage by cover crop in 2016, but these interactions were present in 2017 (Table 2-1). Overall, litter β-glucosidase activity was higher in 2016 compared to 2017 in all cover crop treatments within all sampling dates, with the exception of the last sampling date of each growing season and the HV treatment on June 6, 2016 (DCD 10.5) and June 13, 2017 (11.2). Litter urease activity was highest in the spring tillage in 2017.

#### 2.4.3.1 Litter β-glucosidase activity in 2016 and 2017

In 2016, litter β-glucosidase activity decreased across the decomposition period in HV and CR treatments (Figure 2-3). The mixture treatment followed a similar decreasing trend, but there was a spike in activity on June 28 (DCD 17.5). In all treatments, the lowest activity was measured on the last sampling date of the study at corn physiological maturity on September 23 (DCD 40.8). Enzyme activity was significantly different between treatments at two sampling dates in the 2016 season ($p < 0.05$). First, on June 28 (DCD 17.5), the mixture treatment expressed significantly higher enzyme activity than the HV treatment. Then, on August 1, the HV treatment had significantly lower activity than both the CR and mixture treatments ($p < 0.05$).

Similar to 2016, litter β-glucosidase activity decreased in all treatments over the 2017 decomposition period (Figure 2-4). Due to the significant interaction effect between cover crop and tillage, the 2017 litter β-glucosidase activity is shown in a panel separated by tillage treatment

(Figure 2-4 A, B). Spring tillage residue management treatment resulted in significantly greater β-glucosidase activity on May 22 (DCD 7.1) in all treatments compared to the following four sampling dates ($p < 0.05$). There was not a significant difference between treatments within any of the sampling dates ($p < 0.05$). The no tillage treatment also decreased in all treatments over the decomposition period. The HV β-glucosidase activity in the no tillage treatments were significantly greater than the CR and mixture treatments at the first two sampling dates of the 2017 decomposition period. This trend was not observed in the spring tillage treatment.

Though the dynamics of EA have not been measured in cover cropped agroecosystems, litter decomposition and EA has been assessed in forest ecosystems. In a tropical rainforest system, it has been documented that litter β-glucosidase activity has a positive relationship with leaf decomposition in tropical rainforests (Zhang et al., 2009). The data from my study followed similar trends. In the tropical forest system, litter β-glucosidase activity increased rapidly at the beginning of the decomposition period and then changed to a constant value. In this study, the EA was elevated at the beginning of the decomposition period and then changed to a constant value. This initial increase and subsequent leveling off of β-glucosidase activity was attributed to the recalcitrant nature of the leaf materials in the forest system. The lack of a rapid increase in EA at the beginning of the decomposition period in my agroecosystem could be related to climatic conditions that vary between a tropical rainforest and the Midwest U.S. Corn Belt (Couˆteaux et al., 1995). In addition, the intensive management of agroecosystems plays a factor in the EA dynamics compared to a forested ecosystem.

In agreement with other studies (Kshattriya et al., 1992), our C cycling enzyme activity (β-glucosidase) differed between species of cover crop litter, and litter β-glucosidase activity decreased as cover crop residue C content decreased. Other studies have confirmed this trend of

the C cycling enzyme activity decreasing during a decomposition period (Kshattriva et al., 1992; Dilly and Munch, 1996; Dilly et al., 2007). Most similar to this study, litter β-glucosidase activity was found to decrease over the decomposition period of three grass species in North, Central, and Southern Germany. Additionally, the litter that decomposed more rapidly had a more narrow C:N ratio at the beginning of the decomposition period (2 months) and was associated with high β-glucosidase activity during this period (Dilly et al., 2007). Similar litter β-glucosidase activity dynamics occurred in the 2017 spring tillage treatment of our study when HV had higher activity at May 22 (DCD 7.1) and June 13 (DCD 11.2), but not at later sampling dates. A potential mechanism causing this continual decrease could be that the C utilization efficiency of the microbial community increases as the decomposition period progresses. In addition, this decline in litter β-glucosidase activity could be driven by the decline of C substrate and lack of easily decomposable C compounds (Dilly and Munch, 1996).

#### 2.4.3.2 Litter urease enzyme activity in 2016 and 2017

In 2016, litter urease activity peaked in the CR treatment on August 1 (DCD 27.9) when the corn cash crop was in late vegetative growth stages, and it was significantly greater than the HV treatment ($p < 0.05$) (Figure 2-5). The urease activity in the HV treatment remained constant across the decomposition period and did not differ significantly between any sampling dates. The mixture treatment had increased activity starting on June 28 (DCD 17.5), and the urease activity remained elevated in this treatment until corn physiological maturity on September 23 (DCD 40.8).

Increased litter urease activity was observed in the spring tillage residue management treatment compared to the no tillage treatment over the 2017 decomposition period. In the spring tillage treatment, urease activity peaked in all treatments at the first sampling date on May 22 (DCD 7.1). Following this, the activity in the mixture and HV treatments remained steady until a

decrease in activity was observed at the final sampling date on August 15 (DCD 32.6). The CR activity was at decreased levels on June 13 (DCD 11.2) and July 26 (DCD 29.8) compared to July 3 (DCD 21.8), and the lowest activity occurred on August 15 (DCD 32.6), similar to the other treatments. Similar urease activity trends were observed in the no tillage treatment in 2017 compared to urease activity in 2016. Urease activity was elevated in late July. However, in the no-tillage treatment in 2017, unlike the litter urease activity in 2016, the litter urease activity decreased on August 15.

Few studies have observed the dynamics of urease activity in decomposing litter, but litter urease activity dynamics in 2016 and in the no tillage treatments in 2017 were similar to the trends observed in a litter decomposition study in Germany that had three different grass species in an agricultural system (Dilly et al., 2007). In Northern and Southern Germany, litter urease activity peaked in a decomposing CR treatment in June, and then declined through August, similar to our CR cover crops.

It has been documented that litter chemistry plays a role in litter decomposition, and the chemistry of different litter types changes over the decomposition period based on the decomposer community structure (Wickings et al., 2012). Specifically, even the same litter type exposed to different decomposer communities resulted in differences in litter chemistry, even when the litter was 90% decomposed (Wickings et al., 2012). This highlights the importance of considering climate, litter bag mesh size and construction methods, and litter chemistry when comparing litter enzyme activity and decomposition rates between studies. Also, this signals to the importance of understanding microbial community structure, as well as function.

### 2.4.4 Effect of cover crops on soil β-glucosidase activity

Significant effects of both date and cover crop treatment on soil β-glucosidase activity were observed in 2016 (Figure 2-7) and 2017 (Figure 2-8) ($p < 0.05$) (Table 2-2). There were no significant statistical interaction effects between date, cover crop, or tillage in either 2016 or 2017.

For all treatments, the soil β-glucosidase activity was lowest in April and early May in 2016 (DCD 0.0 - 2.9). In the same year, soil β-glucosidase activity was highest when the corn was at the V6 growth stage on June 6 (DCD 10.5) for both the CR and mixture treatments. At this sampling date, the CR and mixture treatments had significantly higher activity than the HV and control treatments ($p < 0.05$). Soil β-glucosidase activity was greatest for the HV and control treatments on May 23 (DCD 6.4) and June 6 (DCD 10.5). On June 28-September 23 (DCD 17.5 - 40.8), soil β-glucosidase activity in all cover crop treatments remained at somewhat constant levels, which were significantly greater ($p < 0.05$) than that observed in April and early May (DCD 0.0 - 2.9).

In 2017 (Figure 2-8), though not as pronounced as 2016, soil β-glucosidase activity increased during the decomposition period. Similar to 2016, soil β-glucosidase activity was elevated during the early vegetative growth stages of the corn cash crop growing season in all treatments (DCD 4.5 - 29.8). Soil β-glucosidase activity was highest in the CR treatment on May 8 (DCD 4.5) prior to decreasing slightly on May 30 (DCD 9.9) and increasing again on June 28 (DCD 19.8) and July 31 (DCD 29.8). The mixture treatment had highest enzyme activity at May 8 (DCD 4.5). The HV and control treatments had elevated enzyme activity from May 8-July 31 (DCD 4.5 – 29.8), which was significantly greater from the initial (DCD 1.5) and final two (DCD 33.5 and 42.5) sampling dates ($p < 0.05$). Enzyme activity differed significantly between cover crops on three sampling dates ($p < 0.05$). On May 8, 30, and June 28 (DCD 4.5 - 19.8) the CR and mixture treatment had significantly higher enzyme activity than the HV treatment ($p < 0.05$).

In-season β-glucosidase activity dynamics have been reported in the literature with mixed results. Martens et al. (1992) discovered that soil enzyme activity increased on average 2- to 4-fold during a 31-month period when a coarse-loam soil was amended with organic residue treatments. Specifically, when the soil was amended with plant materials such as straw, the soil β-glucosidase activity increased nearly 2-fold compared the fallow control treatment, and the differences in enzyme activity was greatest from April-July during all three years of the study. This aligns with other studies that have determined that the activity of some cellulase enzymes are related to the addition of fresh organic matter (Bolton et al., 1985; Martens et al., 1992; Dick , 1994). In our study, in both 2016 and 2017, at sampling dates in May and June the CR and mixture treatments had higher enzyme activity compared to the control and HV treatments. Higher enzyme activity in the cereal rye-based treatments compared to the HV could be due to the increased rate of decomposition of HV cover crop leading to an increase in available labile C earlier in the decomposition period. In addition, this could be caused by there being more available cover crop biomass in the CR based treatments compared to the HV at termination each year.

Additional studies have observed temporal variation in soil β-glucosidase activity, but could not attribute the variation to organic matter treatments (Debosz et al., 2002). Instead, the increased soil β-glucosidase activity was likely driven by climatic factors and crop growth, which has also been shown to impact soil microbial biomass and activity (Ross, 1987). In our study, soil β-glucosidase activity increased with bare soil temperature during both years of this study leading up to the late vegetative corn growth stages. Additionally, in 2016, the peak activity in early June (DCD 10.5) was 20-30% higher than the peak activity in 2017, which occurred beginning in May (DCD 4.5). This increased activity in 2016 could have been caused by the later onset of soil warming and the colder April compared to 2017. In 2017, the month of April was warmer and

received more precipitation than the same months in 2016 (Figure 2-2). Alternatively, this more pronounced peak in soil β-glucosidase activity in 2016 could have been caused by the increased C substrate available in 2016, as CR and mixture spring biomass available for decomposition was more than double the available amount in 2017.

### 2.4.5 Dynamics of soil urease activity during the decomposition period

There were significant effects of both date and cover crop treatment on soil urease activity (Table 2-2) during the 2016 (Figure 2-9) and 2017 (Figure 2-10) cover crop decomposition periods ($p < 0.05$). Tillage was only a significant effect on urease activity during the 2017 season ($p < 0.05$). In 2016, soil urease activity increased in all treatments with soil temperature until the final sampling date at corn physiological maturity on September 23 (DCD 40.8). This was unlike the trend observed in 2017, which was characterized by higher soil urease activity from April-June (DCD 1.5–19.8) in all treatments compared to July-October (DCD 29.8 – 42.5). In both decomposition periods, there were significant differences between soil urease activities in cover crop treatments within sampling dates ($p < 0.05$). In 2016, the CR treatment had significantly higher activity than the HV and control treatments when the activity for all cover crop treatments peaked on September 23 (DCD 40.8) ($p < 0.05$). In 2017, when the soil urease enzyme activity peaked in April and May (DCD 1.5 – 9.9), the HV treatment was significantly lower than that of the CR, mixture, and control treatments ($p < 0.05$). This lower enzyme activity in the HV treatment could have been caused by the rapid, early season decomposition of the cover crop residue, which has a more narrow C:N ratio when combined with the soybean residue from the previous season compared to the soybean residue alone in the control treatments.

The in-season dynamics of soil urease activity have been documented in the literature, but not in the top 4 cm of the soil during the decomposition period of CR and HV cover crop treatments.

An Oklahoma study examined urease activity in the top 10 cm of the soil of a wheat agroecosystem, and determined that urease activity significantly increased in no tillage and reduced tillage systems when an accumulation of the residue had occurred (Berreto and Westerman, 1988). Similarly, Martens et al (1992) discovered that soil urease activity was significantly increased by the addition of organic matter in the form of straw over a 31-month period. In this 31-month period, soil urease activity peaked one month after the addition of the residue. This aligns with the gradual increase of soil urease activity in our study leading to peak activity in September 2016 (DCD 40.8). Likewise, Bowles et al. (2014) discovered that in an organic agriculture system that underwent cover cropping the activity of N cycling enzymes increased with C availability. This could explain the trends in soil urease activity in 2016 and 2017 in our system. During the 2016 decomposition period, when there was over 1800 kg biomass $ha^{-1}$ of cereal rye residue available for decomposition, soil urease activity was elevated leading up to September (DCD 40.8). In 2017, when there was half as much available litter available for decomposition, the CR soil urease activity peaked earlier in the decomposition period (DCD 1.5 - 9.9). Additionally, West Central Indiana experienced a warmer, wetter April in 2017 compared to 2016. (Figure 2-2; Figure 2-1). This warmer and wetter April could have led to increased cover crop decomposition and potentially increased soil urease activity.

### 2.4.6 Soil inorganic N availability

The interaction effect of sampling date and tillage was significant during both years of the study (Table 2-3) ($p < 0.05$). Tillage had a significant effect on soil inorganic N availability in 2016 (Table 2-3) ($p < 0.05$). The main effect of cover crop treatment did not significantly influence inorganic N availability in the top 4 cm of the soil profile in 2016 or 2017 (Table 2-3) ($p < 0.05$).

In 2016, soil inorganic N availability was significantly greater in the tillage treatment compared to the no tillage treatment at four consecutive sampling dates starting on May 23 (DCD 6.4-27.9) ($p < 0.05$). This coincided with the V6-R2 corn growth stages in 2016. By June 28 (DCD 17.5), the soil inorganic N in the spring tillage plots peaked and there was significantly more inorganic N available at this sampling date compared to any other sampling date ($p < 0.05$). The peak availability of inorganic N in the no tillage plots occurred over the final three sampling dates of the decomposition period from late June through September (DCD 17.5 – 40.8).

The most soil inorganic N was available in late May (DCD 9.9) and June (DCD 19.8) of 2017 in the spring tillage treatments and in late June (DCD 19.8) in the no tillage treatments. May 8 (DCD 4.5) was the only sampling date in 2017 when there was a significant difference in soil inorganic N availability by tillage treatment. At this date, there was more inorganic N in the no tillage treatment ($p < 0.05$).

Overall, the trends of inorganic N availability in 2016 and 2017 were different. In 2017, soil inorganic N values in April and May (DCD 1.5 – 9.9) were two-fold higher than in the same months in 2016. In addition, though the peak inorganic N availability began at the same sampling date (June 28) in 2016 (DCD 17.5) and 2017 (DCD19.8) for the no tillage treatments, the spring tillage treatment had elevated inorganic N availability at the end of May that continued into late June. Lastly, in 2016, the minimum and maximum mean values of soil inorganic N availability ranged from 10-75 mg $kg^{-1}$ soil, whereas in 2017 the minimum and maximum mean values during the decomposition period ranged from 26-65 mg $kg^{-1}$ soil.

The trend in inorganic N availability in both 2016 and 2017 were aligned with the precipitation and soil temperature trends for each year. In 2017, the warmer April could have caused the elevated soil inorganic N levels, and the lower levels of precipitation and temperature

in April 2016 align with the lower levels of inorganic N from April 20-June 6 (DCD 0.0 – 10.5). Trends in inorganic N availability in cover cropped agroecosystems during the decomposition period have not been well documented in the literature, but it has been suggested that N mineralization is tightly linked to microbial demand for C (McGill and Cole, 1981).

## 2.5 Conclusions

This study determined that cover crop and residue management treatments differentially influence soil and litter EA during the cover crop decomposition period. Notably, responses to treatments were evident in 2016, which was the first year after the transition to a no-tillage and cover cropped agroecosystem. As sustainable management practices such as cover cropping and no tillage residue management become more popular among agriculturalists in the Corn Belt, additional studies should be implemented to understand how other species of cover crops impact EA in similar systems to learn more about inorganic N availability following cover crop termination.

It was clear from the study that soil β-glucosidase activity was linked to inorganic N availability. In both the 2016 and 2017 decomposition periods, the peak inorganic N availability for all treatments occurred one sampling date after the peak soil β-glucosidase activity in each treatment. Soil β-glucosidase activity was highest between 4.5 and 9.9 DCDs after cover crop termination. The most soil inorganic N was available between 9.9 and 19.8 DCDs following cover crop termination. In 2016, the DCD when the most soil inorganic N was available occurred on June 28 when the corn was at the VT growth stage. In 2017, the most soil inorganic N was available at DCD 9.9 and 19.8 when the corn was at VE and V6.Though suspected through the assumption that C depolymerization and inorganic N availability are linked, the short term, in-season dynamics

of this phenomenon has yet to be illustrated in the literature. To understand this linkage, additional studies should be considered that control for weather and C substrate availability.

Our study confirms temporal variations in soil and litter enzyme activity during the decomposition period of cover crops in both no tillage and spring tillage residue management in the Midwest U.S. Corn Belt. This provides an indication that several samplings should be completed to calculate an annual EA when comparing EA between research sites or ecosystems. Additionally, seasonal variation in enzyme incubation temperature optima and substrate concentration may be required to most accurately assess changes in EA in different climatic conditions (Bell and Henry, 2011). Our study focused only on two enzymes during the decomposition period; monitoring the dynamics of a broader spectrum of enzymes that are linked to C mineralization and N availability could potentially reveal different results. A C mineralization enzyme that cleaves various C bonds within the microfibril structure earlier in the C mineralization process, such as endoglucanase, may have higher activity earlier in the decomposition period compared to β-glucosidase.

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Table 2-1. Summary table of statistical significant differences in litter enzyme activity. Five sampling dates ranging from May to October, four cover crops, and two tillage systems

Litter Enzyme Activity

| | β-glucosidase | | Urease | |
|---|---|---|---|---|
| | 2016 | 2017 | 2016 | 2017 |
| Date | <0.001 | <0.001 | <0.001 | <0.001 |
| Cover Crop | NS | 0.0026 | 0.005 | NS |
| Tillage | NS | <0.001 | 0.006 | <0.001 |
| Date*Cover Crop | NS | 0.0002 | NS | 0.05 |
| Cover Crop*Tillage | NS | 0.0228 | NS | 0.038 |
| Date*Tillage | 0.003 | <0.001 | NS | <0.001 |
| Cover Crop*Tillage*Date | NS | <0.001 | NS | 0.004 |

Significance reported within column for each year. $\alpha < 0.05$.

$Log_{10}$ transformation was used for litter enzyme activity each year.

Table 2-2. Summary table of statistical significant differences in soil enzyme activity. Seven sampling dates ranging from April to September, four cover crops, and two tillage systems.

Soil Enzyme Activity

| | β-glucosidase | | Urease | |
|---|---|---|---|---|
| | 2016 | 2017 | 2016 | 2017 |
| Date | <0.001 | <0.001 | <0.001 | <0.001 |
| Cover Crop | 0.025 | 0.002 | 0.009 | 0.008 |
| Tillage | NS | <0.001 | NS | 0.031 |
| Date*Cover Crop | NS | NS | 0.033 | 0.021 |
| Cover Crop*Tillage | NS | NS | NS | NS |
| Date*Tillage | NS | NS | NS | NS |
| Cover Crop*Tillage*Date | NS | NS | NS | NS |

Significance reported within column for each year. $\alpha < 0.05$.

Table 2-3. Summary table of statistical significant differences in soil inorganic N. Seven sampling dates ranging from April to September, four cover crops, and two tillage systems.

| Inorganic Nitrogen | | |
|---|---|---|
| | 2016 | 2017 |
| Date | <0.001 | <0.001 |
| Cover Crop | NS | NS |
| Tillage | <0.001 | NS |
| Date*Cover Crop | NS | NS |
| Cover Crop*Tillage | NS | NS |
| Date*Tillage | <0.001 | 0.04 |
| Cover Crop*Tillage*Date | NS | NS |

Significance reported within column for each year. $\alpha < 0.05$.

