ABSTRACT

Title of Thesis: INVESTIGATING THE EFFECT OF MANAGEMENT ON AGRICULTURAL ORGANIC NITROGEN CYCLING AND ALTERNATIVE NITRIFICATION PATHWAYS

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This project studied the influence of agricultural management strategies (such as tillage and fertilizer choice) on nitrogen (N) cycling pathways. Soil samples and leachate samples from a series of experimental plots at the Wye Research and Education Center were analyzed using a combination of traditional chemical N measures (DON, PMN, NO_x, NH_3, TN, and microbial biomass C & N) and novel mass spectrometry techniques (FT-ICR-MS) to characterize shifts in organic matter composition and quantity over time and under three different cover cropping regimes. Analysis of these samples indicate that the DOM (dissolved organic matter) composition of leachate changed significantly with increasing sampling depth. However further research is needed to fully investigate the potential impacts of cover cropping and time on soil and leached DOM and DON. Soil samples were also collected at the Farming Systems Project in Beltsville, MD and at several of the University of Minnesota's Outreach farms. These samples were analyzed for their abundance of *16S, nirK, nirS, nxrA,* and *amoA AOB* to characterize the nitrifying and denitrifying microbial communities under a combination of management strategies. While the findings were not significant, they indicate that fertilization and tillage may have an impact on the nitrification and denitrification communities.

INVESTIGATING THE EFFECT OF MANAGEMENT ON AGRICULTURAL ORGANIC NITROGEN CYCLING AND ALTERNATIVE NITRIFICATION PATHWAYS

By

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Table of Contents

List of Tables

Chapter II

List of Figures

Chapter I

Chapter II

Chapter III

Appendices:

Chapter 1 - Introduction and Literature Review

Nitrogen (N) cycling has been widely researched for decades as it is an important limiting nutrient in temperate ecosystems, is crucial for plant development, and is imperative for sustaining crop productivity (Hou et al., 2012; Leghari et al., 2016; Wagner et al., 2015). Early developments in the field of study led to the discovery of the Haber-Bosch process in the early 1900s, allowing for the successful synthesis of inorganic ammonia (NH_3) (Rouwenhorst et al., 2021). This in turn revolutionized agricultural practices internationally, as N could now be easily supplemented in nutrient deficient fields. However, over time concerns of the environmental impacts of inorganic N additions and over-fertilization came into focus. By the mid-20th century, eutrophication was recognized as a common water pollution problem in North America and Europe (Rodhe, 1969). Additional studies of the impact of fertilization have indicated that additional N in agricultural fields is closely tied to approximately 75% of the United States' production of nitrous oxide (N_2O), a powerful greenhouse gas with 300x the warming potential of carbon dioxide (CO_2) (US EPA, 2021a). The research within this thesis provides a better understanding of nitrification and less researched N cycling pathways, and the impact that common agricultural management strategies (tillage and fertilization regimes) have on organic N pools and transformations.

The first component of this research focuses on organic N held within and leached from conventionally tilled (CT) agricultural soils in Maryland, USA. Organic N refers to the fraction of compounds in soil organic matter (SOM) that contain N, and accounts for a small but active portion of agricultural soils (Chantigny, 2003; McGill et al., 1986). For decades, research into SOM focused on the study of humic substances: complex compounds thought to represent the largest (~60%) pool of SOM and to be the endpoint of SOM degradation (Adey & Loveland,

2007; Trevisan et al., 2010). These substances (fulvic acid, humic acid, and fulvin) were thought to control SOM cycling, improving soil fertility and structure, and affecting plant root structure and nutrient uptake (Trevisan et al., 2010). However, in 2015 a paper by Lehmann & Kleber challenged the common understanding of SOM cycling. They put forward that humic substances were much less prevalent in most soils, and that the majority of studied humic substances were a lab artifact resulting from the traditional required alkaline extraction process. Instead, SOM exists as progressively decomposing compounds within soil, a subset of which (such as free amino acids) can be utilized by plants and microorganisms as a source of C and N (Kleber & Johnson, 2010; Lehmann & Kleber, 2015). Furthermore, recent studies indicate that smaller, labile soil organic nitrogen (SON) may be an important source of N for plants regardless of inorganic N additions. The long-term understanding was that microbes and plants utilized inorganic over organic N sources, and that organic N was only important in in nutrient limited environments (Healey et al., 2004). Organic N dynamics in forests indicate that this might not always be the case, and microbes may utilize organic N more often than previous assumed (Kalbitz et al., 2011). Further research is needed to understand these dynamics and the role of organic N as a potential nutrient source.

There are several common methods to characterize organic N in soils and water. Briefly:

1) **Dissolved Organic Nitrogen (DON).** This is the most common method of determining the quantity of small organic molecules present in leachate and water samples. It is calculated by first analyzing filtered samples (0.45um) for NO_3-N and NH_4-N (the sum of which represents the pool of dissolved inorganic N (DIN)) and the total concentration of N in the sample (TN). DON concentration is then determined as the difference between TN and DIN. The inorganic fractions are conventionally analyzed via flow injection analysis while

methods to determine TN vary. Common methods include UV oxidation, dialysis pretreatment, persulfate digestion, and catalytic thermal decomposition (Lee & Westerhoff, 2005; Tirendi et al., 2002; Shimadzu Corporation, 2010). DON may be responsible for up to 70% of soluble N in N-limited soils (Prendergast-Miller et al., 2015), and accounts for a significant portion of leached N despite its utilization by plants and microbes. There is a wide range of leached DON values in the literature and the amount of DON is strongly impacted by land use (i.e.: forest vs. pasture vs. agriculture) (Van Kessel et al., 2009). One study of Australian cotton fields reported that 40% of leached N from the soil was DON (MacDonald et al., 2017), while another study of Chilean forests indicated that leachate samples contained 94-96% leachate (Hedin, 1995). The conversion of land to agricultural use can also influence leached DON. A study of the Chesapeake Bay observed a significant positive relationship between the area of upstream cropland and organic N in stream water samples (Jordan et al., 2010). Additional studies indicate that leached agricultural urea and poultry litter runoff are both major contributors of N in local waterways, and agricultural watersheds can be considered as areas of DON production (Davis et al., 2016; Osburn et al., 2016)

2) **Extractable Organic Nitrogen (EON) or Soil DON**. These terms refer to the pool of N compounds that can be extracted from soils and is calculated in a similar manner to leachate or water DON. Soils are extracted with a given solution – common extractants include KCl, K_2SO_4, $CaCl_2$, and water – and analyzed for NO_3-N, NH_4-N, and TN. Soil DON is then calculated as the difference between extractable TDN and DIN (Chantigny, 2003). Both storage and extraction can have a strong impact on a soil sample's EON (Ros et al., 2010). Similar patterns have been observed between leached DON and EON under a variety of land

uses. As with leached DON, studies have reported higher concentrations of water extractable ON (WEON) in forest soils when compared to crop land. Agricultural WEON represents a small fraction of soil total N (25 ugL^{-1} – 10mgL_{-1}, or 0.1-3%) and can be strongly impacted by field management strategies (Ros et al., 2009). Studies have shown that WEON increases with the addition of N fertilizer (+118% in EON following fertilizer application), and the incorporation of crop residues can increase EON by 22% (Chantigny, 2003; Ros et al., 2009, 2010).

3) **Potentially Mineralizable Nitrogen (PMN).** This refers to the subset of organic N that is readily mineralized to plant available, inorganic N (NO_3-N and NH_4-N). PMN is commonly determined by incubating a soil sample in a microcosm and analyzing the change in NH_4-N concentration before and after incubation. The additional NH_4-N that is produced during incubation is attributed to the mineralization of OM and used to estimate the amount of readily mineralized N present in the SOM (Waring & Bremner, 1964). There are several methodologies to determine PMN, with varying incubation lengths (7, 28, and 56 days) and conditions (aerobic and anerobic). The 7-day anaerobic protocol is the most common as it is a simple methodology and takes the least amount of time to analyze. Studies of PMN indicate that it is impacted by agricultural management strategies. A 2018 meta-analysis of 43 independent studies indicated that additional N fertilizer can lead to an average increase in PMN; systems fertilized with inorganic N reported 22% higher PMN and systems fertilized with manure reported 34% higher PMN when compared with non-fertilized systems (Mahal et al., 2018). Conservation practices can also lead to an increase in PMN. No-till farming systems have reported an average 11% increase in PMN compared to conventionally tilled systems, while studies of leguminous cover crops reveal 211% greater PMN when compared

to systems without cover crops. Furthermore, studies of diverse rotations of three or more crops indicated 44% greater PMN compared to continuous cropping systems (Mahal et al., 2018).

4) **Microbial Biomass C and N**. This is the amount of C and N present within the soil microbial community, and can be used to assess the size of the soil microbial community (Lori et al., 2017). It is determined by calculating the difference between K_2SO_4 extractable total carbon (TC) and TN before and after chloroform fumigation (Vance et al., 1987). By fumigating a fresh soil sample, the microbes are killed and lysed. It is assumed that any net change in N or C can be attributed to the microbial community, but a conversion factor is used in final reporting of microbial C and N (DeLuca et al., 2019), because empirical evidence showed that not all cells are lysed and the extractability of microbial C and N in the fumigated samples varies among soil types (Dictor et al., 1997). Microbial biomass can account for 1-5% of SOM and is impacted by agricultural management strategies (e.g.: tillage and cover cropping) in agricultural systems (Cookson et al., 2008). A meta-analysis of 56 studies researching the impact of organic farming techniques indicates that organic systems had an average 41% greater microbial biomass C and 51% greater microbial biomass N compared to conventional systems (Lori et al., 2017).