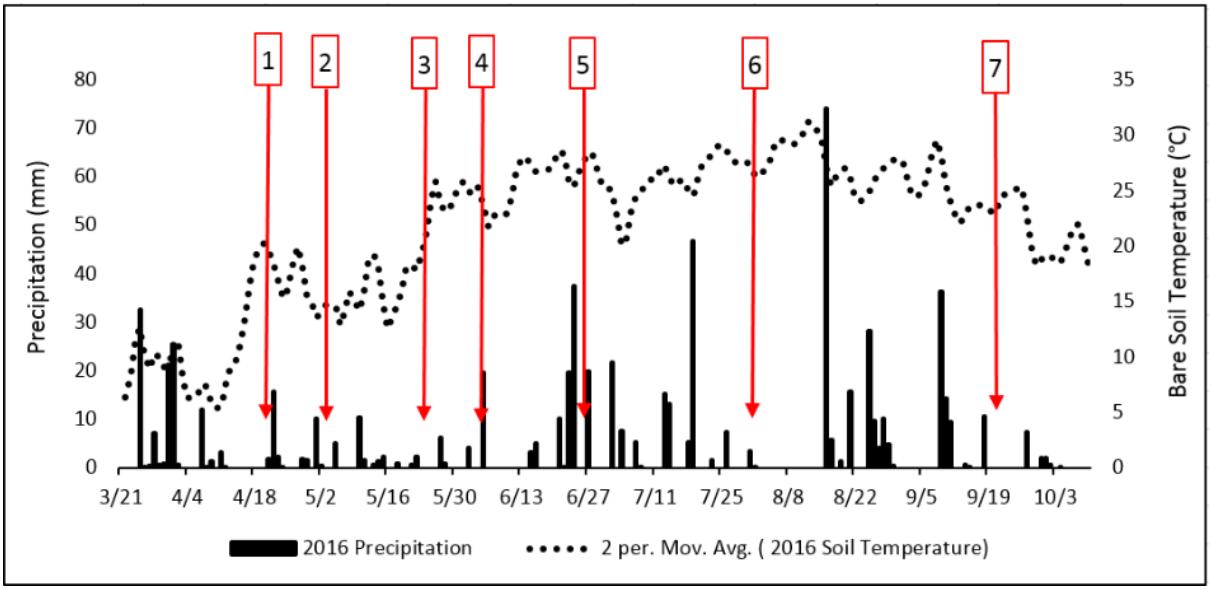


Figure 2-1. Precipitation (mm) and bare soil temperature (°C) averages from March 21-October 10, 2016. Numbers in the red boxes connected to red arrows correspond to the seven soil sampling dates in 2016.

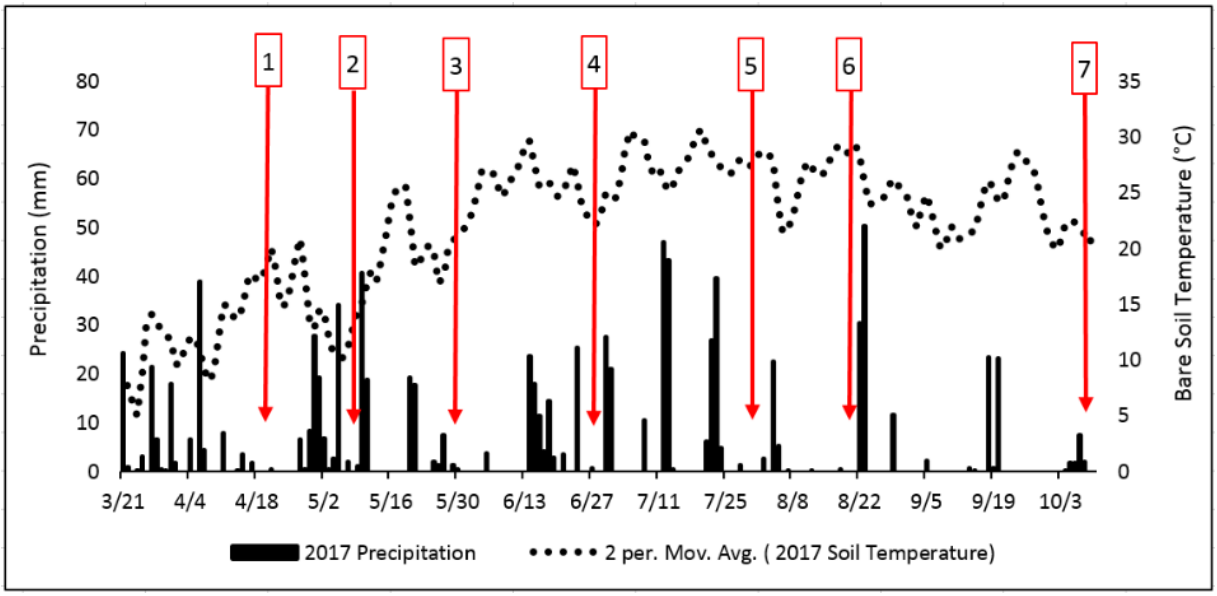


Figure 2-2. Precipitation (mm) and bare soil temperature (°C) averages from March 21-October 10, 2017. Numbers in the red boxes connected to red arrows correspond to the seven soil sampling dates in 2017.

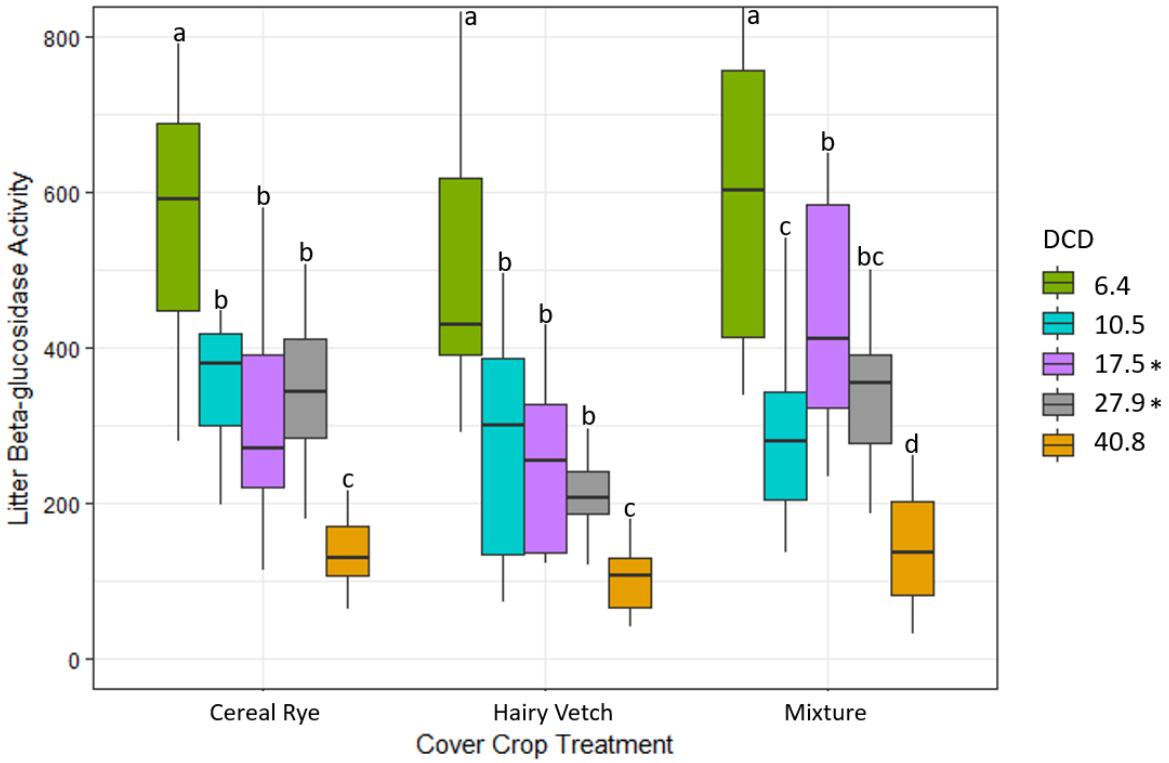


Figure 2-3. Measured average levels of litter β-glucosidase activity (μmoles *para*-nitrophenol released g litter$^{-1}$ hr$^{-1}$) in each cover crop treatment during the 2016 corn growing season. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant statistical difference in enzyme activity between cover crop treatments within two DCDs (*). At DCD 17.5 enzyme activity in the mixture treatment was significantly higher than in the hairy vetch treatment. At DCD 27.9, the cereal rye and mixture treatment enzyme activity was significantly higher than the hairy vetch.

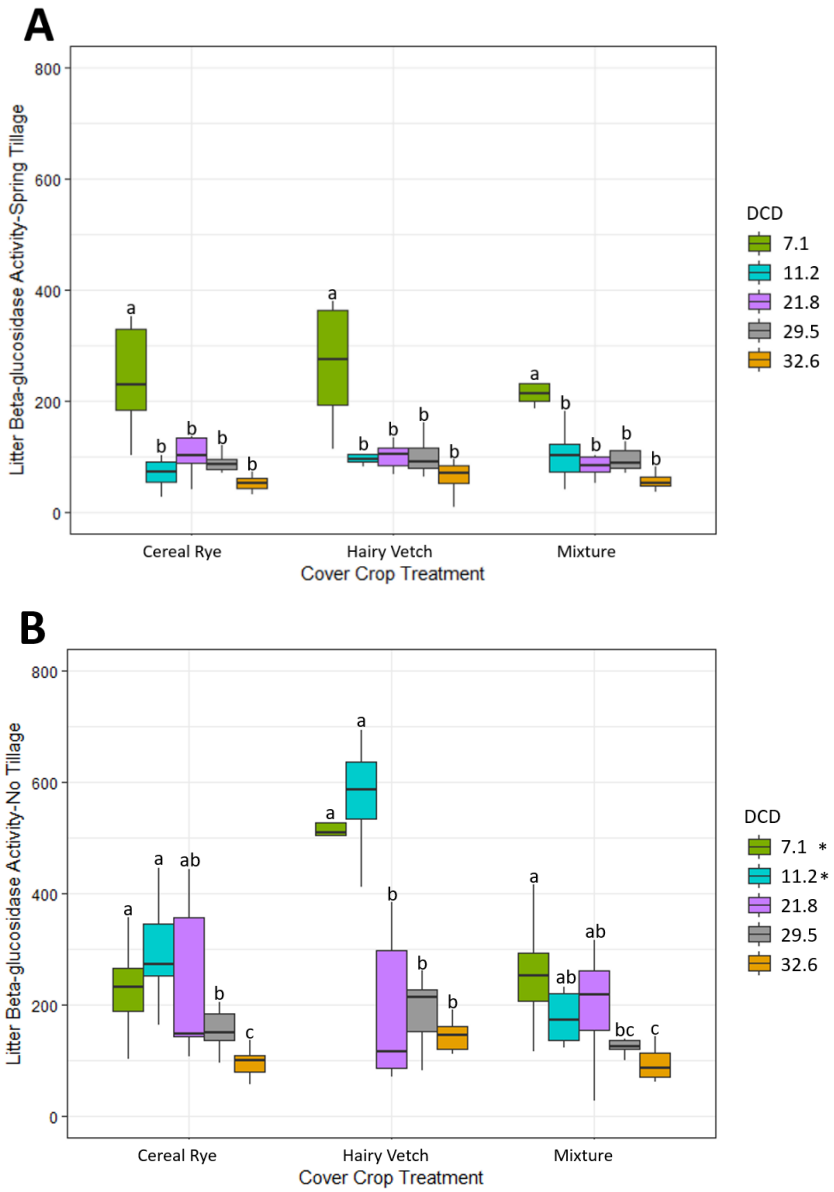


Figure 2-4. Measured average levels of litter β-glucosidase activity (μmoles *para*-nitrophenol released g litter$^{-1}$ hr$^{-1}$) in each cover crop treatment during the 2017 corn growing season in the (A) spring tillage and (B) no tillage treatments. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a statistical significant difference between cover crop treatments within two DCDs in the no tillage system (*). At DCD 7.1, the enzyme activity of the hairy vetch treatment was significantly higher than the enzyme activity in the cereal rye and mixture treatments. At DCD 11.2, the enzyme activity of the hairy vetch was significantly higher than the cereal rye and mixture and the cereal rye activity was significantly higher than the mixture.

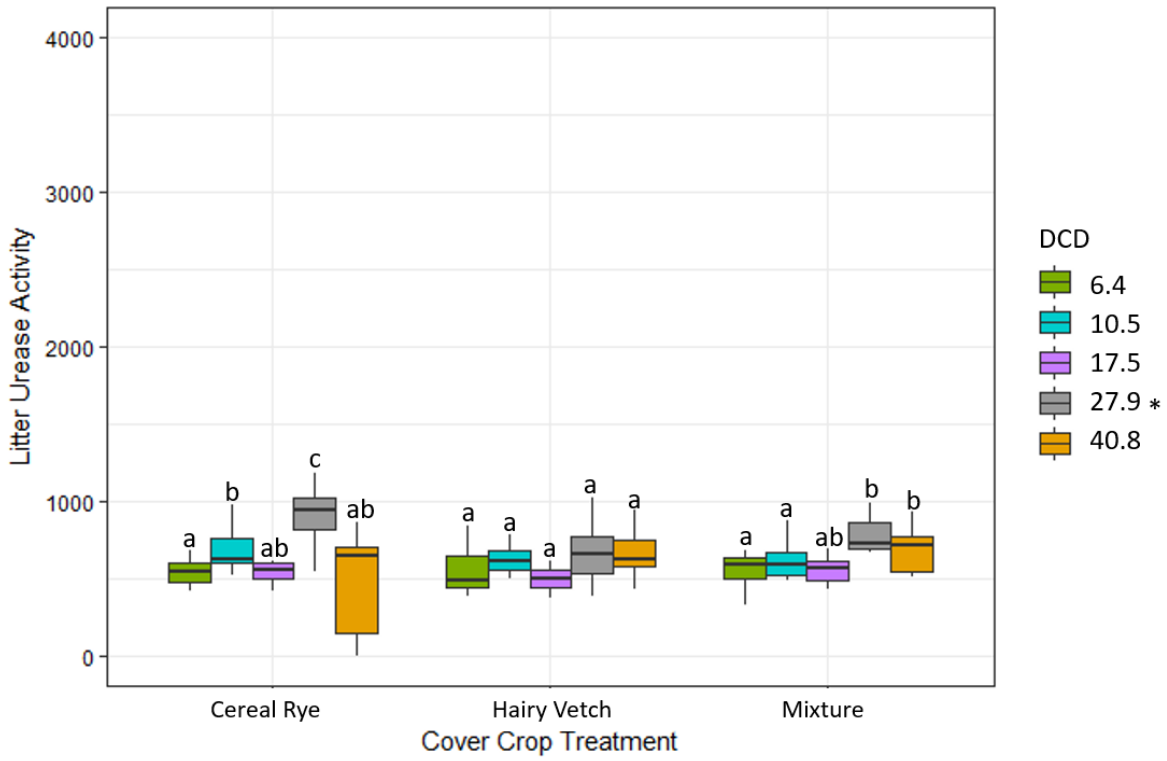


Figure 2-5. Measured average levels of litter urease activity (µg $NH_4$-N g $litter^{-1}$ $hr^{-1}$) in each cover crop treatment during the 2016 corn growing season. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant statistical difference between cover crop treatments within DCD 27.9. (*) The enzyme activity in the cereal rye treatment was significantly higher than that of the hairy vetch treatment.

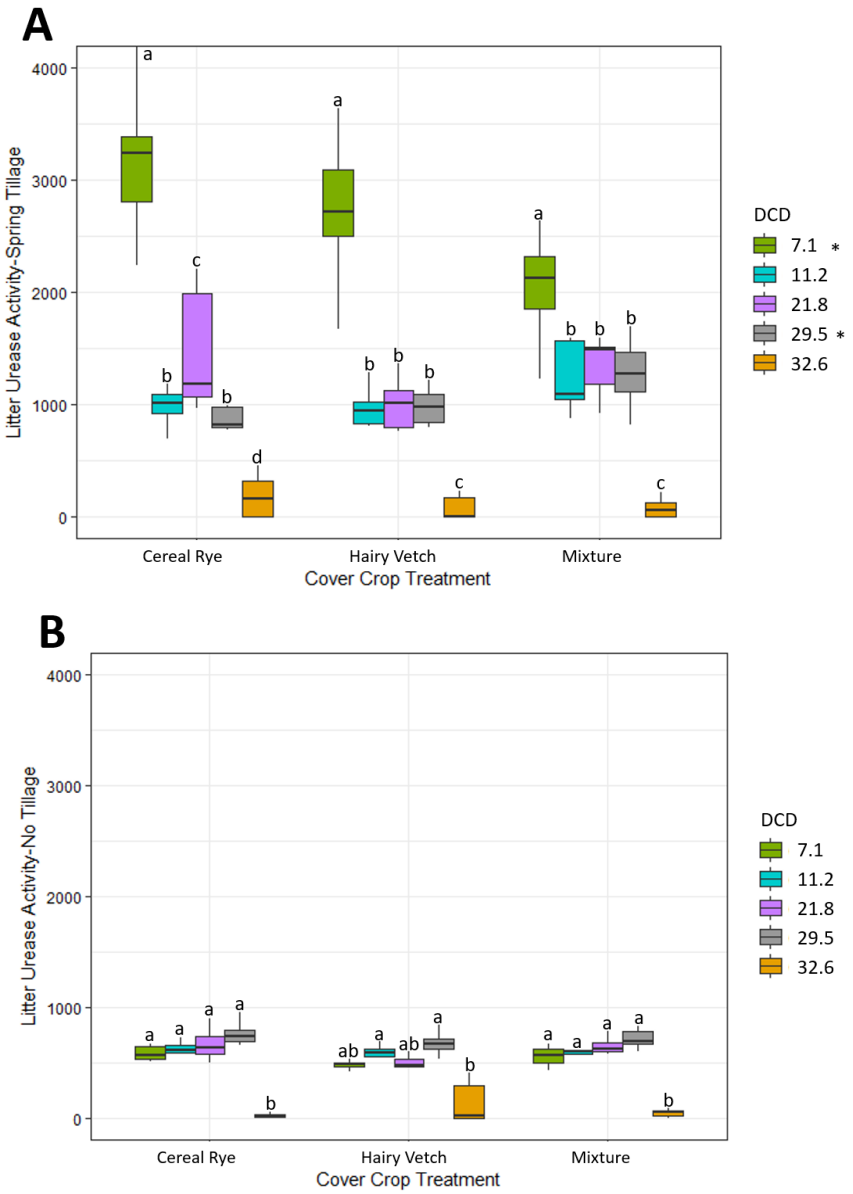


Figure 2-6. Measured average levels of litter urease activity (µg $NH_4$-N g $litter^{-1}$ $hr^{-1}$) in each cover crop treatment during the 2017 corn growing season in the (A) spring tillage and (B) no tillage treatments. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant difference between cover crop treatments within two DCDs in the spring tillage treatment (*). At DCD 7.1, the cereal rye treatment had significantly higher activity than the mixture treatment. At DCD 29.5, the mixture enzyme activity was significantly higher than the hairy vetch and cereal rye.

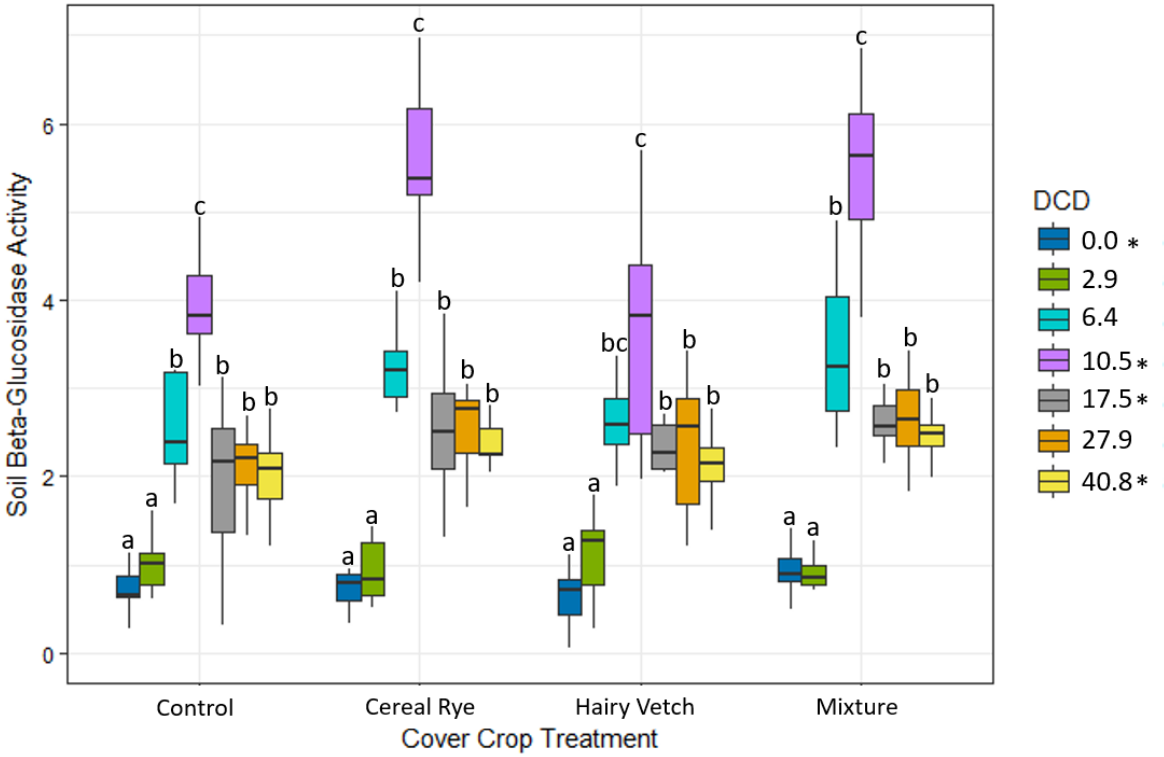


Figure 2-7. Measured average levels of soil β-glucosidase activity (μmoles *para*-nitrophenol released g dry soil$^{-1}$ hr$^{-1}$) in each cover crop treatment during the 2016 corn growing season. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant difference between cover crop treatments within four DCDs (*). At DCD 0.0, the mixture treatment activity was significantly higher from the hairy vetch treatment. At DCD 10.5, the cereal rye and mixture treatments had significantly higher enzyme activity than the hairy vetch and control treatments. At DCD 17.5, the mixture treatment activity was significantly higher than the control treatment. At DCD 40.8, the cereal rye and mixture treatments had significantly higher activity than the control and the mixture had significantly higher activity than the hairy vetch.