The second study in this thesis focuses on the composition of the N-cycling microbial community. N-cycling has traditionally been thought of as consisting of several microbially mediated, sequential transformations (Figure 1.1) (Kuypers et al., 2018).

Figure 1.1. Diagram of Agricultural N-cycling Dynamics

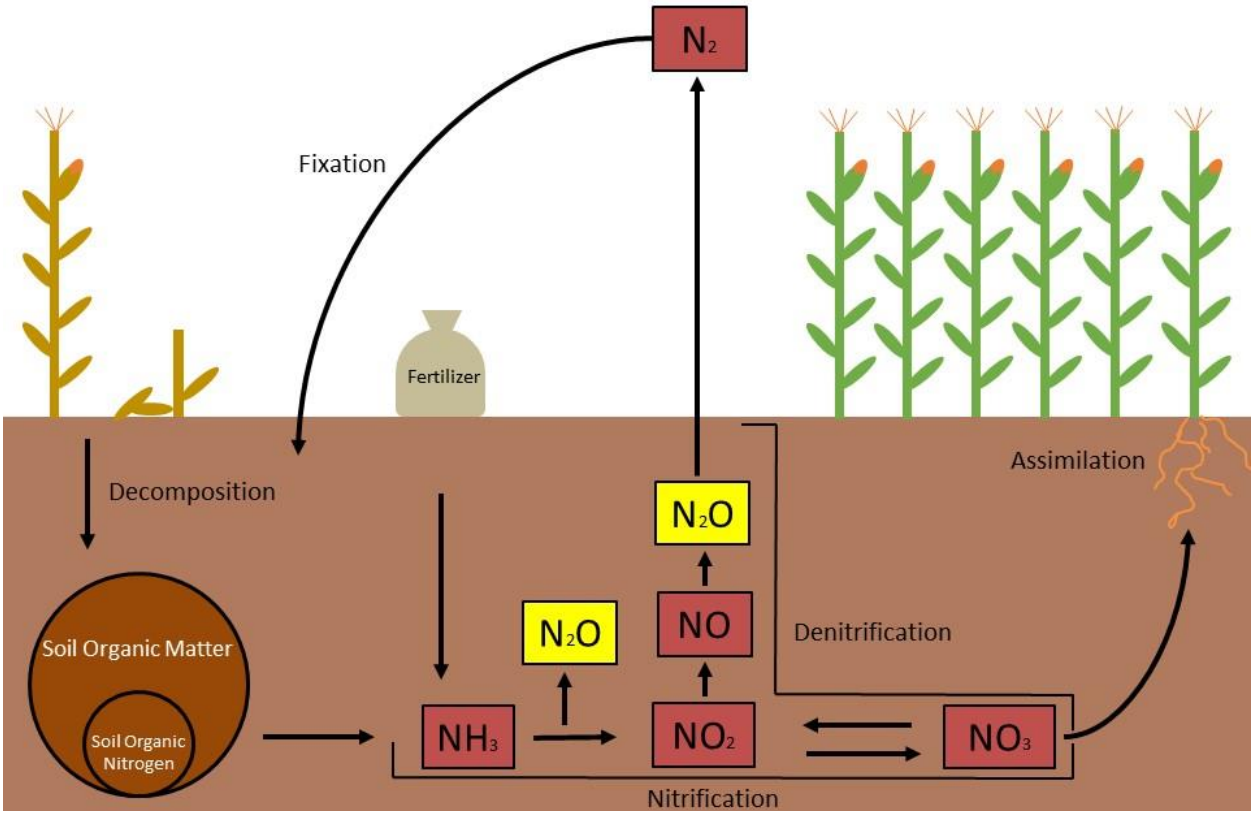

Figure 1.1. The various microbially mediated N transformations that occur in agricultural fields. Emphasis is placed on potential N_2O leaks.

Nitrification:

Nitrification is the transformation of NH_4^+ to NO_3^- and is traditionally considered a two-step process mediated by different bacterial communities.

1) Ammonia Oxidation – ($NH_4^+ \rightarrow NO_2^-$). This step begins with NH_4^+ which can come from a variety of sources (decomposition and mineralization of plant residue, microbially mediated fixation of atmospheric N, or supplemental N fertilizer) (Kuypers et al., 2018). The subsequent transformation is mediated by bacteria (*Nitrosomonas & Nitrosospira spp*.) or archaea (*Nitrososphaera & Nitrosoarchaeum spp*.) with the ability to produce the enzyme ammonia monooxygenase (*amoA*), which transforms NH_4^+ into NH_2OH. Nitrous oxide (N_2O) can be produced as a by-product of this reaction if oxidation is incomplete. The bacteria or archaea also can produce the enzyme hydroxylamine oxidoreductase (*hao*) which converts the resulting NH_2OH into nitrite (NO_2^-) (Peng et al., 2021; Yin et al., 2018).
2) Nitrite Oxidation – ($NO_2^- \rightarrow NO_3^-$). This step is meditated by a different subset of bacteria (*Nitrobacter & Nitrospira spp.*) that can produce the nitrate oxidoreductase enzyme (*nxr*), which converts NO_2^- into plant available (nitrate) NO_3^- (Cabello et al., 2009).

Denitrification:

Denitrification refers to the transformation of NO_3^- to N_2 and is also microbially mediated. Denitrifiers generally contain all the enzymes necessary for complete denitrification and, though there is a wide variety of denitrifying genera, most denitrifying microbes fall into one of the following classes: *Alphaproteobacteria* (32%), *Betaproteobacteria* (28%), and

Gammaproteobacteria (28%) (Jones et al., 2008). There are several intermediate transformations:

1) Nitrate reduction – (NO_3^- → NO_2^-). During this step, NO_3^- is transformed into NO_2^- via the enzyme nitrate reductase (*nar*) (Alvarez et al., 2014). This step requires anerobic conditions as enzyme synthesis is inhibited by the presence of oxygen.
2) Nitrite reduction – (NO_2^- → NO). During this step NO_2 is converted to nitric oxide (NO) using the enzyme nitrite reductase (*nir*). There are two common forms of *nir* that are often targeted when studying the denitrifying community: *nirS* which encodes for a heme form of the enzyme, and *nirK* which encodes for the less common copper form of the enzyme (Alvarez et al., 2014).
3) Nitric oxide reduction (NO→ N_2O). During this step NO is converted to nitrous oxide (N_2O) by nitric oxide reductase (*nor*). This step is responsible for many of the N-N bonds present in nature, and often occurs quickly after nitrite reduction as NO is toxic to cells (Alvarez et al., 2014).
4) Nitrous oxide reduction (N_2O → N_2). During this step N_2O is converted into dinitrogen (N_2) via nitrous oxide reductase (*nosZ*). As with the other denitrification enzymes, this activity is strongly inhibited by the presence of O_2. Given that this step transforms N_2O, *nosZ* gene expression has been frequently studied in relation to N_2O emissions, and lower rates of gene expression are correlated with increases in emission rates (Kong et al., 2021).

Recent studies of *Nitrospira sp*. have redefined our understanding of nitrification. In 2015, unique samples of *Nitrospira sp*. with the ability to completely oxidize NH_4^+ to NO_3^- were isolated in in aquaculture filters (Van Kessel et al., 2015). In the years since its discovery, it has

been isolated from a plethora of systems (i.e.: wastewater treatment plants, freshwater ecosystems, and both agricultural and forest soils) (Xu et al., 2020). Comammox *Nitrospira sp*. cannot be classified based on *16S* rRNA sequencing, instead functional gene markers need to be amplified and sequenced. Previous studies have identified two clades of comammox *Nitrospira* (Clade A and Clade B), with slight niche differences between the two clades (Koch et al., 2018; Xu et al., 2020). There are still unknowns about comammox *Nitrospira sp.,* including their role in agricultural N-cycling. So far studies indicate that *Nitrospira sp*. may play an important role in oligotrophic environments but might be out competed by traditional N-cycling bacteria in fertilized soils (Sakoula et al., 2021; Xu et al., 2020).

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CHAPTER II

Title: Dissolved organic matter and dissolved organic nitrogen composition shifts with sampling depth and management strategies in agricultural fields

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Abstract:

For decades, soil organic matter (SOM) research focused on the dynamics of complex organic acids (i.e.: fulvic and humic acids). Recent studies have shown, however, that these compounds are lab artifacts and that dissolved organic matter (DOM) exists as smaller, free molecules within the soil matrix. It also accounts for up to 70% of N in surface waters (Lehmann & Kleber, 2015; Osburn et al., 2016). Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS) and traditional chemical N measures (NO_3-N, NH_4-N, total dissolved N (TN), total dissolved C (TC), microbial biomass C and N, dissolved organic N (DON), and potentially mineralizable N) were used to determine the chemical composition and amount of leached and extractable DOM and DON in agricultural fields. Using a series of Repeated Measure ANOVAs and Pearson correlations, we investigated the impact of three cover cropping strategies (cereal cover, a mixed cereal and leguminous cover, and fallow) and sampling date on the DOM and DON pools. We also studied the impact of sampling depth on the composition of leached DOM

and DON and found that the relative percentage of recalcitrant compounds and the photodegradability of DOM and DON increased with depth. Traditional chemical soil N measures (K_2SO_4 extractable TN ($F=11.437$, $p=0.043$), water extractable TN ($F=10.676$, $p=0.04$), and DON ($F=10.901$, $p=0.046$)) significantly increased with sampling date and, though not significant, the percentage of labile DOM declined slightly from June to late July. Though not statistically significant, plots with a mixed cover crop reporting slightly higher percentages of soil protein and leached free amino sugars compared to the other cover class treatments. Further study that includes later sampling dates are needed to determine the significance of temporal and cover-based trends, but that this line of research could have lasting impact on how we understand organic N cycling dynamics and could eventually be used to inform N fertilizer and cover-cropping recommendations.

Introduction:

Nitrogen (N) is frequently a limiting nutrient in plant growth, and as such is often supplemented in agricultural soils through inorganic (nitrate (NO_3^-) and ammonia (NH_3^+)) and organic (manure) fertilizer additions (Bundy & Meisinger, 1994). Early agricultural research focused on developing technologies and strategies for feeding the world's ever-growing population. These successful efforts resulted in the increase in international food productivity during the second half of the 20th century, with an estimated 12-13% increase in the food supply of several African and East Asian nations between 1960 and 1990 (Pingali, 2012). However, over fertilization has also acidified agricultural soil (Žurovec et al., 2021) and leached N into local waterways (Dybowski et al., 2020) causing unforeseen environmental issues.

These developments have highlighted the need to better understand agricultural N dynamics and has led to an increase in studies of organic N dynamics and the role of organic

matter as underestimated source of N in agricultural fields. Research focused on organic nutrient availability has underscored the positive impact of cover-cropping and legumes on soil health and organic C and N accumulation (Ebelhar et al., 1984; Mitchell, 1977; Utomo et al., 2010; Wei et al., 2018). Other studies have investigated alternative tillage and no-till practices, noting substatntial increases in mineralizable N (Mahal et al., 2018) and microbial soil diversity under minimum tillage (Li et al., 2020). Despite the growing wealth of studies investigating the impacts of management on organic matter (OM) cycling, many of these studies rely on traditional chemical analysis (i.e.: extractable dissolved organic matter (DOM) and dissolved organic N (DON), potentially mineralizable N (PMN), and Bradford Protein Assays). While these measures provide insight into the size and reactivity of OM pools, they do not provide information on the chemical composition of OM or the mechanims of OM cycing.

Advancements in NMR (nuclear mass resonance spectroscopy) and mass spectrometry provide a more complete picture of the composition of DOM and DON samples. Studies have also been published utilizing Fourier Transform Ion Cyclotron Mass Spectrometry (FT-ICR-MS) to characterize OM in wetlands, freshwater, and marine environments, and is a powerful tool for understanding the complexities of leached OM and soil OM (D'Andrilli et al., 2015; Tfaily et al., 2015). This form of analysis can capture thousands of compounds a given sample and can determine the molecular formula for each (Wilson & Tfaily, 2018). However, FT-ICR-MS data is qualitative in nature and, while it provides incredibly detailed insight into the composition of organic compounds within a sample, it cannot quantify the size of the DOM or DON pool. As such, FT-ICR-MS is often paired with traditional, quantitative DOM measures to contextualize the dataset.

Early FT-ICR-MS research began in 1973 and was primarily utilized to characterize complex organic mixtures with a focus on sources of fuel such as petroleum and pyrolysis biomass derived liquids (Marshall & Chen, 2015; Yan et al., 2016). In the decades since, FT-ICR-MS has been used to study OM in peatland and wetland soils as well as glacial, marine, and freshwater ecosystems (D'Andrilli et al., 2015; Hanson et al., 2018; Solihat et al., 2019; Wilson & Tfaily, 2018). Preliminary studies indicate that it has potential to be a powerful tool to understand agricultural organic N cycling and could be used to inform sustainable soil management techniques (Kwiatkowska-Malina, 2018). However, few studies have applied FT-ICR-MS analysis to explore agricultural N cycling.

Our study was designed to investigate this knowledge gap and apply both traditional chemical N and OM measures with FT-ICR-MS techniques to investigate the chemical composition of soil organic N throughout the growing season in Maryland corn fields. We also examined the potential impact of cover cropping on the amount and composition of soil organic N and organic N leached from agricultural fields. We had several hypotheses:

1) The presence of N-fixing leguminous cover crops are known to increase available N (Kermah et al., 2018), and will increase soil organic N. This will subsequently lead to a higher proportion of labile N-containing compounds in leachate and soil samples from the plots with a mixed leguminous and cereal cover crop than under a cereal cover or left fallow.
2) The OM present in the soil would act as a source of nutrients for the growing corn and we will observe a decrease the relative proportion of labile N-containing compounds throughout the growing season.

3) Leaching has repeatedly been reported as a major pathway of DON loss in agricultural systems, and studies have shown that up to 61% of lost DON can be attributed to lignin, a highly photodegradable organic compounds (Benner & Kaiser, 2011; Li et al., 2018). As such, we hypothesize that leached DOM and DON would be primarily comprised of lignin and that deeper samples would have a higher proportion of recalcitrant, highly photodegradable, and lignin-like compounds.

Experimental Procedures:

Sample Site and Collection:

In Summer of 2019, composite 0-20cm soil samples were collected from a series of 30x60 ft (9.14m x 18.28 m) research plots located at the Wye Research and Education Center (REC). This UMD Research Station was established in Queenstown, MD in 1982 and has had a long history of agricultural research on Maryland's Eastern Shore (College of Agriculture and Natural Resources, 2021). The study site itself is level (0-2% slope) and soils are characterized as Mattapex-Butlertown (MqA) silt loam with a 0 – 2% slope (Soil Survey Staff).

A subset of six plots were selected from a subset of a plots on 6-acre long-term study established in 2019, all of which were under corn (*Zea mays* L.) production during the 2019 field season and soybeans (*Glycine max* L.) the previous year. All the plots were conventionally tilled (CT) and received inorganic fertilizer. On 3/18/2019 the 6-acre field received a broadcast application of 12000 lbs of high Calcium lime at a rate of 2000 lbs/ ac. Urea ammonium nitrate (UAN) fertilizer was applied twice: 15 gals (or 49lbs N) was applied during planting on 6/4/2019, and 240 gals (782 lbs N) was applied via sidedress application on 6/19/2019. The plots

differed in cover crop treatments: two received a mixed cover of crimson clover (*Trifolium incarnatium* L.) and rye (*Secale cereale* L.), two received a cereal rye cover, and two were left fallow over the winter.

Soil samples were taken twice during the summer: shortly before planting (June 3rd) and when the corn had reached the V6 stage (July 18th). To collect each composite soil sample, the study plot was divided into a grid of nine 10ft x 20ft (3.04m x 6.09m) rectangles (Figure 2.1). From there, 3 individual 0-20cm cores were collected from each of the grid quadrants. This resulted in 27 cores per plot, which were combined and sieved (4mm) together while in the field. In total, 12 composite soil samples were collected over the growing season. Prior to planting, suction cup lysimeters were installed in the center of each of the plots at 3 different depths (30cm, 60cm, and 90cm) (Figure 2.2) and the resulting 18 lysimeters were sampled three times during the growing season (June 3rd, June 18th, and July 16th). Due to varied field conditions and precipitation, samples were not able to be collected from each lysimeter at every sampling date (Figure 2.3). Over the field season, 37 leachate samples were collected in total. All samples were returned to the lab and refrigerated at 4^{o}C for later analysis.

Chemical Analysis:

Within 24 hours of sampling, 5g dry equivalent of each refrigerated, field moist soil sample was placed in 12, 50mL centrifuge tubes (Figure 2.1). Soils in three of those tubes were extracted with water by adding 50mL of deionized water to the samples and periodically shaken to resuspend the soil particles for 24 hours. The supernatant was collected via vacuum filtration through 40um filter paper, acidified to pH<2 using sulfuric acid, and stored at 4^{o}C for later analysis.