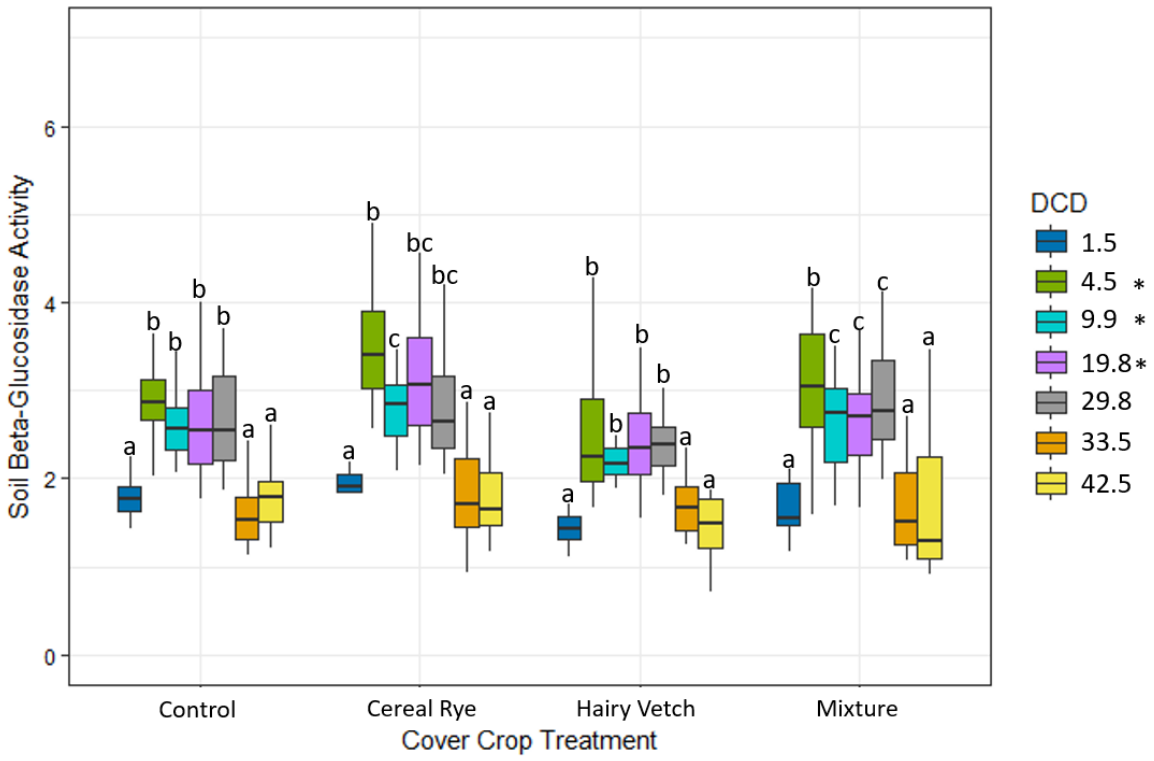


Figure 2-8. Measured average levels of soil β-glucosidase activity (µmoles *para*-nitrophenol released g dry soil$^{-1}$ hr$^{-1}$) in each cover crop treatment during the 2017 corn growing season. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant statistical difference between cover crop treatments within three DCDs (*). At DCDs 4.5 and 19.8, the cereal rye and mixture treatments had significantly higher enzyme activity than the hairy vetch treatment. At DCD 9.9, the cereal rye and mixture treatments had a significantly higher enzyme activity than the hairy vetch treatment and the mixture had a significantly higher activity than the control.

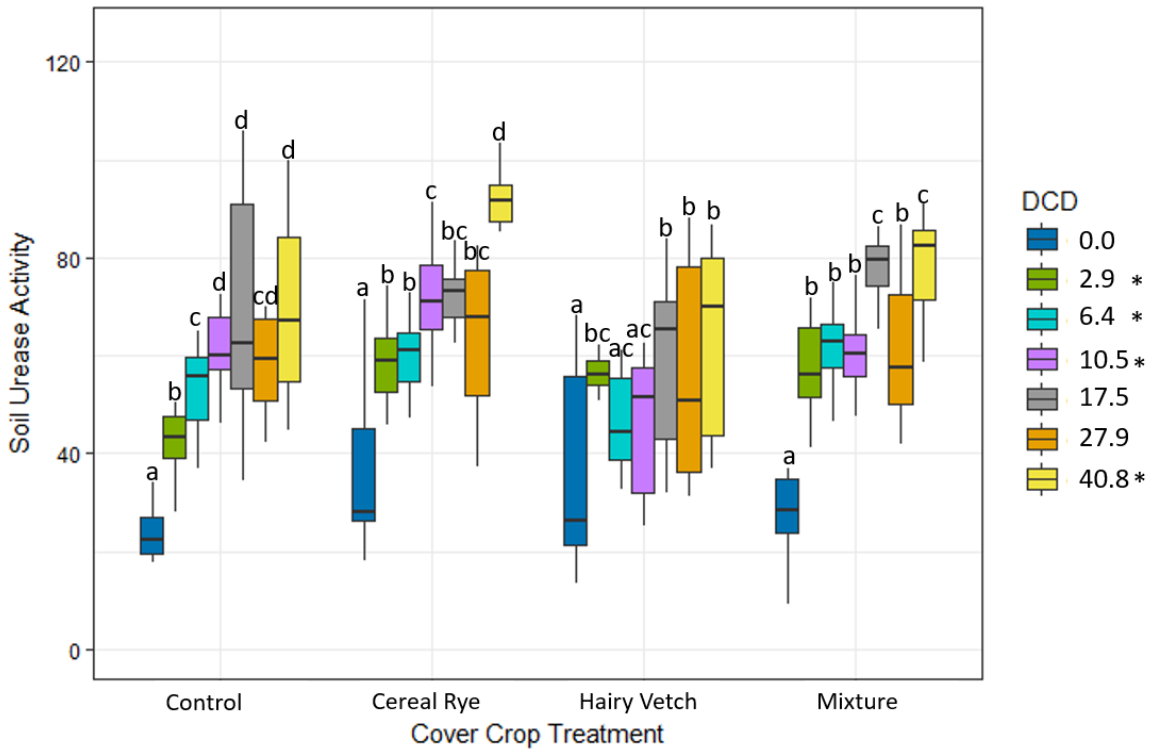


Figure 2-9. Measured average levels of soil urease activity (μg $NH_4$-N g dry $soil^{-1}$ 2 $hr^{-1}$) in each cover crop treatment during the 2016 corn growing season. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant difference between cover crop treatments within four of the DCDs (*) At DCD 2.9, the control had significantly lower activity than the other treatments. At DCD 6.4, the cereal rye and mixture treatment had significantly higher activity than the hairy vetch. At DCD 10.5, the hairy vetch treatment had significantly lower activity than the other three treatments, and the cereal rye had significantly higher activity than the mixture. At DCD 40.8, the cereal rye treatment had significantly higher activity than the hairy vetch and control.

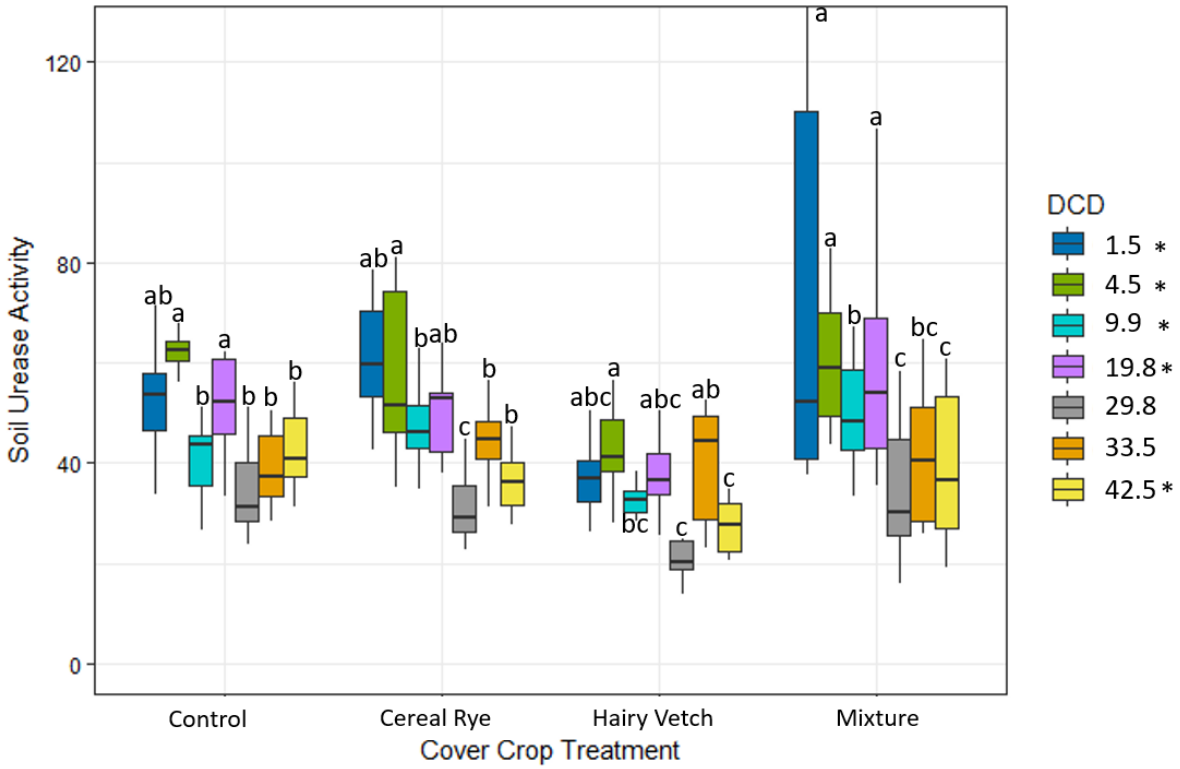


Figure 2-10. Measured average levels of soil urease activity (µg $NH_4$-N g dry $soil^{-1}$ 2 $hr^{-1}$) in each cover crop treatment during the 2017 corn growing season. Cover crop treatments are on the horizontal axis and enzyme activity is on the vertical axis. Bars with the same letter are not significantly different from each other within cover crop treatment over the decomposition period as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. There was a significant difference between cover crop treatments within five DCDs (*) At DCDs 1.5 and 4.5, the hairy vetch treatment had significantly lower activity than the other three treatments. At DCD 9.9, the cereal rye and mixture treatments had significantly higher activity than the hairy vetch and control, and the control had significantly higher activity than the hairy vetch. At DCDs 19.8 and 42.5, the hairy vetch had significantly lower activity than the control and mixture.

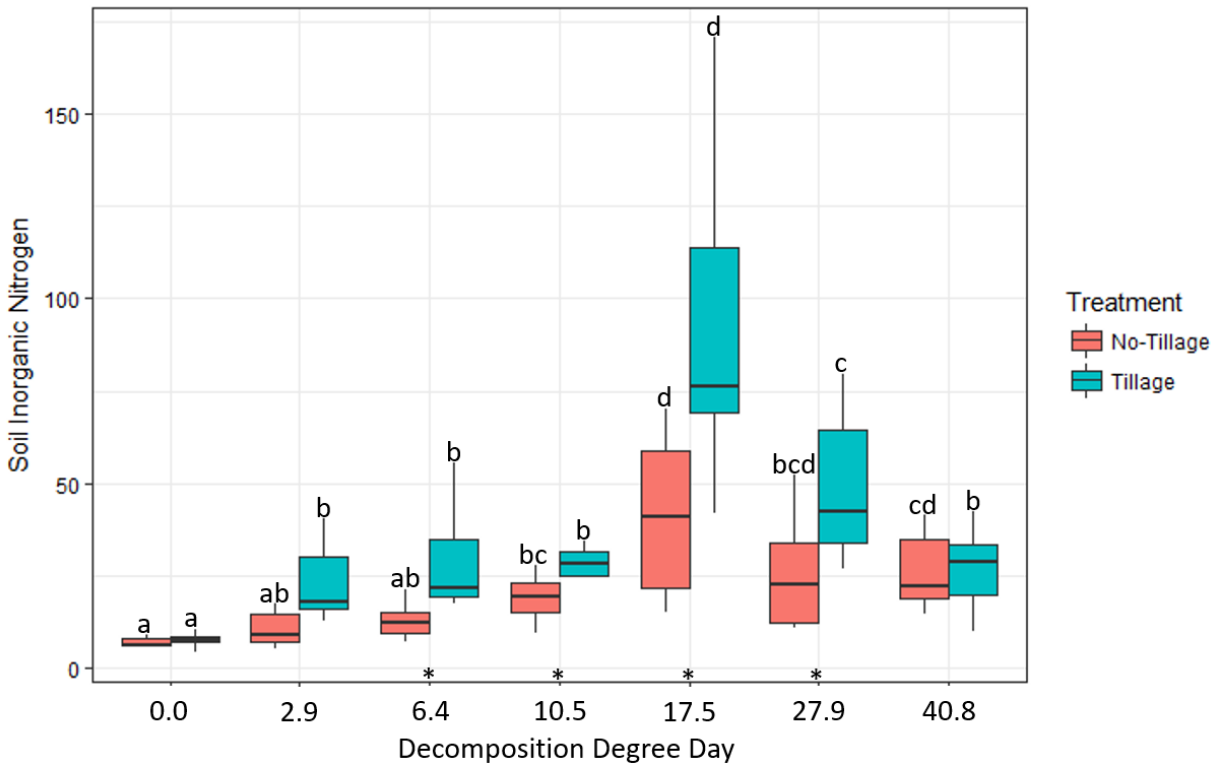


Figure 2-11. Average soil inorganic nitrogen (mg inorganic N kg soil $^{-1}$) for tillage and no-tillage residue management systems during the 2016 corn growing season. Decomposition degree days (DCDs) are on the horizontal axis and inorganic N is on the vertical axis. Bars with the same letter are not significantly different from each other within tillage treatment over the growing season as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. Significant differences within each sampling date are signified by an asterisk (*) directly above the sampling date on the x-axis.

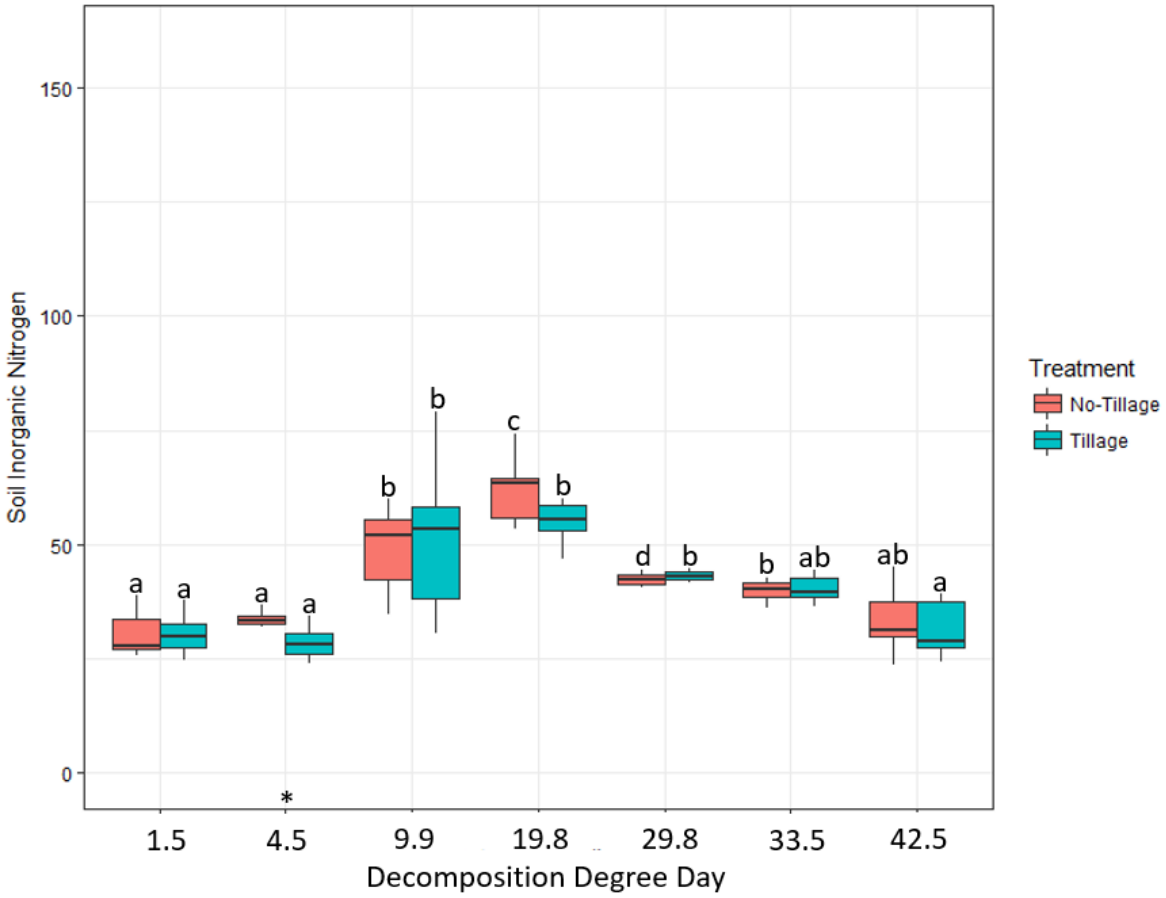


Figure 2-12. Average soil inorganic nitrogen (mg inorganic N kg soil$^{-1}$) for tillage and no-tillage residue management treatments during the 2017 corn growing season. Decomposition degree days (DCDs) are on the horizontal axis and inorganic N is on the vertical axis. Bars with the same letter are not significantly different from each other within tillage treatment over the growing season as determined using PROC MIXED and a repeated measure model ($p < 0.05$). The *whiskers* indicate the lower (25%) quartile and upper (75%) quartile. Significant differences within each sampling date are signified by an asterisk (*) directly above the sampling date on the x-axis ($p < 0.05$).

# CHAPTER 3. THE DECOMPOSITION OF DIFFERENT WINTER COVER CROPS INFLUENCES THE SOIL MICROBIOME

A version of this chapter has been submitted to the Journal of Soil Biology and Biochemistry for review.

## 3.1 Abstract

Cover crop adoption in the U.S. Corn Belt region is a rapidly emerging management practice in corn (*Zea mays*) agroecosystems. However, little is known about the impact of the inclusion of cover crops on the soil microbiome and its relation to the decomposition of the cover crop residue during the cash crop growing season. Therefore, this study sought to determine the impact of cover crop species and residue management practices on soil microbial community composition and structure during winter cover crop decomposition over the corn growing season. Cover crop treatments included hairy vetch (*Vicia villosa* Roth), cereal rye (*Secale cereal*), a hairy vetch/cereal rye mixture, and a no cover crop control. Residue management practices included no-tillage and a 15 cm reduced spring tillage following cover crop termination. Soil samples were collected at five dates during cover crop decomposition that corresponded to an accumulated number of decomposition degree-days (DCD) from cover crop termination. Soil bacterial communities were characterized using the small subunit (16S) rRNA gene sequences determined using the Illumina MiSeq system and sequences were analyzed using the QIIME pipeline. Distance matrices among samples were created through β-diversity analyses using phylogenic and non-phylogenetic metrics. Permutational multivariate analysis of variance (PERMANOVA) of the distance matrices revealed that the main effects of decomposition degree-day, cover crop, and residue management were significant determinants of soil microbial community composition ($p < 0.05$), and the effect of cover crop treatment increased as cover crops decomposed. Linear

discriminate analysis effect size (LEfSe) analysis indicated that as cereal rye began to decompose and soil β-glucosidase (EC 3.2.1.21) activity increased, the relative abundance of bacteria previously identified as cellulolytic including *Agromyces*, *Streptomyces*, and *Bacillus* contributed to the difference among the cover crop treatments (LDA > 2.0). Data generated from this study leads to a deeper understanding of bacterial responses to cover crop decomposition in maize agroecosystems, which could aid agriculturalists with the development of sustainable nutrient management plans to promote beneficial microorganisms.

**Abbreviations**

Decomposition degree day (DCD), Cereal rye (CR), Hairy vetch (HV), Operational taxonomic unit (OTU), Principal Coordinate Analysis (PCoA), Canonical correspondence analysis (CCA), PermDISP (Permutational analysis of multivariate homogeneity of group dispersions), PERMANOVA (Permutational multivariate analysis of variance)

## 3.2 Introduction

The incorporation of organic matter amendments and reduced tillage residue management treatments into nutrient management plans is a growing trend among agriculturalists in the United States (Cover Crop Survey, 2017) as the debate surrounding the sustainability of intensive conventional, agriculture practices continues. Cover crops, vegetation most commonly grown between cash crop plantings, serve as an organic matter amendment to the agroecosystem. Likewise, reduced tillage residue management, such as not tilling soils prior to planting cash crops, is used to improve soil health. It is well understood that cover crops increase soil organic carbon (Liu et al., 2005), and studies have demonstrated that organic matter amendments such as cover crops may increase nutrient use efficiency in an agroecosystem (Radicetti et al., 2016;

Tonitto et al., 2006; Torstensson et al., 2005). Additionally, benefits such as reduced erosion from wind and water have been documented for this management practice (Panagos et al., 2015; Alliaume et al., 2014). Cover crops and reduced tillage residue management increases the amount of labile carbon added to the agroecosystem (Ladoni et al., 2016; Johnson et al., 2013; Sindelar et al., 2014), and the microbial community mediates the decomposition of the organic matter amendment. One of the primary energy sources acquired from the cover crop decomposition is glucose, which is derived from cellulose (Hobbie and Hobbie, 2013). A succession of enzymes, cellulases, depolymerize cellulose until its byproducts can enter the microbial cell. Once inside the cell, these molecules are further hydrolyzed to glucose by β-glucosidase (EC 3.2.1.21). This resulting glucose serves as a vital energy source in microbial metabolism (Eivazi and Tabatabai, 1988). Therefore, in this study, the activity of this enzyme is used as a means to measure decomposition.

It is well established that cover crops and reduced tillage impact soil chemical, physical, and biological properties. Several studies have examined how organic amendments impact the soil microbial community. Phospholipid fatty acid (PLFA) analyses have found that cover crops increased total PLFA concentrations compared to the control plots (Finney et al., 2017). Likewise, the addition of organic matter amendments to agroecosystems has led to increases in total microbial activity (Fließbach et al. 2007), including increased biomass (Cong et al., 2005) and enzyme activity (Stark and Condron 2008). Recently, high-throughput sequencing of bacterial ribosomal genes has been used to survey the impact of cover crops to agroecosystems (Fernandez et al., 2016; Zheng et al., 2018). Still, it is unknown how cover crops and reduced tillage treatments influence soil microbiome diversity and the abundance patterns of members of the microbial community during the cover crop decomposition period.

Past studies using high-throughput sequencing techniques have examined the soil microbial community dynamics in response to organic matter amendments by selectively sampling during the year (Fernandez et al., 2016), but have not captured the short-term dynamics of the soil microbiome during the cover crop decomposition period and critical growth stages of the subsequent cash crop. In addition, no studies have concurrently measured the influx of cover crop carbon and the enzyme activity representing the functional capacity of the soil microbial communities' ability to decompose the cover crop residue after cover crop termination. Lastly, no studies have examined these dynamics in the first year of the transition to a no-tillage residue management and cover cropping.

Therefore, this project had two objectives: i) to investigate the influence of cover crop species on the soil microbial community structure and composition during the decomposition period relative to a no cover crop control, and ii) to determine if residue management influences the soil microbial community structure and composition during the decomposition period of different cover crop species. It was hypothesized that cover crop species and residue management system would influence the soil microbial community diversity and composition throughout the decomposition period. This study increases our understanding of how the soil microbial community responds to carbon inputs during the decomposition of winter cover crops, leading to the identification of bacteria that are highly responsive to management practices during the cash crop growing season and having the largest effect in differentiating cover crop species. Understanding the abundance patterns of the microbiota that are influenced by management practices has the potential to help optimize agriculture management practices to promote beneficial microorganisms as the adoption of management practices such as cover cropping and reduced tillage residue management treatments continues.

## 3.3 Methods

### 3.3.1 Site description and experimental design

The study site was located in Tippecanoe County, Indiana at the Purdue University Agronomy Center for Research and Education (ACRE) (40°28'16"N; 86°59'32"W) on a silty clay loam soil. Precipitation and air temperature measurements were collected from the Indiana State Climate Office (https://iclimate.org). The winter of 2015/2016 was the first year the cover crop and residue management systems were implemented. Prior to these cover crop and tillage regimes, the site was managed in a corn-soybean annual rotation with spring tillage prior to corn planting every other growing season. The experiment had a split-plot design with three field replications. There were six treatments used: three cover crops, no cover crop controls, and two tillage practices. Each plot was 12 rows wide and approximately 80 m in length. Cover crops were planted on September 28-30, 2015, and treatments included hairy vetch (*Vicia villosa*), cereal rye (*Secale cereale*), and a hairy vetch/cereal rye mixture. The hairy vetch (HV) and cereal rye (CR) seeding rates were 33 and 56 kg $ha^{-1}$ drilled, respectively. The hairy vetch/cereal rye mix (HV/CR) seeding rate was 16/44 kg $ha^{-1}$ drilled. Cover crop fall biomass samples were taken on November 23, 2015 and spring biomass sampling occurred on April 16, 2016. The cover crops grew over the winter and were chemically terminated on April 14, 2016. Following termination, anhydrous ammonia was applied at a rate of 78 kg $ha^{-1}$ of N. On April 25, 2016, the spring tillage plots were disk tilled twice to a depth of approximately 15 cm. On April 26, 2016, the same plots were cultivated to prepare the seedbed. The no tillage plots were not disk tilled. On April 26, 2016 maize was planted in all plots.

### 3.3.2 Litter bags for residue decomposition measurement

Cover crop biomass was collected prior to termination on April 15, 2016 (DCD 0.0). Cover crop residue was collected from 1 $m^2$ quadrants randomly placed in each plot. Biomass was oven dried at 105°C until constant weight was reached to determine winter cover crop dry biomass accumulation. Additional cover crop biomass samples were collected on the day after termination and air-dried to be used in litter bags (Organization for Economic Cooperation and Development; Melkonian et al., 2017; Jahanzad et al., 2016; Varela et al., 2017). Nylon mesh (1 mm) litter bags were created and sealed with silicon glue. The mixture, CR, and HV litterbags were filled with 22 g, 20 g, and 10 g of biomass, respectively. The 22 g of biomass in the mixture bag contained 20 g of cereal rye and 2 g of hairy vetch residue to simulate the amount of hairy vetch residue in the mixture treatments in the field. To simulate the tillage treatment, litter bags were buried approximately 15 cm. Litter bags applied to no tillage residue management treatments were attached to the surface between corn rows with lawn staples. The bags were retrieved on approximately a biweekly basis starting two weeks after cover crop termination, and linear interpolation was used to estimate biomass remaining in the litter bags on soil sampling dates that were different from litter bag collection dates. The cover crop residue remaining in the litter bags at each sampling date was weighed to determine mass loss after air drying. Additional litter from each bag was analyzed for total N and C concentrations using the FLASH 2000 Elemental Analyzer (dry combustion method).

### 3.3.3 Soil sampling

Soil samples were collected five times over the cover crop decomposition period from May-August 2016, and each soil sampling date corresponding with a decomposition degree-day from the time of cover crop termination. Decomposition degree-days (DCD) were calculated

according to Ruffo and Bollero (2003). During each sampling, 15 randomly distributed soil cores were removed per plot at a depth of 4 cm using a 4 cm diameter soil probe. The cores were homogenized during sampling to create a composite bulk sample for each plot. The bulk samples were transported from the field to the lab on ice in a cooler. Approximately 150 and 100 g of each bulk sample was stored at -20°C for DNA extraction and 4°C for enzyme assays, respectively. An additional subsample of the bulk soil was air dried and used for physiochemical analyses.

### 3.3.4 Soil physiochemical analysis

Soil moisture was determined by oven drying a subsample of the fresh, 2 mm sieved soil at 105°C for 48 hr. Extractable $NO_3^-$N and $NH_4^+$-N were measured with 2 g of soil sample and 20 mL of 1 M KCl extract. The slurry was shaken for 1 hr and the supernatants were analyzed on a SEAL AQ2 analyzer using USEPA methods 132-A Rev.1 and 103-A Rev. 4 (USEPA, 1993).

### 3.3.5 Soil β-glucosidase activity

Soil β-glucosidase assays (μmoles *para*-nitrophenol released g dry $soil^{-1}$ $hr^{-1}$) were completed as described in section 2.3.5 according to Eivza and Tabatabai (1988).

### 3.3.6 Soil DNA extractions

DNA was extracted from the soil subsample from each plot at the five sampling dates, totaling 120 samples. Total genomic DNA was extracted using approximately 500 mg of soil and the FastDNA™ Soil Spin Kit (MP Biomedicals, LLC) and FastPrep-24 instrument (MP Biomedicals, LLC) following the manufacturer's instructions. Quality of the extracts was assessed using gel electrophoresis and a NanoDrop 1000 spectrometer (260/280 OD ratio). DNA concentration was determined using a NanoDrop 3300 (Thermo Scientific, Wilmington, DE) fluorospectrometer.

### 3.3.7 Bacterial 16S rRNA gene amplification and sequencing

Primers that target the V3-V4 region of 16S rRNA genes were used for amplification with a PCR mix containing Q5 High Fidelity DNA Polymerase (New England Biolabs). In total, the mixtures contained 25 uL of Q5 High Fidelity DNA Polymerase, 0.75 µL of 25 µM forward primer IL-V3-343F (TAC GGR AGG CAG CAG), 0.75 µL of 25 µM reverse primer IL-V4-803R (CTA CCR GGG TAT CTA ATC C), 1.25 µL of bovine serum albumin (BSA), 5 µL of DNA template at 2 ng $\mu L^{-1}$ (10 ng), and 17.25 µL of PCR grade $H_2O$ to a final volume of 50 µL. The first thermal cycling condition were: 5 min at 95°C for initial denaturation, followed by 20 cycles of 94°C for 30 sec, 58°C for 20 sec, 72°C for 20 sec with the final extension time of 10 min at 72°C. After the initial amplification, the PCR amplicons were purified using Agencourt AMPure XP kit (Beckman-Coultur) to remove any DNA that is < 100 bp. After purification, 24 µL of each of the initial PCR amplicons (in PCR grade $H_2O$ after clean-up) was combined with 25 µL of Q5 High Fidelity DNA Polymerase, 0.75 µL of 25 µM i5-TS-DI-50x primer, and 0.75µL of 25 µM i7-TS-DI-70x primer. These primers included the Illumina sequencing adaptors and index sequences to differentiate samples. The second thermal cycling conditions were 5 min at 95°C for initial denaturation, followed by 5 cycles of 94°C for 30 sec, 58°C for 20 sec, 72°C for 20 sec with the final extension time of 10 min at 72°C. PCR amplicons were quality checked with gel electrophoresis and were quantified by flourometry after staining with the Quantiflour dsDNA Assay Kit (Promega). Amplicons for each sample were pooled in equal molar quantities and were delivered to the Purdue Genomics Core Facility where library sequencing was completed on the Illumina MiSeq instrument (Illumina, San Diego, USA) using a 500 cycle sequencing kit and the 2 x 250 pair end cycle sequencing mode.

### 3.3.8 Statistical analysis and bioinformatics

#### 3.3.8.1 Enzyme activity and cover crop decomposition

Statistical analysis of soil β-glucosidase activity and cover crop decomposition was conducted using PROC MIXED (SAS Institute Inc., Cary, NC). Sampling date, cover crop (main plot) and tillage (sub-plot) were each considered fixed effects and field replication (block) was treated as a random effect. Sampling date was modeled as a repeated measure using the ARH (heterogeneous first-order autoregressive structure) variance-covariance matrix. The effects of cover crop and tillage on soil β-glucosidase activity and cover crop decomposition within sampling date were determined with the SLICE statement. The Tukey-Kramer adjustment was used to consider all pairwise comparisons.

#### 3.3.8.2 Processing sequence reads

Sequence reads were processed to remove primers and low-quality sequences, and then the pair ends were merged using the Panda software (Masella et al., 2012). Sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline version 1.9.1 (Caporaso et al., 2010). The OTUs were selected using the pick_open_reference_otus.py script. The Greengenes database (version 13_8) was used for taxonomic assignment (McDonald et al., 2012). Sequences were clustered by OTUs using the UCLUST algorithm at 97% identity (Edgar, 2010).

#### 3.3.8.3 Bacterial community diversity

QIIME was used for statistical analysis. Good's coverage was used to determine sequence coverage of the bacterial communities. Soil microbial community α-diversity, richness and evenness, was investigated using the Chao1 and Simpson's Evenness Indices, respectively, and the Shannon Diversity Index. α-diversity analysis was completed using the

alpha_diversity.py script. To compare communities, β-diversity comparisons were completed using phylogenetic based weighted Unifrac distances (Hamady, Lozupone, and Knight, 2010) and non-phylogenetic Bray-Curtis distances. Visual differences among samples were determined using principal coordinate analysis (PCoA). Statistical differences in β-diversity among soil microbial communities were tested with PERMANOVA (permutational multivariate analysis of variance) (Anderson, 2001), and the PERMANOVA assumption of equal variance between groups was tested with PermDISP (permutational analysis of multivariate dispersions) (Anderson, 2006). All PERMANOVA and PermDISP tests were completed with 999 permutations using the compare_categories.py script.

#### 3.3.8.4 Linear discriminate analysis effect size (LEfSe)

The LEfSe (Linear Discriminate Analysis Effect Size) (Segata et al., 2010) algorithm was used to identify biomarkers that are most likely to characterize the differences between cover crop treatments within each of the sampling dates by coupling tests for statistical significance with tests for biological consistency. LEfSe uses a non-parametric factorial Krustal-Wallis sum-rank test to determine which features are differentially abundant. Then, a set of pairwise tests, using an unpaired Wilcoxon rank-sum test, is used to compare among subclasses, which helps avoid a high false positive rate and reports only features that are biologically relevant. A "one-against-all" strategy for multi-class analysis was used when comparing among subclasses and alpha was set to 0.05. A linear discriminate analysis estimates the effect size of the differentially abundant features.

#### 3.3.8.5 Phylogenetic investigation of communities by reconstruction of unobserved states

The functional composition of the microbiome was predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States http://picrust.github.com, (Langille et al., 2013)). The open reference OTU table generated by

QIIME output script pick_open_reference_otus.py was used for analysis. Before analysis, de-novo OTUs were removed using the filter_otus_from_otu_table.py QIIME script and the newest available reference collection: gg_13_5_otus.tar.gz. Samples with zero read counts were removed earlier in the QIIME pipeline. The resulting closed reference OTU table was normalized by copy number to correct for multiple 16S rRNA gene copy number, resulting in a better reflection of organism abundance. The metagenome was predicted and the hierarchical data was collapsed to the desired functional level. Metagenomic profile results were visualized using the STAMP (Statistical Analysis of Metagenomic Profiles) software package (version 2.1.3) (Parks and Beiko, 2010).

#### 3.3.8.6 Canonical correspondence analysis

Canonical Correspondence Analysis (CCA) (ter Braak and Verdonschot, 1995) was created using the vegan package (Oksanen et al., 2017) in the R project (http://www.R-project.org/) to determine the associations between bacterial genus relative abundance and environmental variables. Variable included in the model included DCD, cover crop treatment, residue management treatment, soil nitrate, soil ammonium, soil moisture, soil β-glucosidase activity, soil organic matter, and soil organic carbon. An analysis of variance (ANOVA) was used to determine which variables were significantly impacting bacterial genus relative abundance in the model ($p < 0.05$). A variance inflation factor (VIF) < 1.95 was used to detect variable redundancy.

## 3.4 Results

### 3.4.1 Quantification of soil β-glucosidase activity during cover crop decomposition

Cover crop biomass collected at cover crop termination revealed that there was 1967, 1858, and 418 kg $ha^{-1}$ of biomass in the mixture, cereal rye, and hairy vetch field plots, respectively.

According to the litter bag study, from the time of cover crop termination (decomposition day (DCD) 0.0)) to DCD 2.9 approximately 28-34% of the total cover crop biomass was released from the litter bags in the spring tillage residue management treatment, regardless of cover crop treatment (Figure 3-1A). In the no tillage system, there was approximately a third less cover crop decomposition during the same period, between 16-23% (Figure 3-1B). Additionally, between 65-70% and 40-45% of the total cover crop biomass available at termination had been released from the litter bags by DCD 6.4 in the spring tillage and no tillage systems, respectively.

Direct enzyme assays showed there was β-glucosidase activity during the decomposition period from DCD 2.9-27.9. The quantified activity of this enzyme increased from DCD 2.9 to 10.5 in both spring tillage (Figure 3-1A) and no tillage (Figure 3-1B) residue management treatments. Enzyme activity was statistically greater in all treatments at DCD 6.4 relative to activity at DCD 2.9, except for the hairy vetch in the no tillage treatments. At DCD 10.5, significantly higher enzyme activity was measured for the cereal rye and mixture treatments than the hairy vetch and control treatments in both residue management treatments

### 3.4.2 Microbial community composition

The number of sequences per sample ranged from 85,831 to 342,349 with an average of 162,890 $\pm$ 57,089. The sequences were rarefied to an equal abundance of 85,831 reads per sample, and all 120 samples were included in the analysis. After quality checking, 1003 OTUs were identified across all samples. Good's coverage for the samples ranged from 90-92%. Over the decomposition period, the OTUs observed represented 45 unique bacterial phyla. The soil bacterial community was dominated by three phyla, Proteobacteria (29-33%), Actinobacteria (22-25%) and Acidobacteria (20-24%).

### 3.4.3 Influence of cover crop species on soil microbial community structure

#### 3.4.3.1 Soil microbial community a-diversity differed during the decomposition period and within cover crop treatments

The Shannon Diversity Index, an α-diversity metric of taxa richness and evenness, revealed that the samples from the initial soil collection on DCD 2.9 had a significantly lower average than the following four sampling dates when sequences from all treatments were grouped together ($p < 0.05$) (Table 3-1). Additionally, the Simpson's Evenness Index revealed that the DCD 2.9, 10.5, and 17.5 samples had a significantly lower evenness than the DCD 27.9 samples ($p < 0.05$) (Table 3-1). The Chao1 richness index indicated that DCD 2.9 had a significantly lower richness (Chao1) than the DCD 10.5 and 17.5 sampling dates but was similar to DCD 6.4 and 27.9 ($p < 0.05$) (Table 3-1).

Within cover crop treatment, the only differences in average species richness over the decomposition period occurred in the hairy vetch treatment. The Chao1 richness index indicated that DCD 2.9 samples had a significantly lower richness than DCD 10.5, but not DCD 6.4, 17.5 or 27.9 ($p < 0.05$) (Figure A1).

#### 3.4.3.2 Soil microbial community β-diversity differed between cover crops during decomposition

PCoA of a weighted Unifrac distance matrix revealed a clear separation of communities by DCD along PC1 (29.00%) (Figure 3-2). The PermDISP test revealed that dispersion differences between the DCD groupings were significant ($p < 0.001$). The dispersion of the samples from DCD 2.9 was significantly different from that of DCD 6.4-27.9 and DCD 27.9 was different from DCD 10.5 ($p < 0.01$). A separation of microbial communities by DCD was also visible in the PCoA using a Bray Curtis distance matrix (data not shown). Comparing the rarefied sequences within each sampling date grouped by cover crop treatment, a PCoA of weighted Unifrac distance matrix

illustrated that communities did not statistically separate by cover crop treatment at DCD 2.9 ($p > 0.05$) (Figure 3-3), but there was a significant separation of communities at DCD 6.4-27.9 ($p < 0.05$). At DCD 10.5 (Figure 3-4) cover crop samples separated along PC1 (22.34%). PCoAs of DCDs 6.4, 17.5, and 27.9 can be found in the appendix (A.2-4). PermDISP tests revealed that there was not significant differences in dispersion among communities within each DCD ($p > 0.05$).