Figure 2.1: Soil Sample Collection and Preparation for Analysis

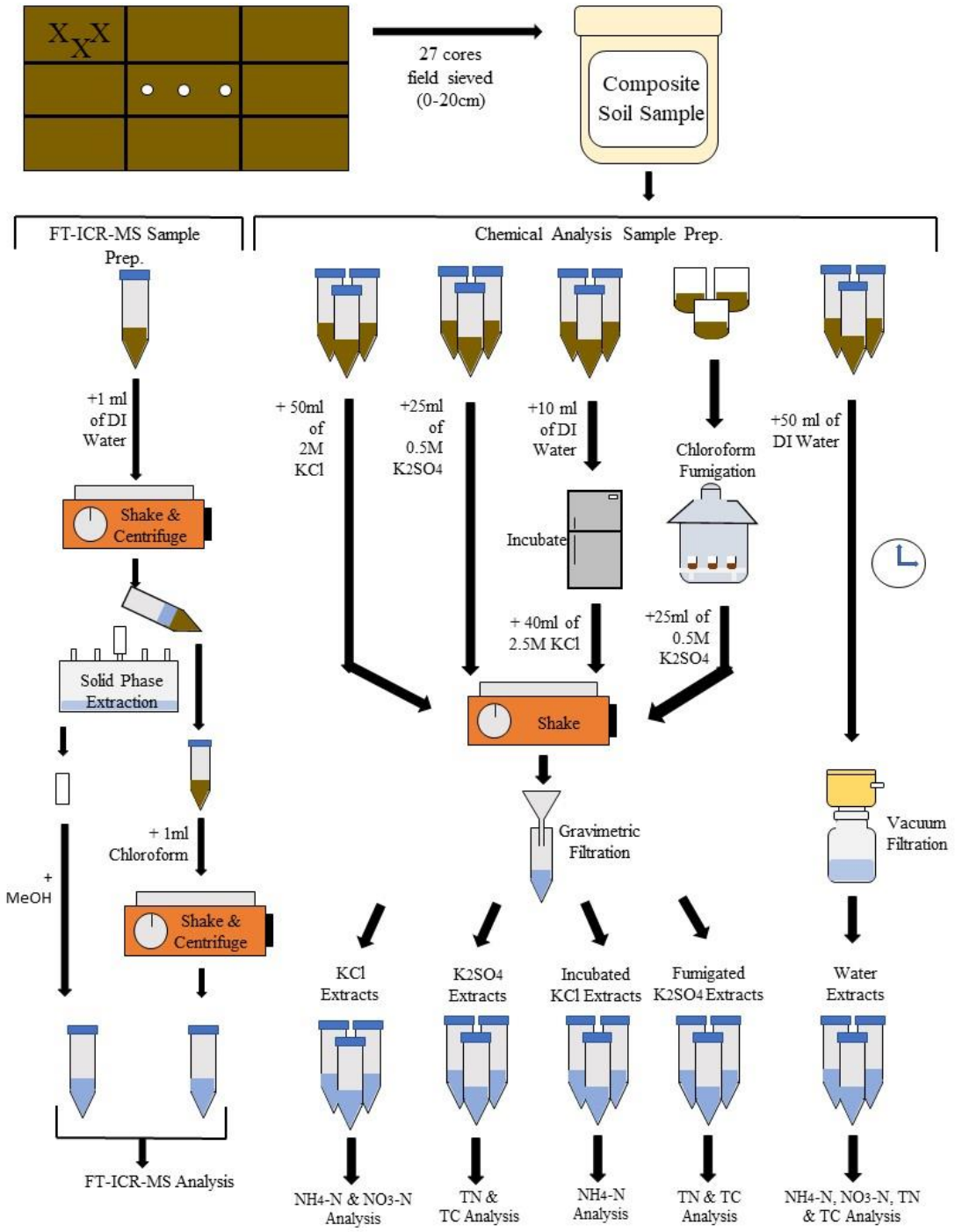

Figure 2.1. The soil sampling method and sample preparation for chemical analysis (water extractable NH_4-N, NO_3-N, TN & TC, KCl extractable NH_4-N and NO_3-N, K_2SO_4 extractable TN & TC, PMN, and Microbial Biomass C & N). Soil DON was calculated by subtracting the DIN from the total N concentration in the soil water extracts. PMN was calculated by determining the difference in NH_4-N between the anaerobically incubated KCl extracts and fresh field moist soil samples. Soil microbial biomass C and N was determined by determining the average difference in C and N between the fumigated and fresh field moist K_2SO_4 extracts and correcting for extraction efficiency by dividing the value by 0.45. A portion of each composite soil sample was shipped to our collaborators in Arizona for FT-ICR-MS analysis.

Figure 2.2: Leachate Sample Collection and Preparation for Analysis

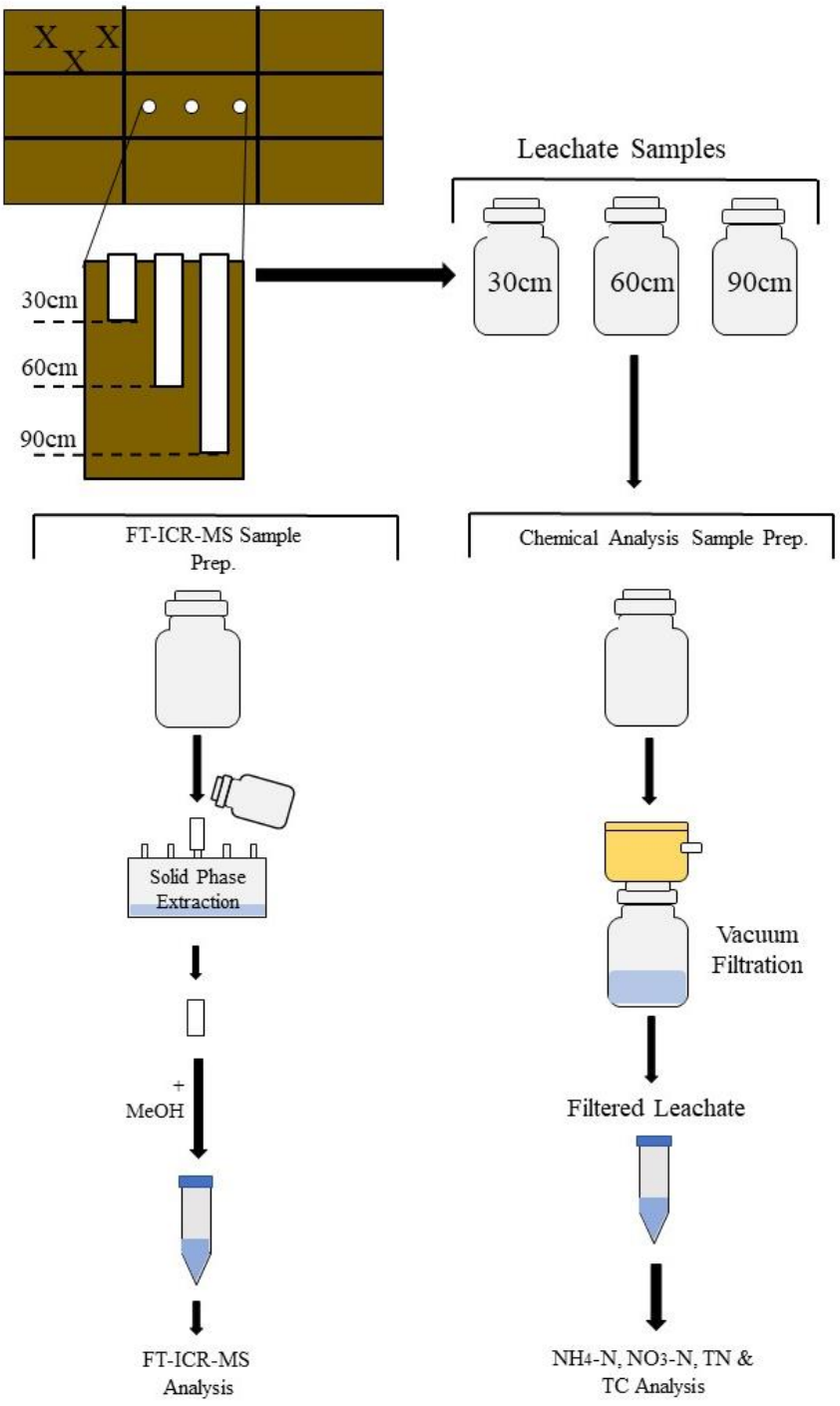

Figure 2.2. The leachate sampling method and sample preparation for chemical analysis (NH_4-N, NO_3-N, TN & TC). A portion of each leachate sample was shipped to our collaborators in Arizona for FT-ICR-MS analysis.

Figure 2.3: Precipitation at the Wye REC (Summer 2019)

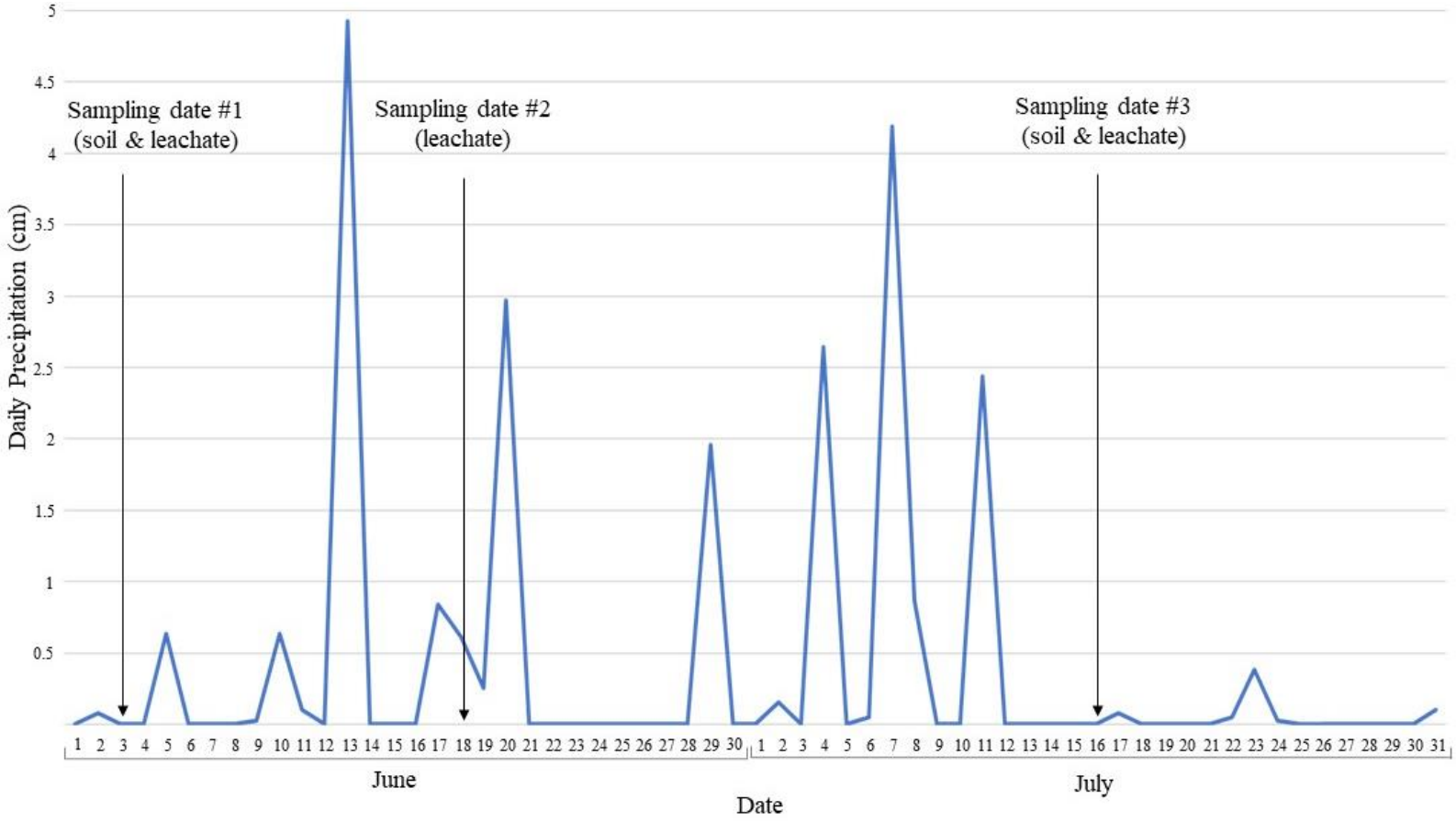

Figure 2.3. Daily precipitation data gathered using a NOAH IV Precipitation Gauge at the Wye Research and Education center during the summer of 2019. The three sampling dates are noted on the graph with arrows, indicating the date as well as what types of samples (soil or leachate) were collected on the given date.