### 3.4.4 Predicted abundance of the soil β-glucosidase gene during the decomposition period

According to the predicted KEGG Orthology (KO) (Kanehisa et al., 2012) profile, the gene that encodes for soil β-glucosidase was relatively, but not statistically ($p > 0.05$), more abundant during DCDs 6.4, 10.5, and 17.5 when sequences were grouped by DCD (Figure A5). This predicted presence of the soil β-glucosidase gene followed the same trend of the quantified soil β-glucosidase activity during the decomposition period.

### 3.4.5 Influence of residue management system on soil microbial community structure

#### 3.4.5.1 Soil microbial community α-diversity was impacted by residue management treatment

Samples from the no tillage residue management treatment had significantly greater average species evenness (Simpson's) than the spring tillage treatment in the mixture cover crop ($p < 0.05$). (Figure A6). There was not a significant difference in average species richness (Chao1) between the residue management systems within cover crop treatments.

#### 3.4.5.2 Soil microbial community β-diversity was impacted by residue management treatment

A PERMANOVA test of a weighted Unifrac distance matrix with all samples from the decomposition period grouped by residue management treatment resulted in a significant effect of residue management treatment ($p < 0.05$) (Figure A7). However, a significant PermDISP test ($p < 0.05$) of the same distance matrix indicates a failure to meet the assumption of homogeneity

of variance, and therefore the PERMANOVA significance was likely caused by variation of the samples. Furthermore though, when grouping samples by DCD, there was a significant impact of residue management treatment at DCD 6.4 as samples separated along PC1 (28.86%) (Figure 3-5) ($p < 0.05$) and there was a trend for samples to separate on a PCoA by residue management treatment at DCD 10.5 (Figure A8), 17.5 and 27.9, but there was not a significant statistical separation between treatments ($p > 0.05$). There was not an impact of residue management treatment at DCD 2.9 ($p > 0.05$).

### 3.4.6 Transformations in the composition of the soil microbial community during the decomposition period

The LEfSe analysis revealed that there were more taxa with larger effect sizes associated with the hairy vetch treatment at DCD 2.9 compared to the other cover crop treatments (Figure 3-6). The taxa with the largest effect sizes in the hairy vetch treatment were *Rhodoplanes* and *Bacillus* (LDA score > 4.5). A shift occurred at DCD 6.4, and there were more taxa with larger effect sizes associated with the cereal rye treatment (Figure 3-7). This trend of more taxa with larger effect sizes in the cereal rye-based treatments continued through DCD 27.9. At DCD 6.4, *Agromyces* and Enterobacteriaceae were the most significant taxa in the cereal rye treatment (LDA score > 4.0) (Figure 3-7), and at DCD 10.5 Pseudomonadales was the most significant taxon, followed by Enterobacteriaceae (LDA score > 4.0) (Figure A9). At DCD 17.5, the most significant taxon was *Devosia* in the cereal rye treatment (LDA score > 4.5) (Figure A10). At DCD 27.9, the largest effects (LDA score > 5.0) were for *Solibacteres* in the hairy vetch treatment and Acidobacteria in the mixture treatment (Figure A11).

### 3.4.7 Canonical correspondence analysis illustrates that cover crop and DCD are significant factors contributing to the soil microbiome

This CCA model included the variables DCD, cover crop treatment, residue management system, soil nitrate, soil moisture, and soil β-glucosidase activity. Each of these variables had a variance inflation factor (VIF) < 1.95, indicating that the variables were not redundant with each other. The environmental metadata of soil organic matter, organic carbon, and ammonium where not significant variables ($p > 0.05$) and therefore not included in the model. The CCA indicated that the most variance of genus level observations was explained by measured variables and treatment effects ($p < 0.001$), with the first two components explaining 52% (CCA1) and 19% (CCA2) of the variance of the model (Figure 3-8). DCD had the largest relative importance among variables in the environmental matrix ($p < 0.001$), as illustrated by the length of the DCD arrow loading on CCA1. Soil nitrate level was associated with DCD as indicated by the narrow angle between the arrows. Cover crop treatment had the second largest relative importance in explaining variation in the microbial communities with the second longest arrow loading on CCA2, and cover crop treatment was associated with quantified soil β-glucosidase activity.

## 3.5 Discussion

This study demonstrated the effects of cover crop decomposition and residue management on the soil microbiome during the first year of implementing these agriculture management practices. The structure of the soil microbiome changed during the decomposition period, most notably, after 2.9 DCDs. This was evident through changes in microbial community α- and β-diversity, and these differences increased as the decomposition period progressed. Based on β-diversity analyses, soil microbial communities were significantly different based on cover crop treatment at DCDs 6.4-27.9, but there was not a significant statistical difference at

DCD 2.9. The variation of the soil microbiome structure at the beginning and end of the decomposition period was greater than the variation during peak cover crop decomposition. In addition, the soil microbial community was impacted by residue management treatment, and the impact was greatest at DCD 6.4 during peak cover crop decomposition. Finally, a CCA concluded that DCD, cover crop treatment, and residue management treatment were important variables in explaining the variance of the genus level observations.

The significant β-diversity PERMANOVA test of weighted Unifrac distance matrix with samples grouped by DCD revealed that DCD was the main factor driving soil microbiome structure. This was visually apparent in the PCoA, despite that the PCoA of the distance matrix did not follow the assumption of homogeneity of multivariate dispersion through a significant PermDISP test. The significant β-diversity PermDISP test of the weighted Unifrac PCoA with samples grouped by DCD has meaningful biological interpretation. Specifically, the pairwise PermDISP indicated that there was a significant difference in variation or spread among samples (dispersion) at DCD 2.9 compared to DCDs 6.4-27.9 and at DCD 27.9 compared to DCD 10.5. This led to the spread of microbiome samples in the PCoA (Figure 2A) (Wang et al., 2016). It is speculated that the greater variation among samples at the beginning of the decomposition period at DCD 2.9 was a result of weak competition among bacteria. However, as the decomposition period progressed leading up to DCD 27.9, the variation among communities within each DCD decreased, which could be a result of environmental filtering and increased competition and selection for the most fit microbiota. Because there are statistical differences in β-diversity between communities associated with cover crop treatments at DCD 6.4-27.9 ($p < 0.05$), but not at DCD 2.9 ($p > 0.05$), this suggests that DCD 2.9 was a successional transition stage for bacterial populations in our intensely managed agroecosystem. Prior to DCD 2.9, intensive

management practices applied to the cover cropped agroecosystem in synchronization with the beginning of cover crop decomposition included an herbicide application, an anhydrous ammonia application, planting of the cash crop, and a tillage application in the spring tillage residue management treatment plots. It is hypothesized that these management practices acted as stressors on the soil microbiome leading to an unstable community state observed at DCD 2.9 when visual spread among samples was greatest.

To explain the β-diversity patterns, taxa were identified that significantly differed in relative abundance based on cover crop treatment at each DCD. At DCD 2.9, the LEfSe analysis revealed that there were more taxa that differed significantly in the hairy vetch cover crop treatment compared to the other cover crop treatments; at this DCD the hairy vetch treatments were 20-33% decomposed. At DCD 2.9, OTUs from the order Rhizobiales (Proteobacteria) were identified as having a large effect size in the hairy vetch treatment. Of the 17 other taxa from DCD 2.9 associated with the cereal rye, mixture, and control treatments, none of them were from the Rhizobiales order. The identification of Rhizobiales as an indicator bacterial order during peak hairy vetch decomposition could be related to the mutualistic relationship between rhizobia and leguminous plants. In addition, a similar trend was observed with Bacillaceae (Firmicutes), when Bacillaceae (*Bacillus* and *Lysinibacillus*) were found to have a large effect (LDA > 4.0) in only the hairy vetch treatment at DCD 2.9. *Bacillus* has been found to depolymerize cellulose (Rastogi et al., 2010), and cellulase enzymes have been isolated from *Bacillus* strains (Yeasmin et al., 2011).

Starting at DCD 6.4, there were more taxa with larger effect sizes in the cereal rye-based treatments (cereal rye and mixture) at every DCD, and more total biomass had been decomposed in the cereal rye and mixture treatment compared to the hairy vetch at this time. It was

hypothesized that the change in microbial communities was related to the cover crop decomposition, and PICRUSt predicted that the taxa present had β-glucosidase genes. It was predicted that more β-glucosidase genes were present at DCD 6.4 than DCD 2.9. At DCD 6.4, *Agromyces* (Actinobacteria) had the largest impact (LDA score > 4.0) in differentiating cover crop treatments with a higher relative abundance in the cereal rye treatment. *Agromyces* reoccurred as a genus that had a significant impact in differentiating the cereal rye treatment from the other cover crops (LDA score > 4.0) at DCD 10.5 and 27.9, and had the longest bar, indicating the largest effect, at DCD 27.9 compared to any other taxa. The *Agromyces* association with the cereal rye treatment is likely a result of increased cover crop decomposition in cereal rye plots at DCD 6.4 as *Agromyces* has been found to respond to isotopically labeled cellulose (Pepe-Rainey et al., 2016). *Enterobacteriaceae* (Proteobacteria) had the second largest impact in differentiating cover crop treatments at DCD 6.4. Species in the *Enterobacteriaceae* family are proficient in lignocellulosic degradation (DeAngelis et al., 2010). Also having a large effect in differentiating the treatments at DCD 6.4, Burkholderiales (*Polaromonas* and *Achromobacter*) were associated with the cereal rye treatment. *Achromobacter* has been identified as having β-glucosidase activity (Yang et al., 2011; Talia et al., 2012) and to be in cellulolytic environments (Lednická et al., 2000). One commonality between DCD 2.9 and 6.4 was the identification of *Bradyrhizobium* as having a significant impact in differentiating the hairy vetch treatment from the other cover crops. This indicates that these bacteria remain highly abundant in soil of the leguminous cover crop treatment through much of leguminous cover crop decomposition. Overall, it was hypothesized that the change in microbial community composition was related to cover crop decomposition. Then, it was predicted if the taxa present during the decomposition period had the β-glucosidase gene.

There were several uncultured, candidate bacteria identified to be indicator bacteria associated with different cover crop treatments at different time points in the decomposition period. This speaks to the importance of developing a better understanding of the abundance patterns of rare bacteria in agroecosystems. These bacteria, though they may not be central in microbial community networks, may play a key role in the maintenance of ecosystem diversity (Jiao et al., 2017).

The significant PermDISP test revealing a failure to meet the assumption of homogeneity of variance resulted in a rejection of the significant PERMANOA test when all samples from the decomposition period were grouped by residue management. When viewing the PCoA, there was not a visual separation of samples, and therefore the significant PERMANOVA result was likely an effect of the variation among samples in each treatment. However, residue management treatment did have a significant impact on the microbial community β-diversity at DCD 6.4 ($p < 0.05$), but not at other DCDs. This finding confirms the results of other researchers (Degrune et al., 2016; Sengupta and Dick, 2015; Quadros et al, 2012; and Carbonetto et al., 2014). Some studies, without organic matter amendments, have revealed that the effect of tillage on the microbiome structure decreases with time from tillage application as the microbiome naturally recovers from the mechanical tillage disturbance (Degrune et al., 2016). In our study, at DCD 6.4 between 65-70% of the total cover crop biomass available at termination had been released from the litter bags in the spring tillage treatments compared to 40-45% in the no tillage. This indicates that there is an interaction between cover crop decomposition and residue management treatment on the soil microbiome, which has yet to be documented in the literature. Our results also revealed that there was significantly greater average species evenness (Simpson's) in the no tillage system than in the more disturbed spring tillage system in the mixture treatments, but not

in the cereal rye, hairy vetch, and control treatments. This greater average species evenness indicates that the decomposing cereal rye and hairy vetch residue combined to form the cover crop mixture in the no-tillage treatment is selecting for a microbial community with more even distribution among taxa. This aligns with other studies which observed that there is high microbial community α-diversity under less perturbed tillage regimes (Sengupta and Dick, 2015; Quadros et al, 2012; Navarro-Noya et al., 2013).

Here, the transitions in the soil microbiome during the cover crop decomposition period were explored, which revealed a succession of different microbial groups associated with different cover crop treatments. Our data suggest that the composition of the soil microbiome is most impacted by the stage of cover crop decomposition during the cover crop decomposition period. Cover crop species was the second most important variable in explaining the genus level observations. Future studies should investigate how the soil microbiome of different cover crop species influence the root microbiome of cash crops. The initial composition of the bulk soil microbiome determines which microbes are recruited for the cash crop root microbiome early in the cash crop life cycle (Lundberg et al., 2012; Bulgarelli et al., 2012; Haney et al., 2015). Overall, this study highlights the importance of cover crop species on the composition of the soil microbiome during the decomposition period, which is of great interest to agriculturalists implementing sustainable management practices such as cover cropping and residue management practices.

## 3.6 References


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Table 3-1. Average α-diversity of samples during the decomposition period averaged across all cover crop treatments and residue management treatments. Different letters indicate a significant difference between DCDs within an α-diversity metric. ($p < 0.05$).

| | Shannon Diversity | | Simpson's Evenness | | Chao1 Richness | |
|---|---|---|---|---|---|---|
| DCD 2.9 | 11.27 | b | 0.056 | b | 28144 | b |
| DCD 6.4 | 11.51 | a | 0.067 | ab | 29279 | b |
| DCD 10.5 | 11.61 | a | 0.067 | b | 38209 | a |
| DCD 17.5 | 11.51 | a | 0.064 | b | 35487 | a |
| DCD 27.9 | 11.47 | a | 0.074 | a | 28381 | b |

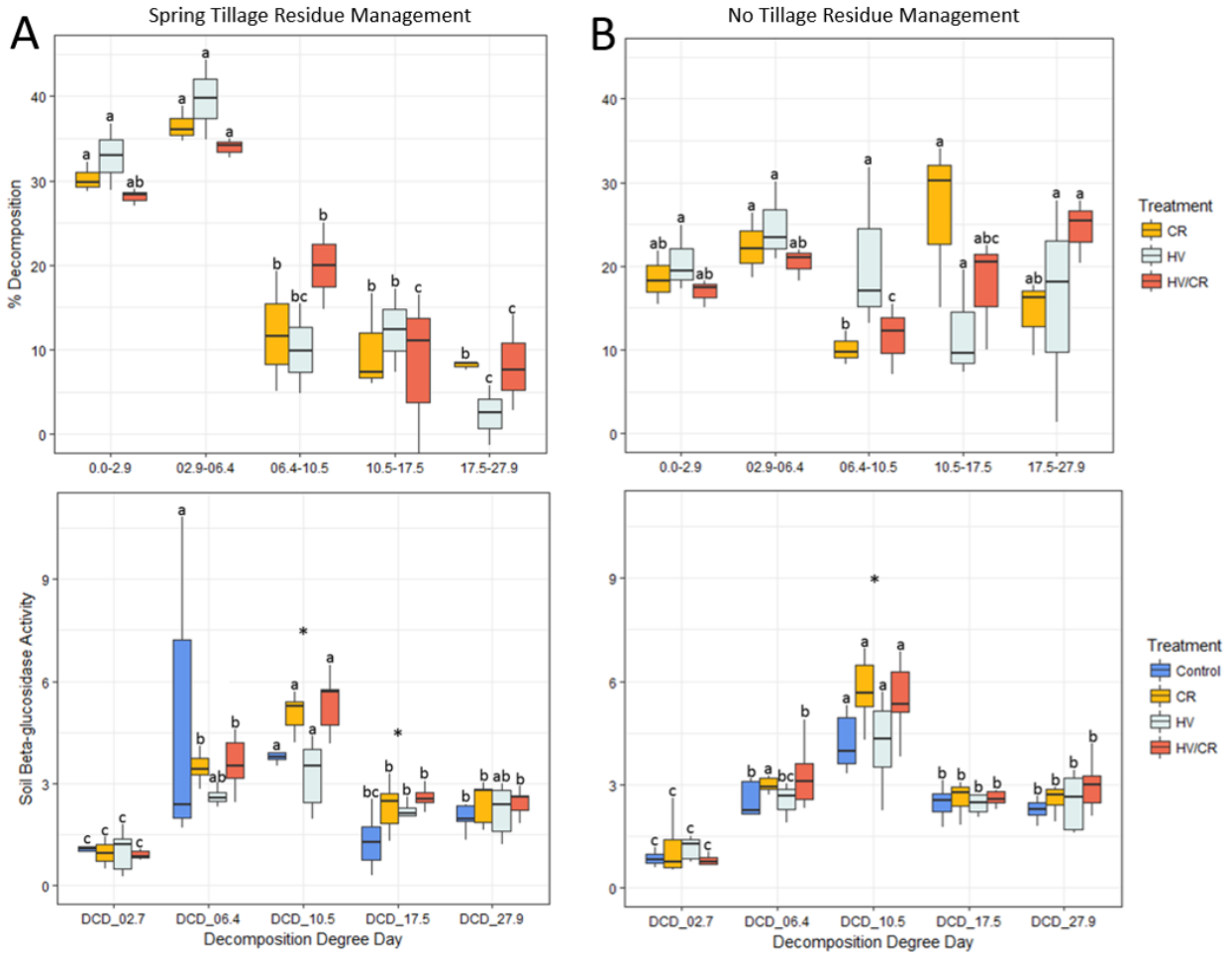


Figure 3-1A. Percent of total cover crop carbon released during the decomposition period (above) and quantification of β-glucosidase enzymatic activities by PNG-substrate (µmole of *para*-nitrophenol released g dry soil $^{-1}$ hour $^{-1}$ ) during the cover crop decomposition period (below) in spring tillage treatments. Within sampling dates, the CR and HV/CR had significantly greater β-glucosidase activity than the HV and Control at DCD 10.5 (*). At DCD 17.5, the CR and HV/CR had significantly greater β-glucosidase activity than the Control (*).

Figure 3-1B. Percent of total cover crop carbon released during the decomposition period (above) and quantification of β-glucosidase enzymatic activities by PNG-substrate (µmole of *para*-nitrophenol released g dry soil $^{-1}$ hour $^{-1}$) during the cover crop decomposition period (below) in no-tillage treatments. At DCD 10.5, the CR and HV/CR treatments were significantly higher than the HV and Control (*) ($p < 0.05$).

The *error bars* indicate the standard deviations and *letters* correspond to differences within cover crop treatments across decomposition degree days ($p < 0.05$). (CR=Cereal Rye, HV=Hairy Vetch, HV/CR=Mixture)

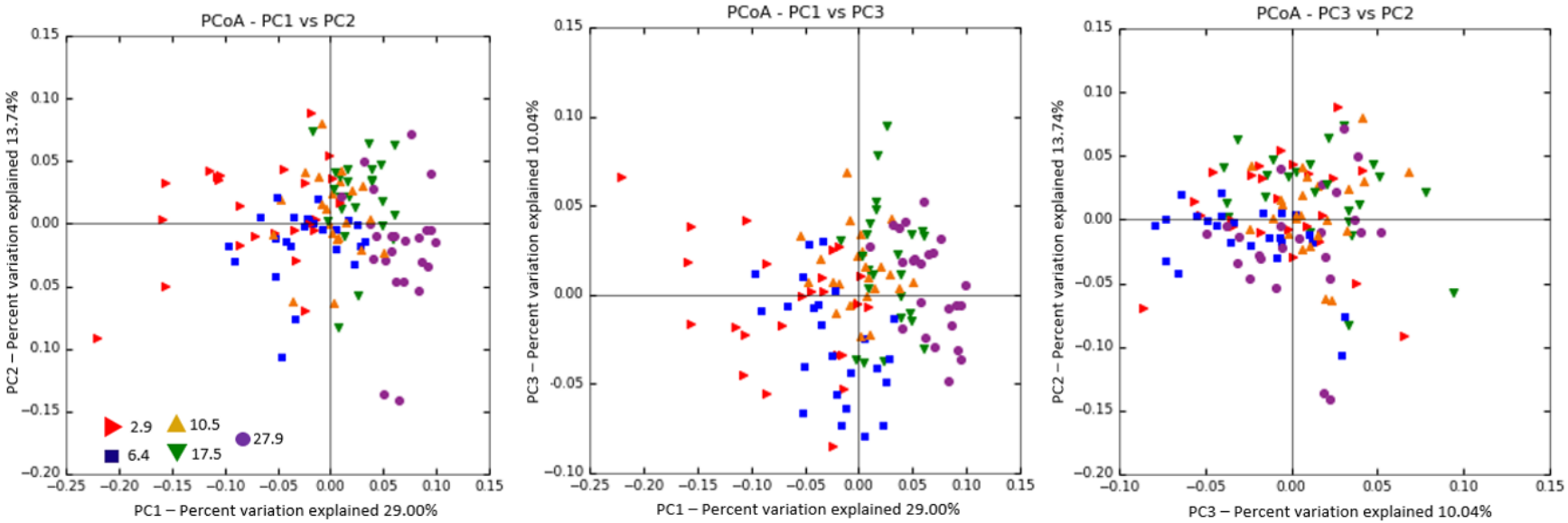


Figure 3-2. Weighted Unifrac distance matrix revealed differences between samples separated by DCD determined using PERMANOVA ($p < 0.001$). The PermDISP was significant ($p < 0.001$).

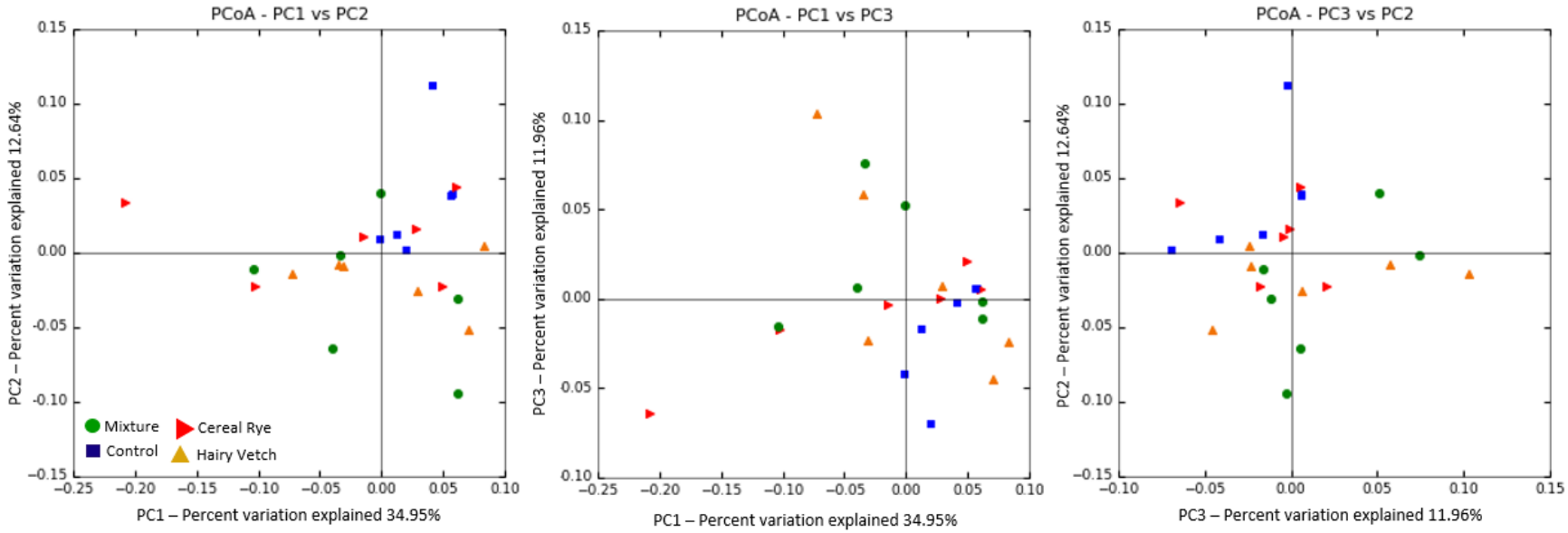


Figure 3-3. Weighted Unifrac distance matrix did not reveal a significant difference between samples grouped by cover crop treatment at DCD 2.9. Differences were determined using PERMANOVA ($p > 0.05$). The PermDISP was NS.

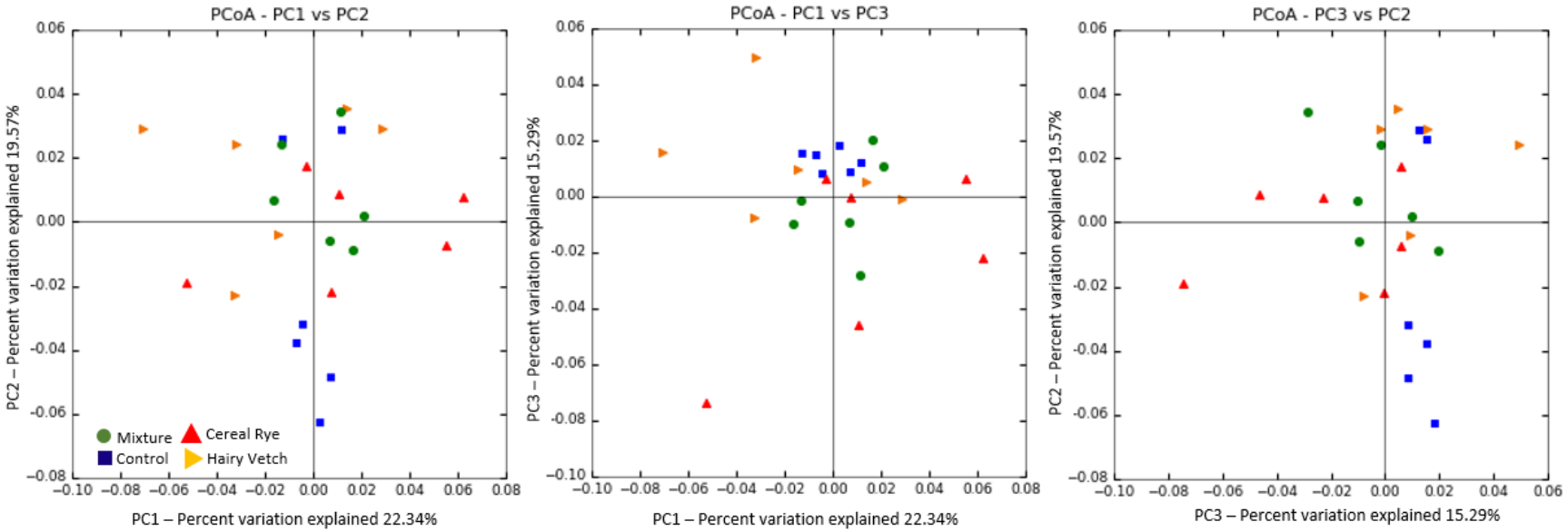


Figure 3-4. Weighted Unifrac distance matrix revealed differences between samples when grouped by cover crop treatment at DCD 10.5. Differences were determined using PERMANOVA ($p < 0.05$). The PermDISP was NS.

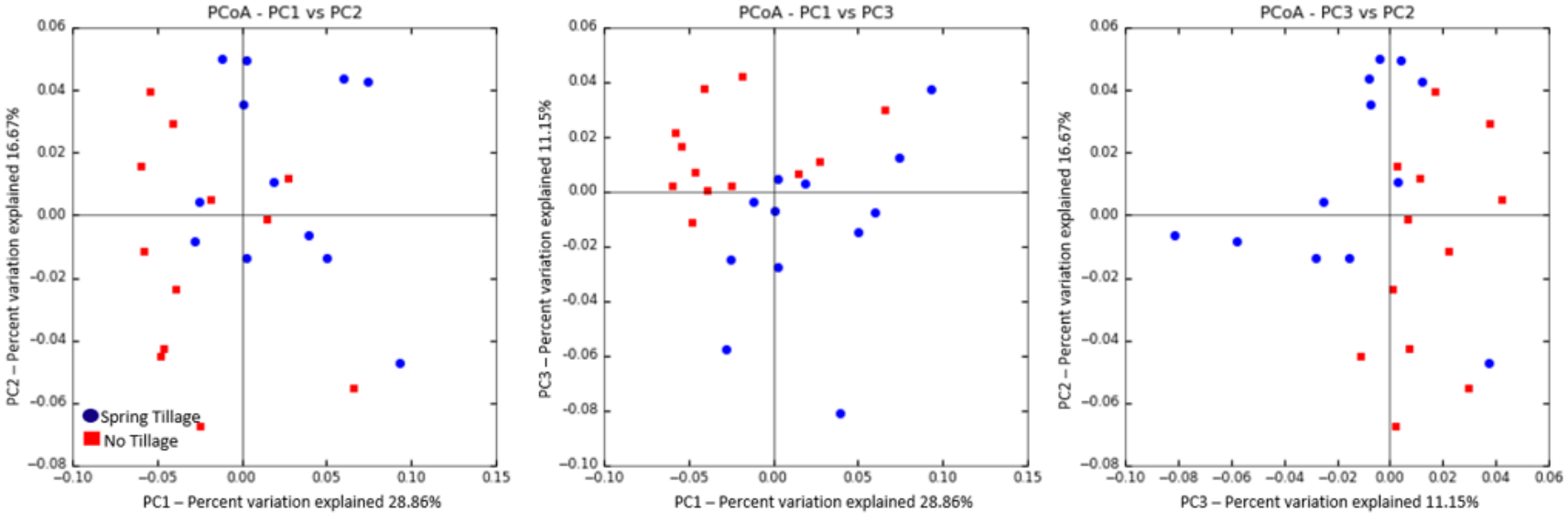


Figure 3-5. Weighted Unifrac distance matrix revealed differences between samples grouped by residue management treatment at DCD 6.4. Differences were determined using PERMANOVA ($p < 0.05$). The PermDISP was NS.

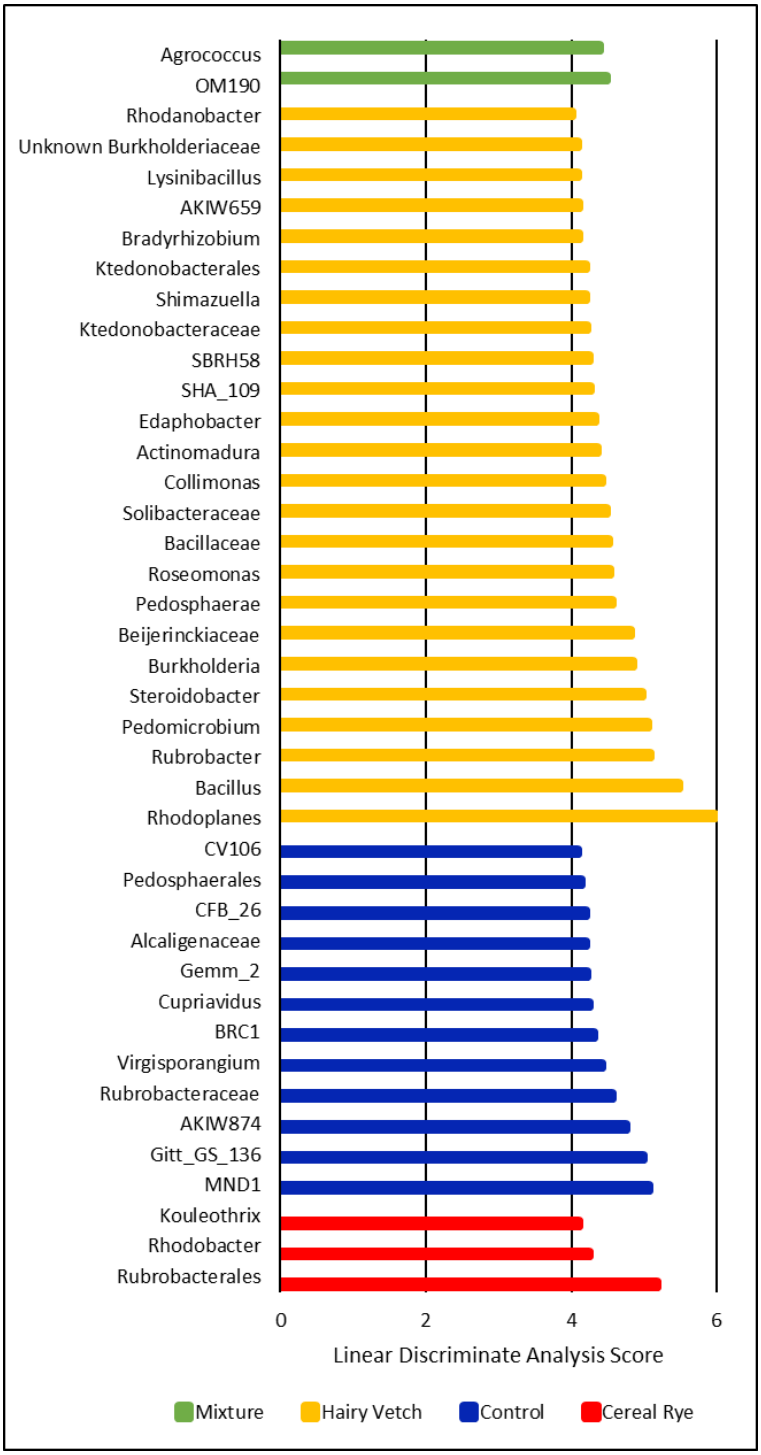


Figure 3-6. Histogram of the results of Linear Discriminate Analysis Effect Size (LEfSe) analysis of cover crop differences at DCD 2.7. Longer histogram bars indicate a larger impact of a certain taxa to the difference between the four cover treatments. P-value < 0.05 considered significant.

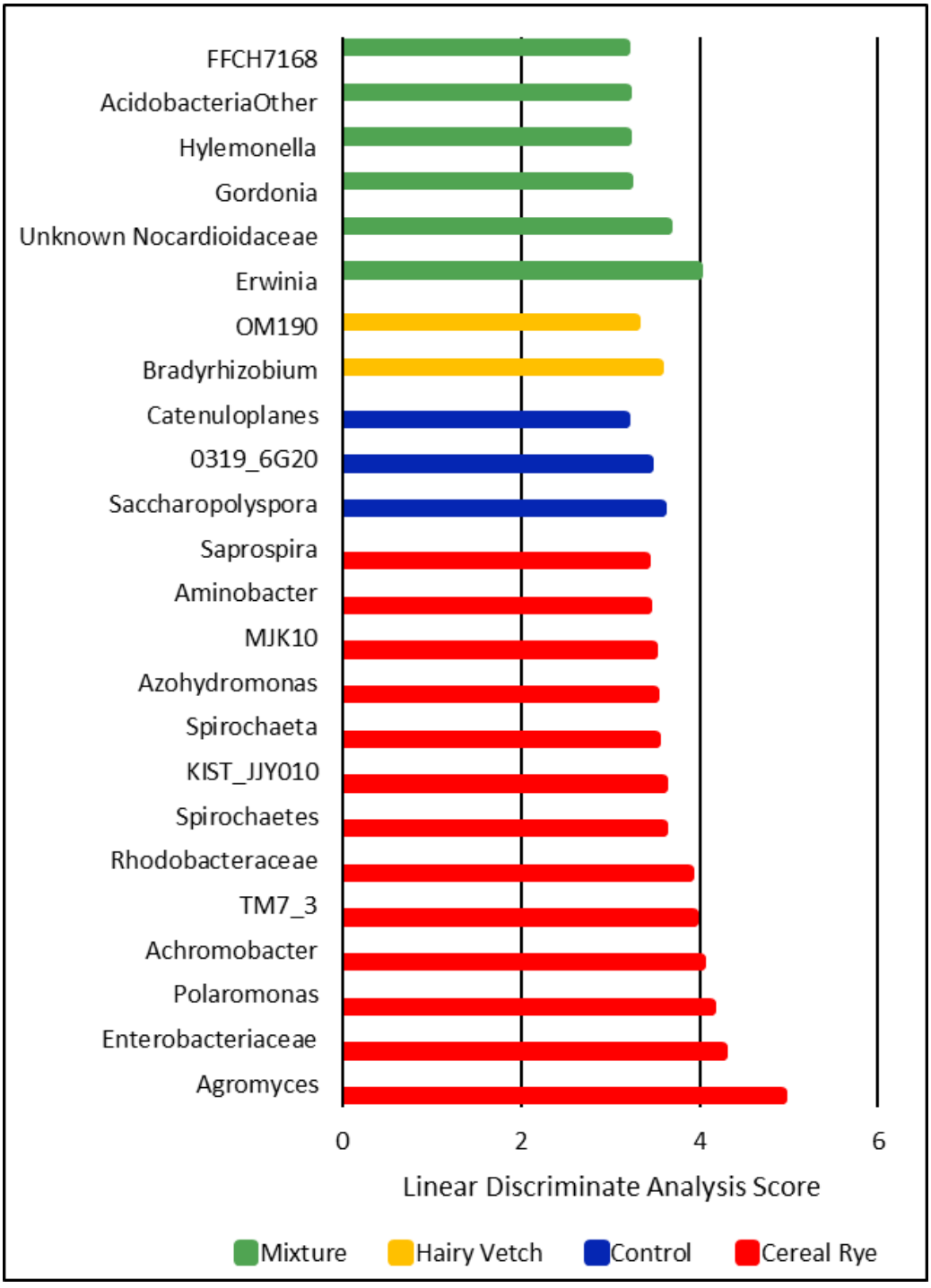


Figure 3-7. Histogram of the results of Linear Discriminate Analysis Effect Size (LEfSe) of cover crop differences at DCD 6.4. Longer histogram bars indicate a larger impact of a certain taxa to the difference between the four cover treatments. P-value < 0.05 considered significant.

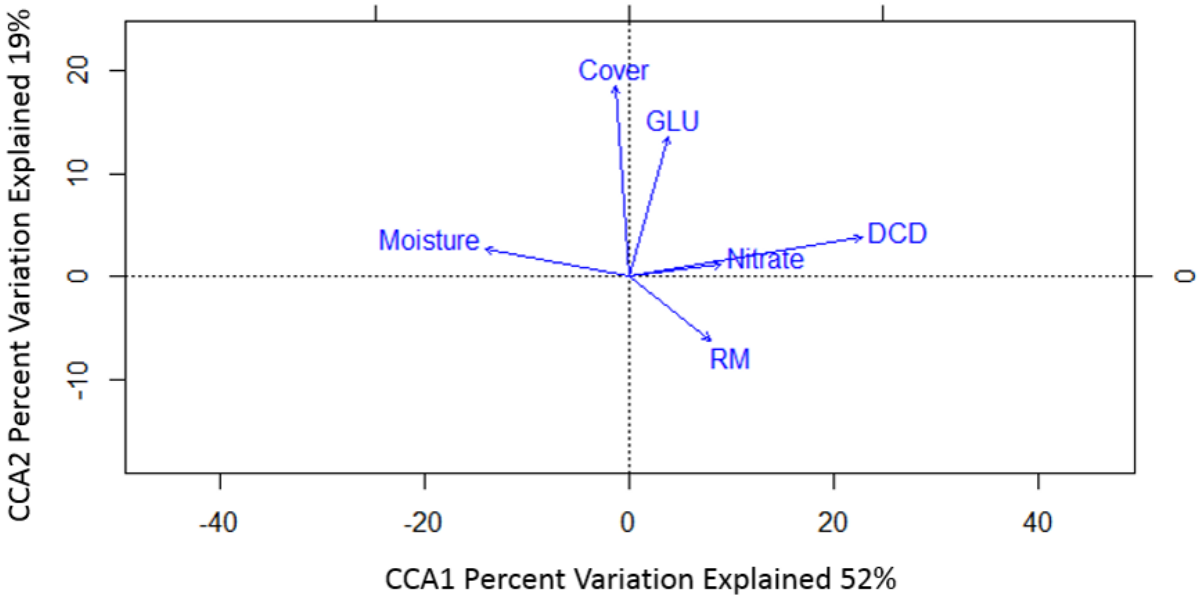


Figure 3-8. Canonical correspondence analysis performed with microbiome relative abundances as the species matrix and the environmental variables of DCD, nitrate, soil moisture, β-glucosidase activity, cover crop treatment, and residue management system. The arrows in the biplot represent the direction of the environmental gradient and the relative length of the arrow indicates the importance of the variable to the model. The angles between arrows indicate the relationship between the environmental variables. Overall $p < 0.001$. Axis 1 and 2 explain 52 and 19% of the total constrained variation. (Cover=Cover crop treatments, GLU=soil β-glucosidase activity, DCD=Decomposition degree day, Nitrate=Soil nitrate, RM=Residue Management Treatment, Moisture=Soil moisture)

# CHAPTER 4. CONCLUSION

## 4.1 Overall Summary

The soil microbial community is often referred to as the "black box" that regulates the decomposition of cover crops and the nutrient cycling in agroecosystems. As cover cropping in the Midwest Corn Belt increases, it is vital to understand nutrient release dynamics, and therefore the soil microbial community dynamics, in cover cropped agroecosystems for the farming community.

In the introduction in Chapter 1, a summary of cover crop benefits, risks, and barriers to adoption were discussed. Though it is known that some cover crops have the ability to scavenge or biologically fix nitrogen prior to cover crop termination, there is a lack of consensus surrounding when, or if, that nitrogen is returned to the soil in an available form for the corn cash crop. Next, the introduction discussed the different nitrogen and carbon cycling transformations that occur in soil systems. This led to a discussion on the enzymatic reactions that drive these transformations, and briefly, the extent to which the activity of these enzymes have been studied in sustainable agroecosystems with this topic discussed heavily in sections 2.4.3 - 2.4.5. Finally, Chapter 1 concludes with a summary of the current knowledge on the soil and litter microbiome, and their interaction with the decomposition of organic material.