The soils in a second another set of three centrifuge tubes were extracted with 50mL of 2M KCl, by shaking for one hour with a fixed speed reciprocal shaker (280rpm) and collecting the supernatant via gravimetric filtration with Whatman 42 filter paper (Vance et al., 1987; Maynard & Kalra, 1993). A third set of 3 analytical soil replicates were incubated anaerobically at 40^{o}C with 10mL of water for 7 days (Waring & Bremner, 1964). The samples were then shaken with 40mL of 2.5M KCl using a fixed speed reciprocal shaker (280 rpm) and the supernatant was collected via gravimetric filtration with Whatman 42 filter paper. A fourth set of 3 analytical replicates was extracted with 25mL of 0.5M K_2SO_4, shaken for one hour with a fixed speed reciprocal shaker (280rpm), and the supernatant was collected via gravimetric filtration with Whatman 42 filter paper. A final set of 3 analytical replicates (5g dry equivalent of field moist soil) was weighed into 50mL glass beakers and fumigated in a desiccator under a vacuum with chloroform for 48 hours to determine microbial biomass C and N. The fumigated soils were then shaken with a fixed speed reciprocal shaker (280rpm) with 0.5M K_2SO_4 for one hour, and the supernatant was collected by gravimetric filtration with Whatman 42 filter paper (Vance et al., 1987). All KCl and K_2SO_4 extracts were stored frozen at -20^{o}C for later analysis.

The water extractions and KCl solutions were analyzed for NO_3 – N and NH_4 – N using a Lachat Quickchem Flow Injection Analyzer (Harbridge, 2007a; Harbridge, 2007b), and all the solutions were analyzed for their total nitrogen (TN) and total carbon (TC) content using a Shimadzu TOC Analyzer (Shimadzu Corporation, 2010) (Figure 2.1). The leachate samples were filtered via vacuum filtration through 40um filter paper, acidified to pH<2 using sulfuric acid, and stored at 4^{o}C until they analyzed for NO_3-N, and NH_4-N using a Lachat Quickchem Flow Injection Analyzer (Harbridge, 2007a; Harbridge, 2007b) and were analyzed for TN and TC with a Shimadzu TOC Analyzer (Shimadzu Corporation, 2010) (Figure 2.2).

Potential mineralizable N was determined by subtracting the average NH_4-N concentration of the non-incubated KCl extractions from the average NH_4-N concentration of the samples that were incubated for 7 days. The resulting change in NH_4-N represents the pool of organic N in the soil that is readily converted into a plant available form (Waring & Bremner, 1964). Microbial biomass C and N was determined by comparing the average C and N values of non-fumigated 0.5M K_2SO_4 extractions with those of the samples that had been fumigated with chloroform and applying the conversion factors in equations 1 and 2 (Vance et al., 1987).

$$\text{Biomass C} = \Delta C / 0.45 \quad (1)$$

$$\text{Biomass N} = \Delta N / 0.45 \quad (2)$$

FT-ICR-MS Analysis:

To prepare the soil samples for FT-ICR-MS analysis, 100mg of soil was weighed into a 2mL capped centrifuge tube. Given the cost and time for FT-ICR-MS analysis, replicates were not used for this portion of the study. Each sample was initially extracted with 1mL of de-ionized water and shaken with an orbital sharker (20rpm) for 2 hours (Figure 2.1) (Tfaily et al., 2015). The solution was allowed to settle and then centrifuged for 10 minutes at 4430g to collect the supernatant. Organic compounds in the resulting solution (Figure 2.1) and the leachate (Figure 2.2) samples were isolated with solid phase extraction (SPE) (Dittmar et al., 2008). To improve ionization efficiency, the DOM isolates were mixed with MeOH, to create a resulting solution that is 33% sample and 66% MeOH. The soil fraction of the water extraction samples underwent a second extraction using 1mL of chloroform and shaken with the orbital shaker (20rpm) for 2 hours (Tfaily et al., 2015).

Figure 2.4: Compound Classification in a Van Krevelan Diagram

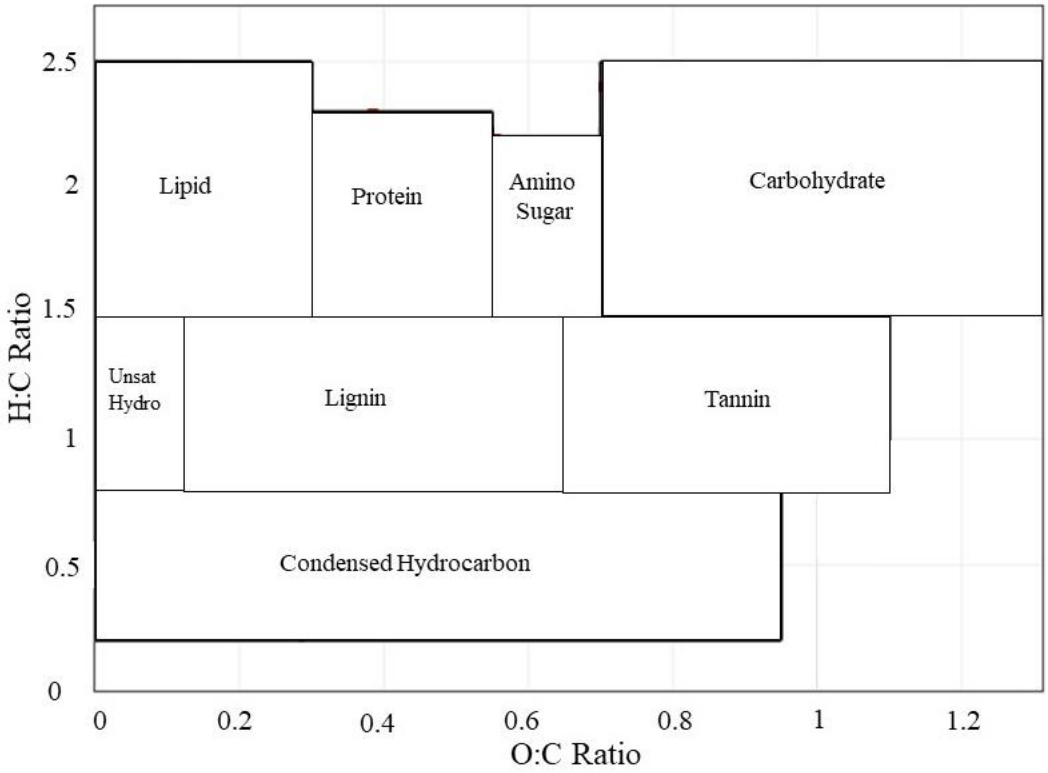

Figure 2.4: Classification ranges depicted for each of the biogeochemical organic compound classes in a standard Van Krevelan diagram.

The resulting solution was centrifuged for 10 minutes at 4430g and injected directly into the FT-ICR-MS. By sequentially extracting the samples first with water and then with chloroform, we were able to capture both the pool of free organic compounds, as well as the semi-polar and non-polar compounds that are captured in a chloroform extraction (Tfaily et al., 2018).

The soil water, chloroform, and leachate samples were ionized with electrospray ionization (ESI) and analyzed with a Bruker 9.4T FT-ICR. The resulting data was processed with Formularity Version 1.0. Using a compound identification algorithm and the FR-ICR-MS peaks, the database assigned a chemical formula and determined the number of C, H, O, N, S, and P atoms present with each compound (Tolić et al., 2017). These matches were used to calculate the O/C ratio and H/C for each of the compounds which, in turn, was used to broadly classify the compounds present into the categories in Table 1 and Figure 2.4 (Tfaily et al., 2015).

Table 2.1. Adapted from AminiTabriz et al, (2020). The ranges were used to determine the biogeochemical classification of the DOM and DON compounds within the samples.