In Chapter 2, effort focused on determining the impact of different cover crop species and reduced tillage on soil inorganic nitrogen availability and enzyme activity during the cover crop decomposition period. Notably, responses to treatments were evident in 2016, which was the first year after the transition to a no-tillage and cover cropped agroecosystem. Specifically, litter enzyme activity decreased over the decomposition period in all treatment combinations for β-glucosidase in both years of the study. Litter urease activity fluctuated more than litter β-

glucosidase activity. In 2016, cereal rye activity peaked at decomposition degree day (DCD) 27.9 while the hairy vetch activity did not fluctuate over the decomposition period. The mixture treatment peaked at DCD 17.5 and remained elevated until DCD 40.8 at corn maturity. In 2017, litter urease activity was highest in the spring tillage treatments at each DCD compared to the no tillage treatment, and activity continually decreased over the decomposition period in all treatments. There was a significant increase in soil β-glucosidase activity in both decomposition periods in all treatments. This increase was related to an increase in soil temperature. In 2016, peak β-glucosidase activity and available cover crop biomass was nearly double that measured in 2017. In 2016, β-glucosidase activity peaked began on DCD 6.4, and in 2017 peak β-glucosidase activity began on DCD 4.5. In both years, peak inorganic N occurred in all treatments within 7 DCD from peak β-glucosidase activity.

Given the results from Chapter 2, which described a fluctuation in microbial community function in our soil systems, Chapter 3 sought to investigate if there was a change in the soil microbial community structure and composition. This study concluded that the structure of the soil microbiome changed during the decomposition period, most notably after DCD 2.9 when a majority of the cover crop decomposition started to occurred. In addition, cover crop decomposition decreased the variation among soil microbial community samples. Furthermore, residue management treatment significantly impacted the microbiome at DCD 6.4 during peak cover crop decomposition. Lastly, a canonical correspondence analysis revealed that DCD, cover crop species, and residue management treatment were important variables in explaining the variance of genus level observations during the decomposition period.

## 4.2 Limitations of Research

The above discussion summarized the knowledge gained from this research, but it is also beneficial to reflect on the limitations of the completed research when finishing the scientific process. While there were many advances in understanding from this project, weaknesses in this research became apparent as the study progress.

A primary weakness in this study is the lack of observation of fungal community dynamics during the decomposition period. It is known that the fungal community is highly involved in decomposition of organic materials. Unfortunately, funding and time constraints resulted in Chapter 3 of this study including primers only designed for bacteria. Along similar lines, it would have been beneficial to replicate Chapter 3 of this study in 2017 to confirm observations from 2016.

Though beneficial, field scale research has limitations. In this study, temperature and precipitation, major drivers of decomposition rates in combination with biotic factors, were attempted to be normalized for by calculating for DCDs that corresponded to sampling dates. In a greenhouse study, temperature and precipitation could be controlled.

Lastly, sampling during both years of the study was conducted based on corn growth stages. Due to large precipitation events in 2017, our corn cash crop was planted nearly a month later than in 2016. This resulted in corn reaching growth stages at drastically different times during the summer of 2017 compared to 2016. To precisely compared sampling dates between growing seasons, samples should have been collected based on decomposition degree days accumulated each year from time of termination, not corn growth stage.

## 4.3 Future Research Suggestions

After this study, there are many avenues for future research to better understand the microbiome of different cover crop species and reduced tillage residue management treatments. Specifically, there are a few key areas of research that could greatly aid in advancing our understanding of cover crop microbiomes.

Though there is immense value in field scale studies in production agriculture research, much of the conclusions derived from this research could be built upon in a study that utilized a more controlled environment, such as a greenhouse. Therefore, different scenarios should be studied in a controlled setting. First, controlling for precipitation and temperature would allow for controlled accumulation of decomposition degree days. This would result in a more precise tracking of inorganic N levels and enzyme activity as decomposition degree days accumulate during the decomposition period. I predict that after several scenarios, it is possible that timing of inorganic N release based on decomposition degree days could be determined for different cover crop species.

Building off the greenhouse study detailed above, DNA-stable isotope probing (DNA-SIP) could be used to gain a better understanding of the bacterial community responsible for decomposing different cover crop litters at different time points during the decomposition period. Incorporating the stable isotope $^{13}C$ into cover crop biomass during cover crop growth, this study would allow for determination of bacterial community members enriched with $^{13}C$, at different times in the decomposition period. This would provide insight into which members of the community are "active" decomposers of the cover crop, versus the current study that allowed for determination of all bacteria in the soil samples, through 16S rRNA gene sequencing.

Another potential area of exploration is the microbiome of cover crop litter, not the bulk soil, as the litter is decomposing. I hypothesize that there is a lower average species richness and

higher average species evenness in the microbial communities that are found on decomposing cover crop litter compared to the communities in the bulk soil surrounding decomposing cover crop litter. This would provide insight into the microbial communities solely responsible for the decomposition of cover crop residue, which have been selected for from the bulk soil based on litter type.

Finally, it is known that plants recruit a root microbiome early in their life cycle, and that this recruited microbiome is largely determined by the composition of the microbiome of the surrounding medium or bulk soil. From this research, it is known that different cover crops support a different soil microbiome during the decomposition period. Therefore, as cover crops are decomposing during corn germination and early vegetative growth stages, the microbiomes of different cover crop species could be impacting the initial microbiome of the corn rhizosphere. A study should be designed that examines the bulk soil cover crop microbiome in conjunction with the corn rhizosphere microbiome during the decomposition period, specifically, frequent sampling intervals from corn planting to corn early vegetative growth stages.

# APPENDIX

## Supplementary Figures

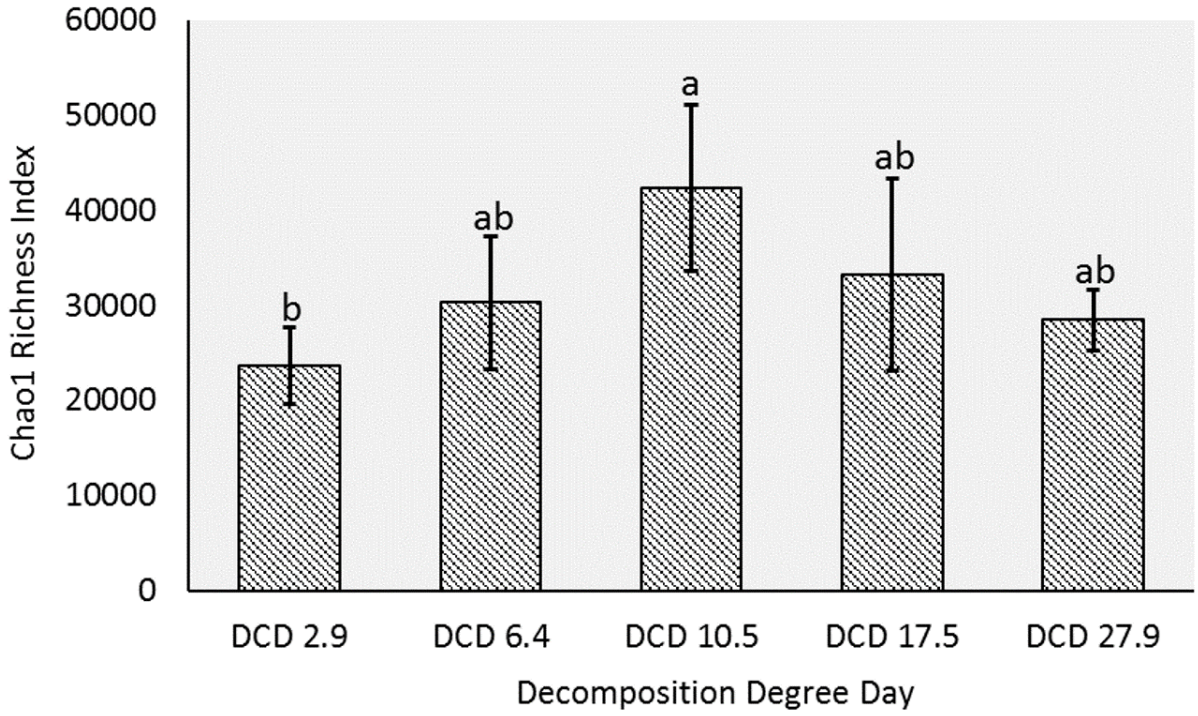


Figure A.1. Chao 1 richness index revealed that the hairy vetch samples from DCD 2.9 had a significantly lower richness than hairy vetch samples from DCD 10.5, but not DCDs 6.4, 17.5, or 27.9 ($p < 0.05$). The *error bars* indicate the standard deviations and *letters* correspond to differences across decomposition degree days.

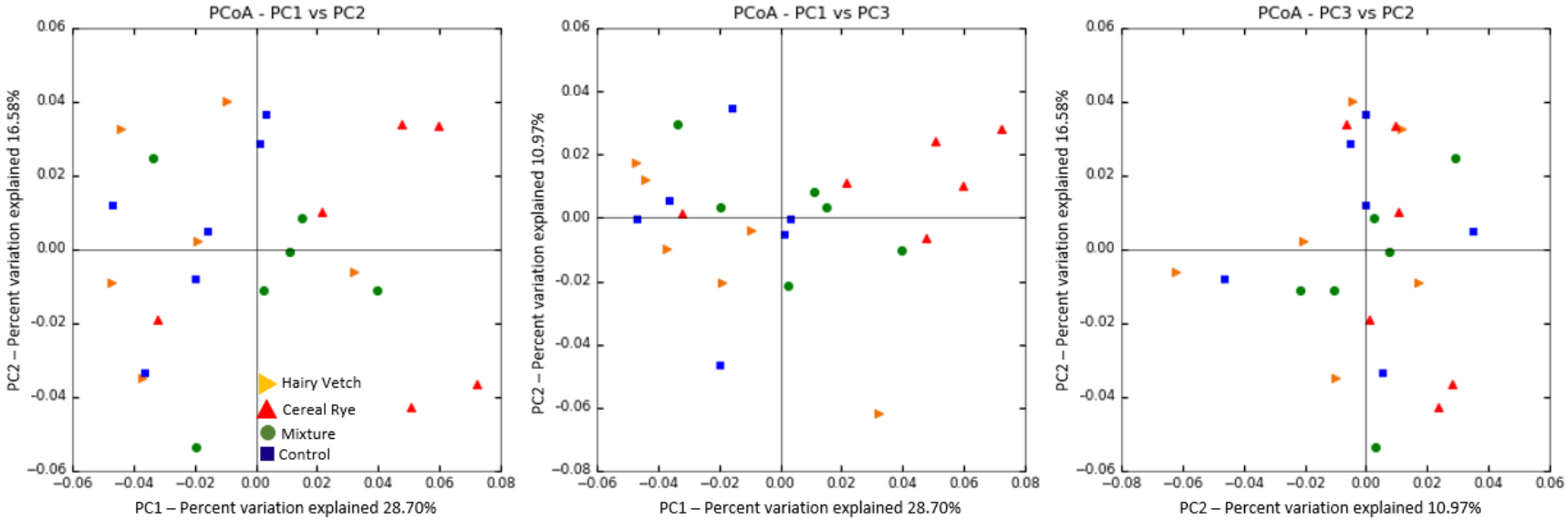


Figure A.2. β-diversity differences (weighted Unifrac) visualized with a PCoA between samples separated by cover crop treatment at DCD 6.4. Differences were determined using PERMANOVA ($p < 0.05$). The PermDISP was NS.

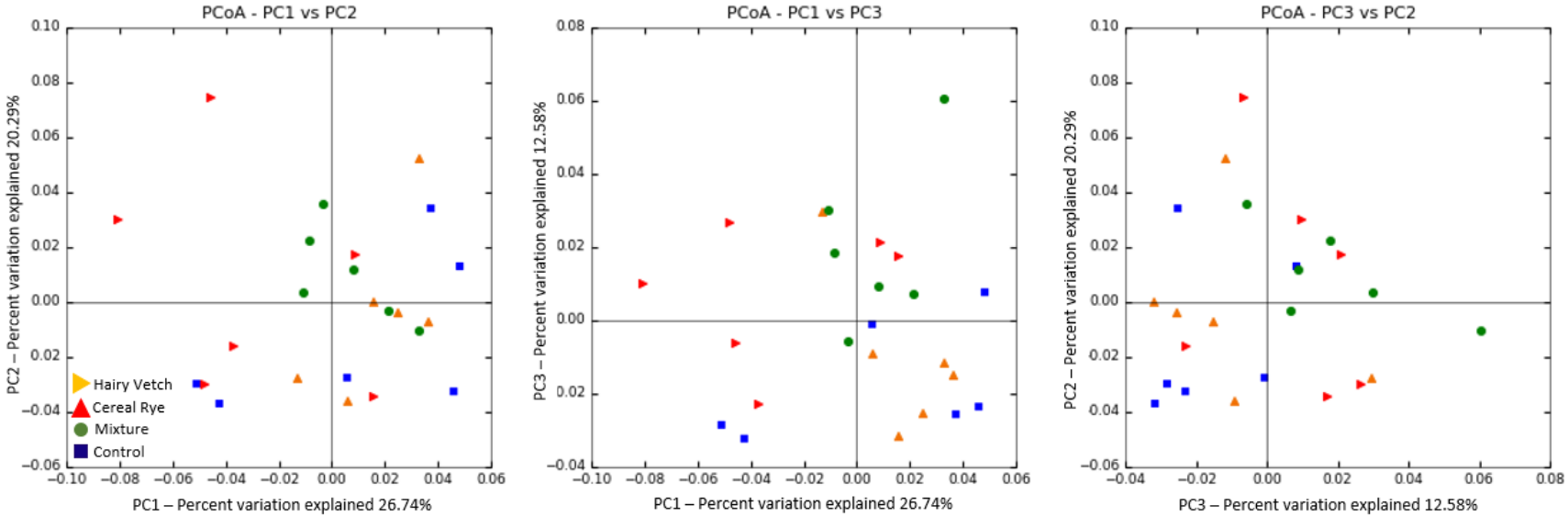


Figure A.3. β-diversity differences (weighted Unifrac) visualized with a PCoA between samples separated by cover crop treatment at DCD 17.5. Differences were determined using PERMANOVA ($p < 0.05$). The PermDISP was NS.

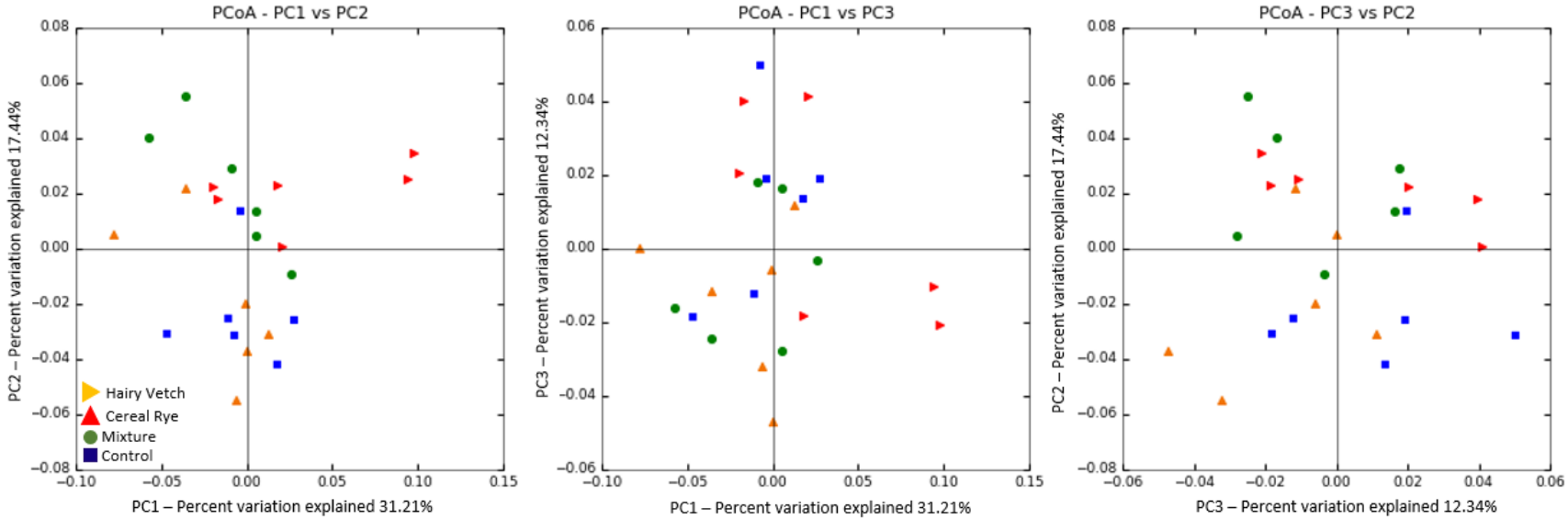


Figure A.4. β-diversity differences (weighted Unifrac) visualized with a PCoA between samples separated by cover crop treatment at DCD 27.9. Differences were determined using PERMANOVA ($p < 0.05$). The PermDISP was NS.

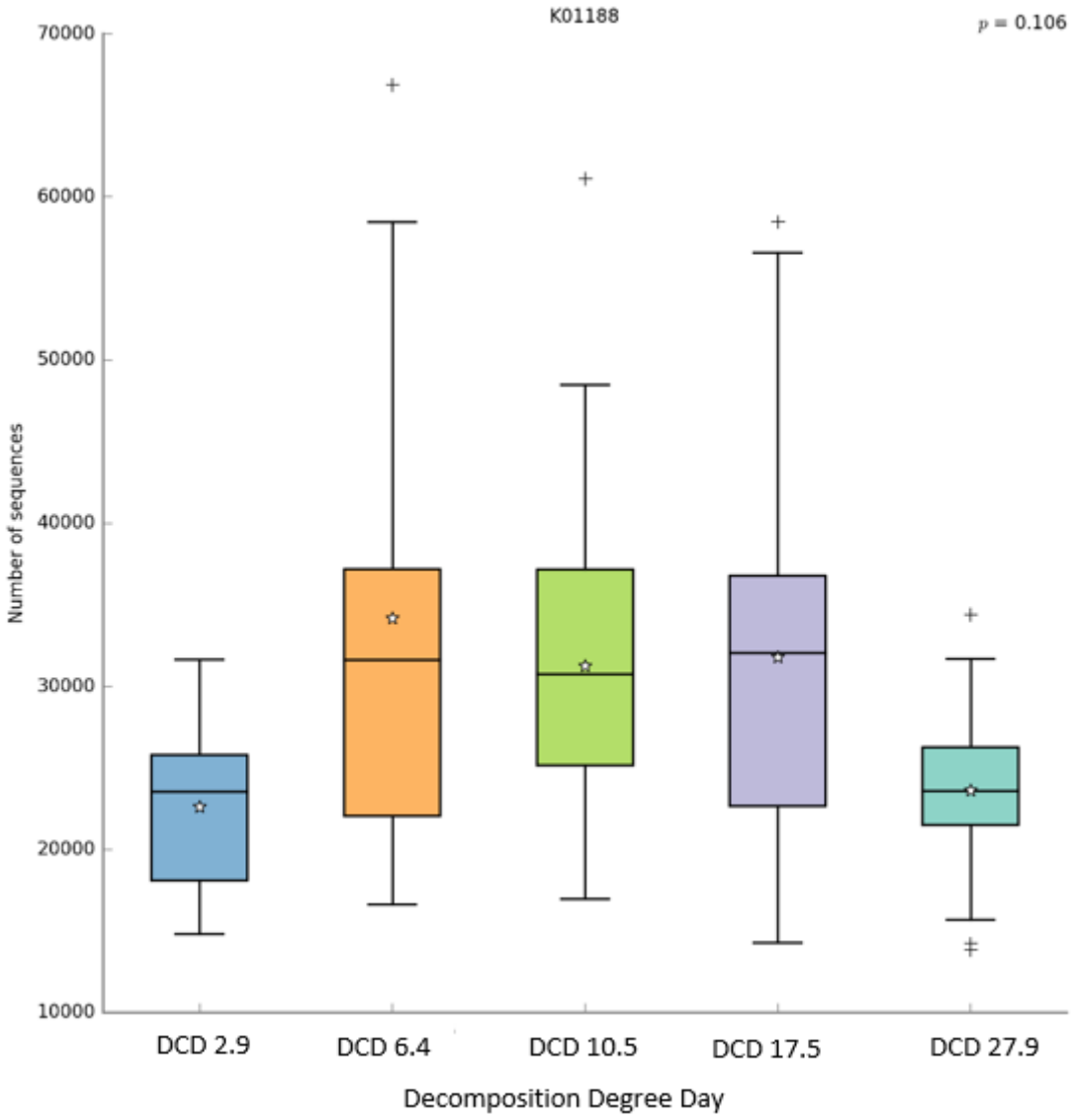


Figure A.5. Predicted KO profile of the soil β-glucosidase gene during the decomposition period ($p > 0.05$). The *error bars* indicate standard deviations.