Compound Class	O/C range	H/C Range
Lipid	0.0 – 0.3	1.5 – 2.5
Unsaturated Hydrocarbon	.0 – 0.3	1.0 – 1.6
Condensed Hydrocarbon	0.0 – 0.4	0.2 – 0.8
Lignin	0.29 – 0.65	0.7 – 1.5
Protein	0.3 – 0.6	1.5 – 2.3
Carbohydrate	0.5 – 0.7	0.8 – 2.5
Amino Sugar	0.5 – 0.7	0.8 – 2.5
Tannin	0.65 –1.00	0.8 – 1.5

The FT-ICR-MS peaks were also used to calculate each sample's double bond equivalency (DBE) and aromaticity index (AI) (Equations 3&4) (Koch & Dittmar, 2006).

$$DBE = 1 + ½ (2C – H + N + P) \quad (3)$$

$$AI = (1 + C – O – S – 0.5H) / (C – O – S – N – P) \quad (4)$$

The number of C atoms can be divided by the number of rings and double C bonds in a compound to determine the relative stability. High DBE/C ratios can indicate the presence of stable, aromatic structures (Koch & Dittmar, 2006). Similarly, the AI of a sample can indicate whether a compound is easily photodegradable; an AI $\geq$ 0.67 indicates the presence of more recalcitrant, aromatic structures. Conversely lability can be indicated by a compound's H/C ratio; highly labile compounds are classified as having a H/C > 1.5 (D'Andrilli et al., 2015).

Data Analysis:

The FT-MS-R Exploratory Data Analysis Tool (FREDA) was used to visualize the spread of the data with Van Krevelan diagrams (Figure 2.4) and conduct Principal Coordinates Analysis (PCoA) on the FT-ICR-MS dataset. The statistical package JMP Pro 15 was used for multivariate analysis. Pearson Correlations, Repeated Measure ANOVAs, and Multiple Regression Analysis were performed to ascertain the impact of sampling date and cover-cropping treatments on the composition of DOM and DON pool. Similar analysis was used to assess the relationship between the FT-ICR-MS data, photodegradability metrics, and traditional N measure gathered through chemical analysis (microbial biomass C and N, PMN, DON, TDN, and KCl and water extractable NO_3-N and NH_4-N).

Results:

Leachate Chemical Analysis:

Repeated Measure ANOVAs to investigate the impact of sampling date and cover cropping on traditional chemical N measures reveal little difference in DIN measures among the cover crop treatments (Table 2.2). Though not significant (p=0.05) there was an increase in NO_3-N (F=3.010, p=0.087) and TN (F=3.619, p=0.058) with date (Figure 2.5a & c).

Table 2.2: The sample number, sampling depth, sampling date and cover cropping strategy along with several traditional chemical N measures for each of the leachate samples.

Leachate Chemical Analysis

#	Depth	Date	Cover	NO_3-N (mg/L)	NH_3-N (mg/L)	TC (mg/L)	TN (mg/L)	DIN (mg/L)	DON (mg/L)
15	30	1	Mixed	0.17	0.04	12.37	4.72	0.21	4.51
18			Cereal	0.05	0.005	18.82	1.20	0.05	1.15
20			Fallow	5.51	0.07	10.93	6.24	5.58	0.66
21		2	Mixed	1.71	-0.004	41.9	6.99	1.70	5.28
22			Fallow	1.51	0.009	14.27	2.63	1.51	1.11
25			Mixed	3.34	-0.003	10.92	4.34	3.34	1.00
28			Cereal	311.72	12.60	5.40		324.31	
31			Mixed	16.35	0.02	6.44	19.27	16.37	2.90
32		3	Fallow	126.88	0.27	3.32	130.3	127.15	3.15
34			Cereal	103.27	0.11	5.53		103.38	
37			Mixed	219.79	5.49	8.22		225.28	
13	60	1	Cereal	0.62	-0.02	2.71	3.00	0.60	2.40
26		2	Mixed	0.09	0.007	11.82	1.57	0.10	1.47
29			Cereal	48.31	0.27	3.84	51.52	48.59	2.93
35		3	Cereal	13.65	0.35	7.99	14.34	14.00	0.34
38			Mixed	21.24	0.13	5.24	22.26	21.37	0.89
14	90		Cereal	0.22	-0.003	2.62	1.30	0.21	1.09
16		1	Fallow	2.67	-0.02	2.73	9.10	2.64	6.46
17			Cereal	1.75	0.04	2.13	5.34	1.80	3.54
19			Cereal	0.90	-0.005	12.79	2.83	0.90	1.93
23		2	Fallow	2.64	-0.03	2.08	2.78	2.61	0.17
24			Cereal	3.41	0.04	2.8	3.61	3.45	0.16
27			Mixed	3.94	0.08	6.81	4.21	4.02	0.19
30		3	Cereal	21.69	0.00	2.13	23.09	21.69	1.40
33			Fallow	7.30	-0.01	1.34	8.666	7.29	1.38
36			Cereal	219.93	6.46	6.72		226.39	
39			Mixed	16.87	0.00	4.48		16.87	

Table 2.3: The plot, sampling date, and cover cropping strategy along with several traditional chemical N measures for each of the soil samples.

Soil Chemical Analysis

Plot	Date	Cover	Avg. Water Extractable (mg/L)						Avg. KCl extractable (mg/L)			Avg. K_2SO_4 extractable (mg/L)		Microbial Biomass	
			NO_3-N	NH_4-N	TC	TN	DIN	DON	NO_3-N	NH_4-N	PMN	TC	TN	C	N
1	1	Cereal	0.17 +/- 0.01	-0.02 +/- 0.01	3.32 +/- 0.19	5.44 +/- 0.06	0.15	5.29	0.17 +/- 0.07	0.54 +/- 0.01	1.29	42.84 +/- 3.37	0.98 +/- 0.73	-69.63	4.93
2		Fallow	0.28 +/- 0.01	-0.03 +/- 0.00	3.65 +/- 0.41	6.35 +/- 0.14	0.25	6.11	0.36 +/- 0.01	0.53 +/- 0.01	2.69	40.71 +/- 2.48	1.66 +/- 0.00	59.93	4.88
3		Mixed	0.23 +/- 0.00	-0.03 +/- 0.00	3.63 +/- 0.30	9.00 +/- 0.18	0.20	8.80	0.29 +/- 0.02	0.57 +/- 0.02	2.39	44.00 +/- 4.29	1.34 +/- 1.05	-30.78	5.75
4		Fallow	0.20 +/- 0.01	-0.03 +/- 0.00	3.39 +/- 0.14	6.78 +/- 0.06	0.17	6.61	0.31 +/- 0.02	0.54 +/- 0.02	1.95	38.56 +/- 1.31	1.66 +/- 0.15	152.96	5.60
5		Cereal	0.18 +/- 0.01	-0.03 +/- 0.01	3.51 +/- 0.20	6.25 +/- 0.05	0.14	6.11	0.23 +/- 0.00	0.54 +/- 0.01	1.79	42.53 +/- 1.80	1.59 +/- 0.06	221.63	7.62
6		Mixed	0.17 +/- 0.02	-0.03 +/- 0.00	3.77 +/- 0.21	6.95 +/- 0.13	0.14	6.80	0.26 +/- 0.01	0.61 +/- 0.02	1.60	40.28 +/- 1.83	1.66 +/- 0.04	290.89	7.20
1	3	Cereal	0.46 +/- 0.12	-0.03 +/- 0.05	3.39 +/- 0.12	10.69 +/- 0.17	0.43	10.26	0.63 +/- 0.04	0.55 +/- 0.05	0.93	20.10 +/- 0.66	2.59 +/- 0.04	223.93	5.03
2		Fallow	0.31 +/- 0.01	-0.01 +/- 0.00	3.76 +/- 0.55	8.27 +/- 0.80	0.30	7.97	0.38 +/- 0.01	0.58 +/- 0.09	1.54	23.53 +/- 0.70	2.14 +/- 0.06	195.78	5.09
3		Mixed	0.95 +/- 0.01	0.00 +/- 0.02	3.44 +/- 0.18	8.42 +/- 0.88	0.94	7.47	1.12 +/- 0.02	0.50 +/- 0.01	0.65	22.47 +/- 1.10	3.22 +/- 0.04	193.78	5.14
4		Fallow	1.99 +/- 0.05	0.00 +/- 0.00	3.18 +/- 0.24	15.16 +/- 2.12	1.99	13.17	2.17 +/- 0.05	0.61 +/- 0.01	0.71	23.69 +/- 0.26	5.42 +/- 0.05	243.70	5.86
5		Cereal	1.41 +/- 0.03	-0.01 +/- 0.00	2.71 +/- 0.08	12.45 +/- 0.23	1.40	11.05	1.57 +/- 0.06	0.57 +/- 0.03	1.03	21.52 +/- 0.98	4.18 +/- 0.03	203.04	4.83
6		Mixed	0.82 +/- 0.14	-0.01 +/- 0.01	2.84 +/- 0.06	9.60 +/- 1.22	0.81	8.79	0.91 +/- 0.00	0.57 +/- 0.02	0.75	21.97 +/- 1.59	3.04 +/- 0.02	-3.52	4.74