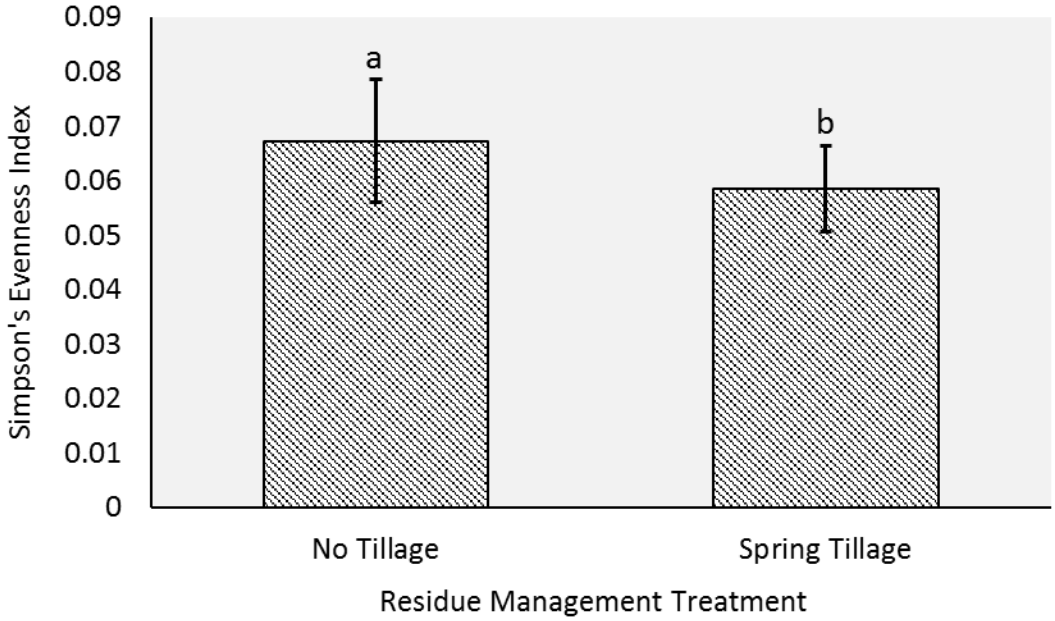


Figure A.6. Samples from the no tillage residue management treatment had significantly greater average species evenness than the spring tillage treatment in the in the mixture treatment ($p < 0.05$). The *error bars* indicate the standard deviations and *letters* correspond to differences between residue management treatments.

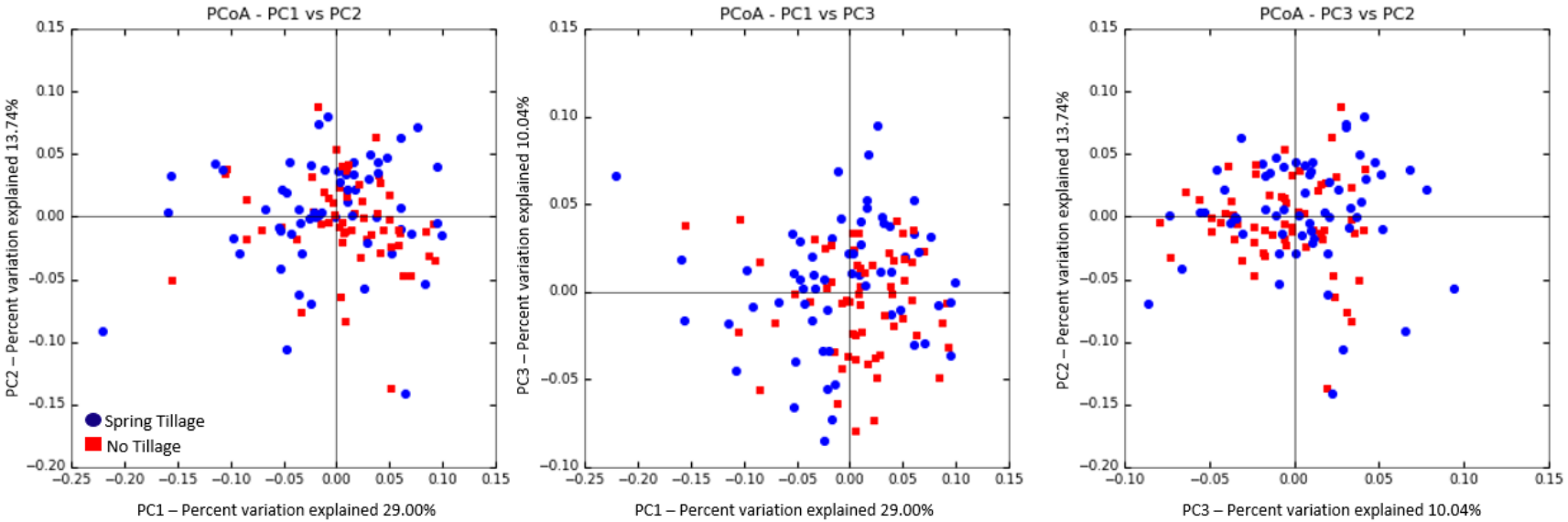


Figure A.7. A PERMANOVA test of a weighted Unifrac distance matrix with all samples from the decomposition period grouped by residue management treatment resulted in a significant effect of residue management treatment ($p < 0.05$). A significant PermDISP test ($p < 0.05$) of the same distance matrix indicates a failure to meet the assumption of homogeneity of variance, and therefore the PERMANOVA significance was likely caused by variation of the samples.

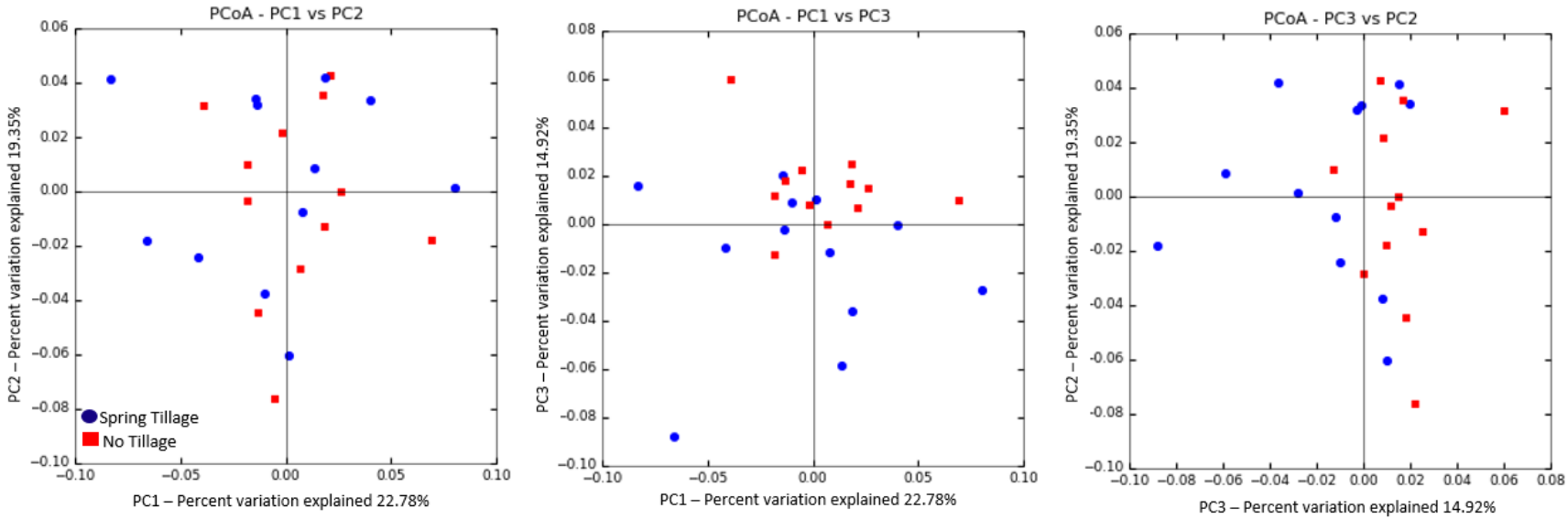


Figure A.8. β-diversity differences (weighted Unifrac) visualized with a PCoA did not separate by residue management treatment at DCD 10.5. Differences were determined using PERMANOVA ($p > 0.05$). The PermDISP was NS.

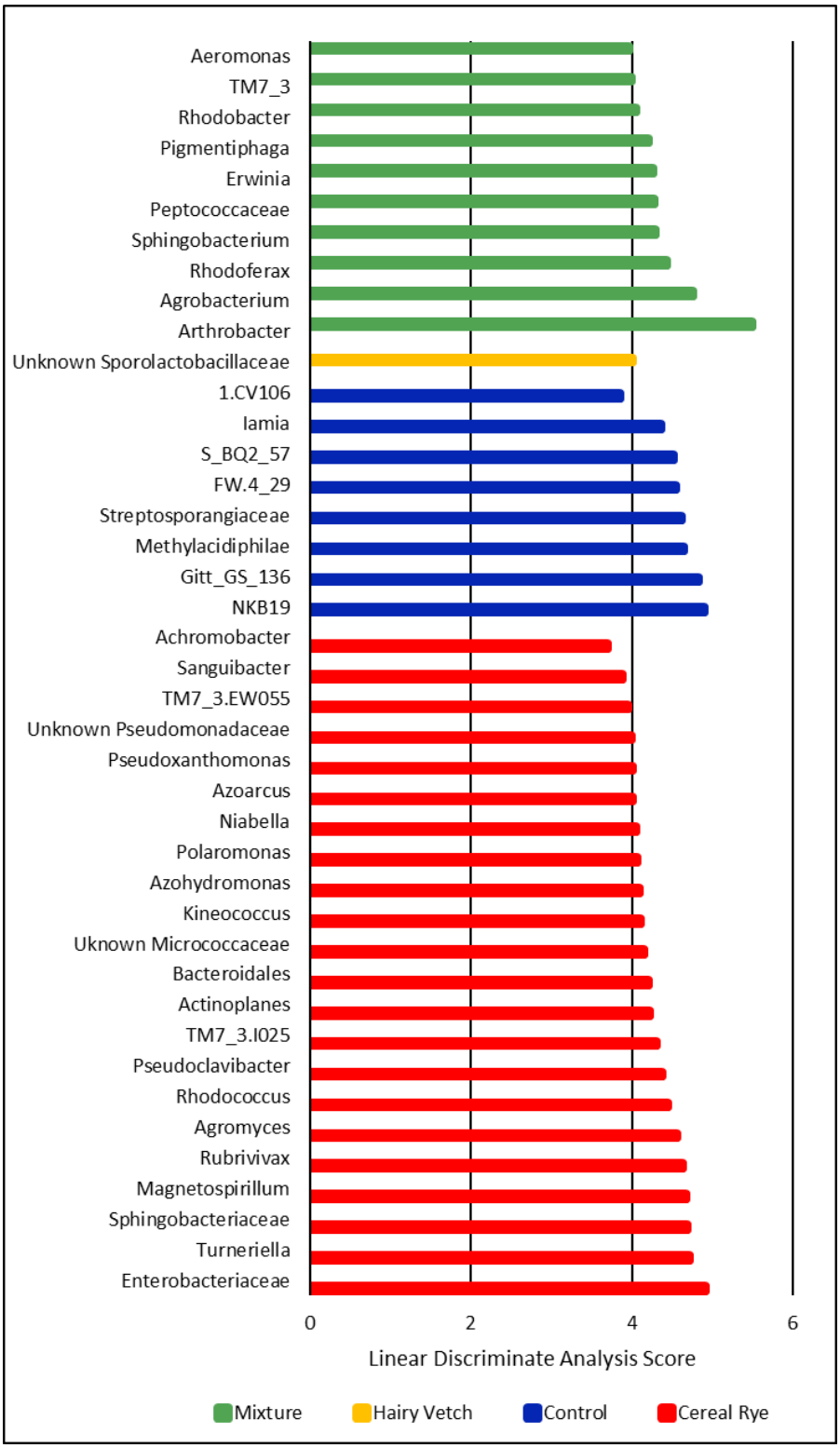


Figure A.9. Histogram of the results of Linear Discriminate Analysis Effect Size (LEfSe) of cover crop differences at DCD 10.5. Longer histogram bars indicate a larger impact of a certain taxa to the difference between the four cover treatments. P-value < 0.05 considered significant.

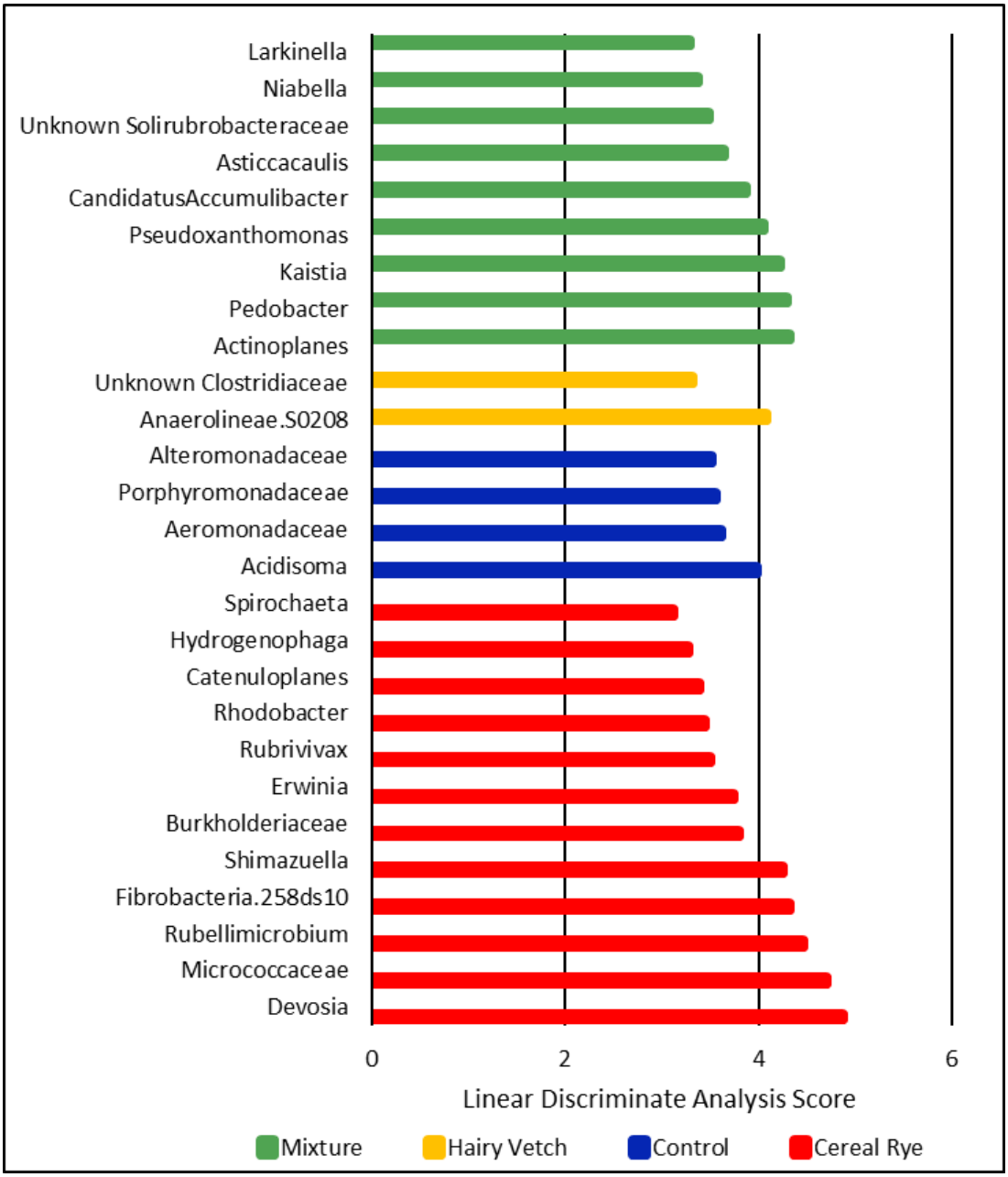


Figure A.10. Histogram of the results of Linear Discriminate Analysis Effect Size (LEfSe) of cover crop differences at DCD 17.5. Longer histogram bars indicate a larger impact of a certain taxa to the difference between the four cover treatments. P-value < 0.05 considered significant.

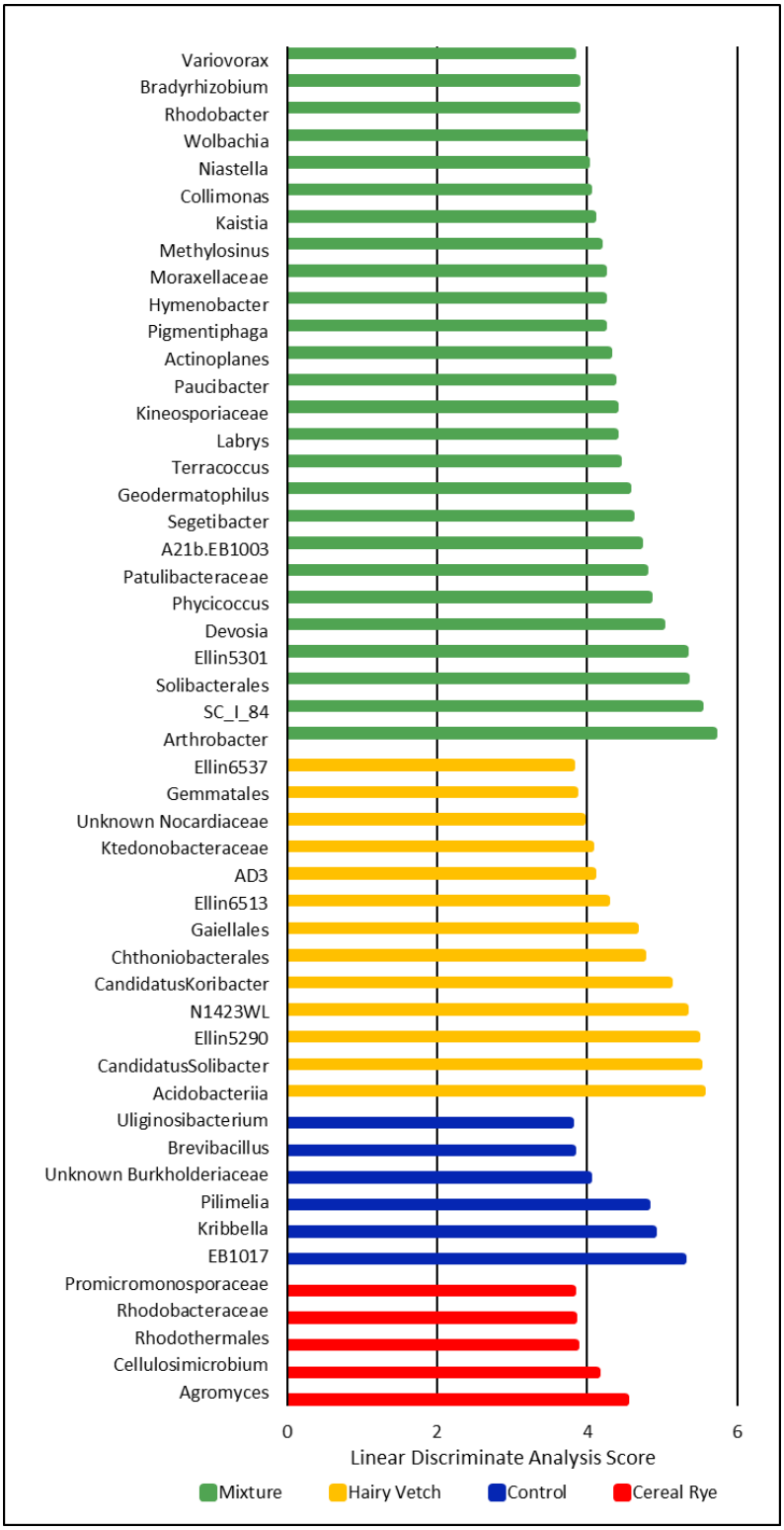


Figure A.11. Histogram of the results of Linear Discriminate Analysis Effect Size (LEfSe) of cover crop differences at DCD 27.9. Longer histogram bars indicate a larger impact of a certain taxa to the difference between the four cover treatments. P-value < 0.05 considered significant.