Figure 2.5: Leachate Chemical Analysis Interaction Plots

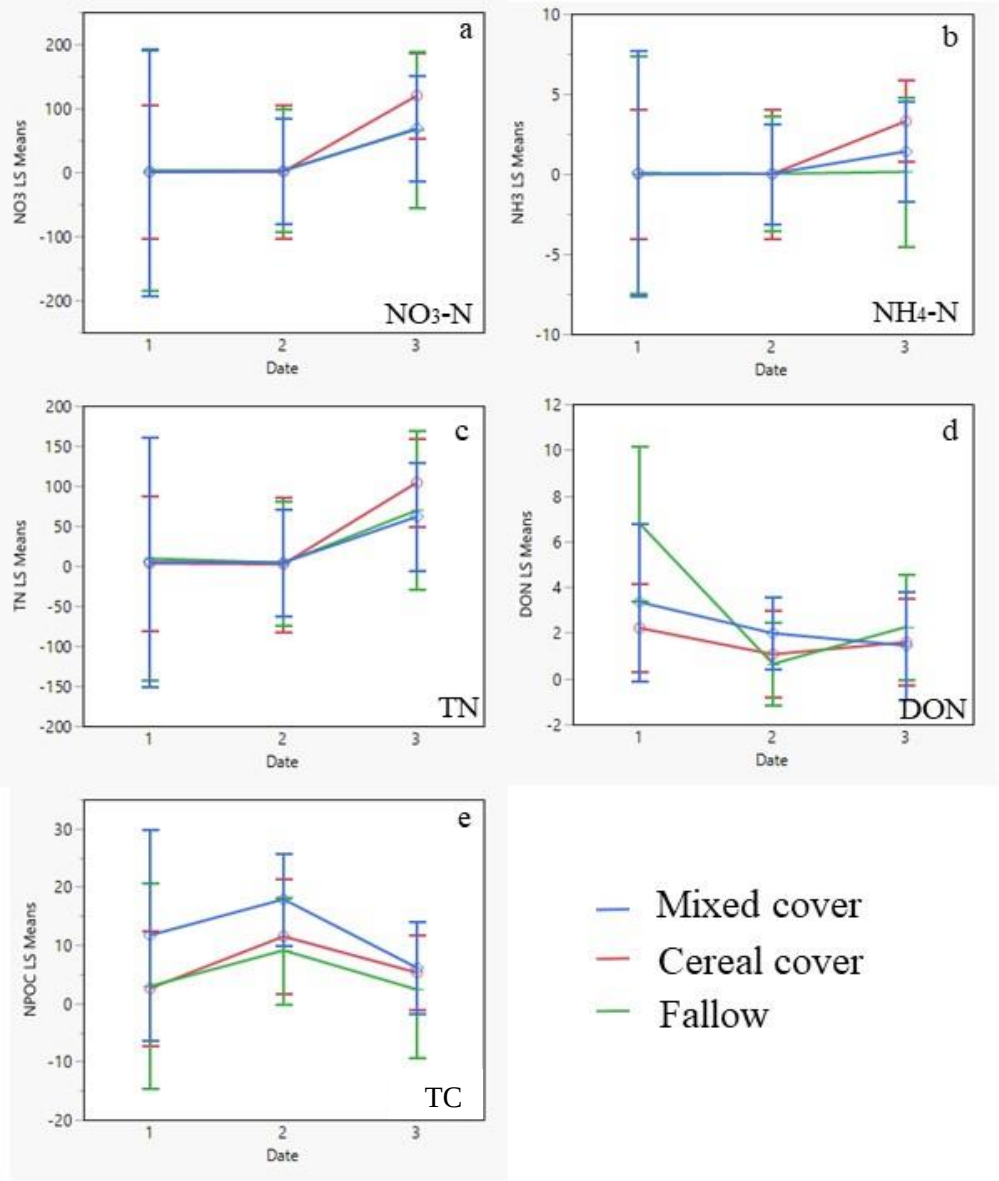

Figure 2.5: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on NO_3-N, NH_4-N, TN, DON, and TC.

Figure 2.6: Soil Chemical Analysis Interaction Plots

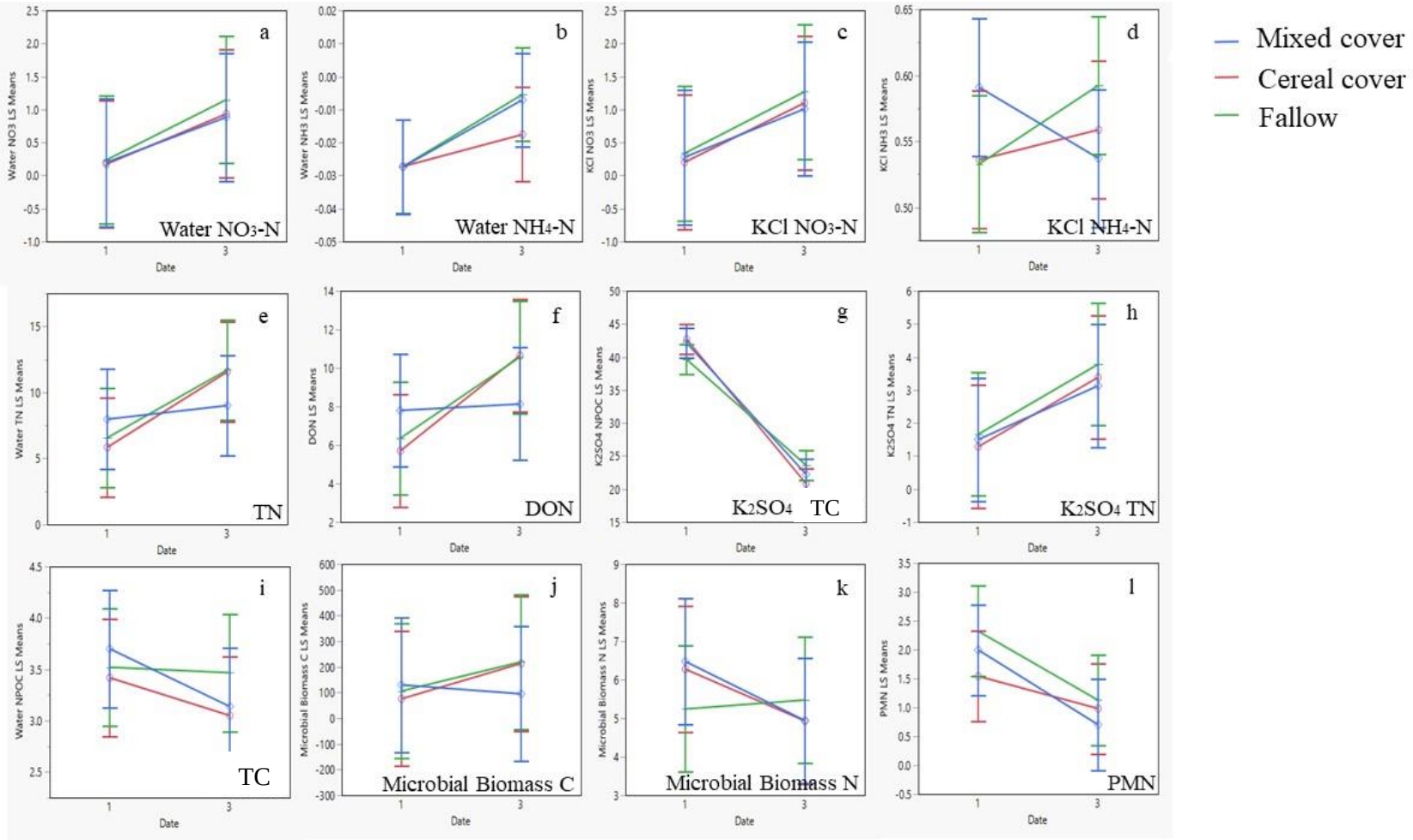

Figure 2.6: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on water extractable NO_3-N, NH_4-N, KCl extractable NO_3-N, NH_4-N, water extractable TN and DON, K_2SO_4 extractable TC and TN, water extractable TC, Microbial biomass C and N, and PMN.

Dissolved organic nitrogen significantly decreased throughout the sampling season (F=5.940, p=0.036) and – though not significant- there was a minor interaction between cover cropping strategy and date (F=0.209, p=0.209) (Figure 2.5d). There was a wider range of DON values at the beginning of the sampling season (Table 2.2), with the highest DON reported in the fallow plots, followed by the mixed and cereal cover plots. Variability between the cover crops treatments decreased over time, and similar DON values were reported across all cover cropping treatments by the third sampling date (Figure 2.5d). Total carbon also varied with date (F=3.039, p=0.084), although there was not a consistent increase or decrease (Figure 2.5e).

Soil Chemical Analysis:

There was a significant increase in K_2SO_4 extractable TN (F=11.437, p=0.043), water extractable TN (F=10.676, p=0.04), and DON (F=10.901, p=0.046) over time (Table 2.3 and Figure 2.6 e, h, & f). Though not significant, there was also an observed increase in soil water extractable DIN measures over time (NO_3-N, F= 5.5782, p=0.0992; NH_3-N, F=10.021, p=0.0507) (Figure 2.6 a & b). Significant decreases were observed in the K_2SO_4 extractable TC pool (F=715.212, p=0.0001) and PMN (F=38.752, p=0.008) (Figure 2.6 g & l). Cover class did not influence the chemical N measures, and there was little change in either Microbial Biomass C or N (Table 2.3 and Figure 2.6 j & k).

Leachate DOM:

All the DOM samples were primarily composed of lignin (48 - 66% of compounds in the DOM pool). There was more variability in relative percentages of the other compounds present (Table 2.4).

Figure 2.7: Leachate DOM Van Krevelan Diagrams

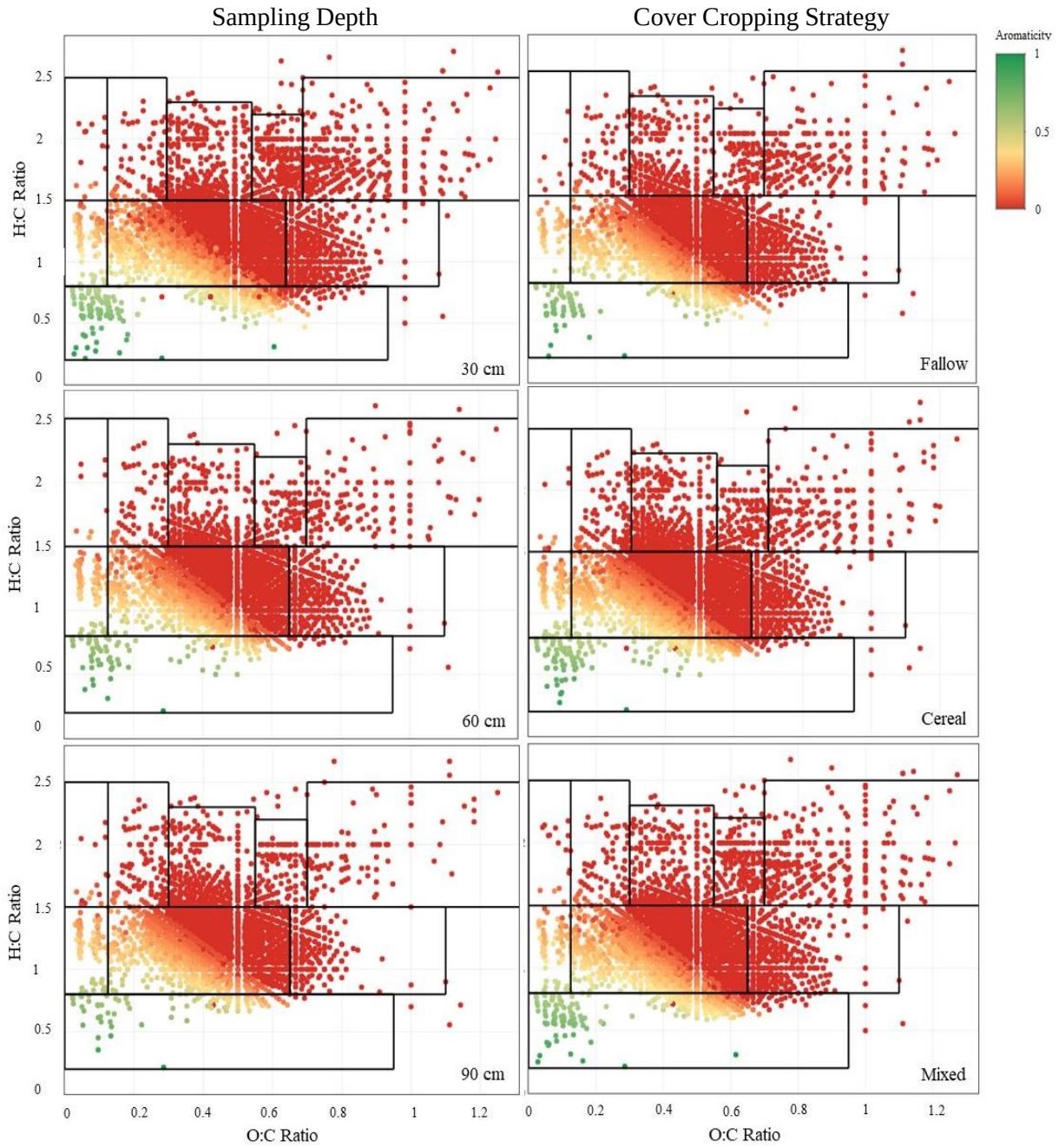

Figure 2.7. The figure is comprised of two sets of Van Krevelan diagrams illustrating the DOM composition of the leachate samples. One separates the DOM based on the sampling depth of the leachate, and one separates the samples based upon the cover cropping strategy of the given sample plot. The graphs are also color coded based upon the aromaticity of the compounds (red indicating highly labile and green indicating highly recalcitrant).

Table 2.4: The sample number, sampling date, cover cropping strategy, DOM FT-ICR-MS peaks (indicating the total number of unique compounds), the relative percentage of each compositional class (lipids, unsaturated hydrocarbons, condensed hydrocarbons, proteins, amnio sugars, carbohydrates, lignin, tannins, and unclassifiable compounds), the average DBE/C ratio and the percentage of compounds with an AI> 0.67.

Leachate DOM Composition

					Relative Percentage										
#	Depth	Date	Cover	Peaks	Lip.	Unsat Hydro	Con Hydro	Pro.	Amino Sugars	Carb.	Lig.	Tan.	Comp. NA	DBE/C	% AI
15	30	1	Mixed	3979	1.0	0.7	4.8	4.9	2.2	4.1	48.4	7.2	26.8	0.5 +/- 0.2	1.0
18			Cereal	3687	1.0	0.8	2.7	4.8	1.8	3.0	52.8	7.3	25.9	0.5 +/- 0.1	0.7
20			Fallow	3574	1.0	0.6	5.2	3.0	1.2	2.8	53.5	9.8	23.0	0.5 +/- 0.1	0.7
21		2	Mixed	4012	0.7	0.6	4.8	3.6	2.6	3.9	49.0	7.8	27.1	0.5 +/- 0.2	0.9
22			Fallow	3866	0.6	0.6	4.5	3.9	1.4	3.5	50.6	9.2	25.8	0.5 +/- 0.2	0.9
25			Mixed	3899	0.6	0.6	2.3	5.5	1.8	2.2	49.3	7.8	29.8	0.5 +/- 0.1	0.7
28			Cereal	3386	1.2	0.6	1.5	8.6	1.8	0.5	57.9	6.5	21.4	0.5 +/- 0.1	0.2
31			Mixed	4347	0.7	0.5	4.5	4.0	2.0	3.5	48.5	7.3	29.1	0.5 +/- 0.2	0.8
32		3	Fallow	3494	0.9	0.5	3.7	4.7	1.4	2.5	55.3	7.4	23.6	0.5 +/- 0.1	0.7
34			Cereal	3573	0.6	0.5	3.0	6.0	1.3	1.9	57.6	8.5	20.7	0.5 +/- 0.1	0.4
37			Mixed	4342	1.1	0.7	2.5	6.3	2.5	1.8	50.6	7.0	27.5	0.5 +/- 0.1	0.6
13	60	1	Cereal	2993	1.9	1.2	0.8	8.5	1.3	1.0	56.6	4.7	24.1	0.4 +/- 0.1	0.2
26		2	Mixed	3540	1.1	0.7	0.9	6.3	2.1	1.5	52.5	8.3	26.7	0.4 +/- 0.1	0.3
29			Cereal	2874	1.2	1.3	0.4	8.4	1.1	0.7	61.9	3.6	21.6	0.4 +/- 0.1	0.1
35		3	Cereal	3248	1.5	0.9	3.2	4.2	1.3	2.9	53.6	8.3	24.1	0.5 +/- 0.1	0.6
38			Mixed	2386	1.6	0.9	0.5	5.7	0.9	0.6	63.4	7.3	19.3	0.5 +/- 0.1	0.1
14	90	1	Cereal	3560	2.0	1.0	1.7	9.3	1.7	1.0	53.8	4.9	24.6	0.4 +/- 0.1	0.3
16			Fallow	1255	3.9	0.6	0.3	12.0	1.9	1.5	52.4	1.8	25.7	0.4 +/- 0.1	0.1
17			Cereal	2767	1.4	1.1	0.6	8.8	1.2	1.3	59.4	4.5	21.8	0.4 +/- 0.2	1.1
19		2	Cereal	1650	1.3	0.6	0.4	6.2	0.4	0.2	66.2	4.0	20.7	0.4 +/- 0.1	0.2
23			Fallow	1189	3.6	0.6	0.3	4.8	1.2	0.9	61.1	2.1	25.3	0.4 +/- 0.1	0.1
24			Cereal	2675	1.6	0.5	0.6	11.0	2.0	0.9	55.6	6.0	21.9	0.4 +/- 0.1	0.3
27			Mixed	1935	2.9	0.8	0.6	7.2	1.3	1.2	59.8	2.0	24.1	0.4 +/- 0.1	0.3
30		3	Cereal	2879	2.1	0.9	1.3	7.4	0.9	0.5	58.3	4.4	24.3	0.4 +/- 0.1	0.1
33			Fallow	1352	3.1	0.7	0.2	4.7	0.4	0.7	59.6	2.5	28.0	0.4 +/- 0.1	0.0
36			Cereal	2541	1.5	1.1	0.6	6.9	0.7	0.6	64.7	3.7	20.3	0.4 +/- 0.1	0.2
39			Mixed	1816	3.0	0.8	0.8	6.7	1.5	1.0	56.0	1.4	28.8	0.4 +/- 0.1	0.3

Table 2.5: The results of Pearson Correlations comparing the sampling depth, the relative percent of highly photodegradable compounds (AI>0.67), the average DBE/C ratio, the amount of DON, TN, TC, NH_4-N, and NO_3-N, and the relative percentage of each compositional class (lipids, unsaturated hydrocarbons, condensed hydrocarbons, proteins, amnio sugars, carbohydrates, lignin, tannins, and unclassifiable compounds) within the leachate DOM samples. Bolded values within the chart indicate statistically significant correlations ($p<0.5$).

Leachate DOM Pearson Correlations

	% AI	Avg DBE/C	DON	DIN	TN	TC	NH_4-N	NO_3-N	Comp. NA	Tan.	Lig.	Carb.	Amino Sugars	Pro.	Con Hydro.	Unsat Hydro.	Lip.
Depth	**-0.62**	**-0.82**	-0.27	-0.14	-0.15	**-0.44**	-0.10	-0.14	-0.22	**-0.81**	**0.61**	**-0.72**	**-0.48**	**0.55**	**-0.80**	0.37	**0.76**
Lip.	**-0.62**	**-0.84**	-0.02	-0.21	-0.22	**-0.46**	-0.12	-0.21	0.09	**-0.84**	0.34	**-0.52**	-0.27	**0.42**	**-0.64**	0.13	
Unsat Hydro	-0.28	-0.25	-0.08	0.01	0.01	-0.27	0.04	0.01	-0.31	-0.33	**0.41**	**-0.39**	-0.35	0.28	**-0.42**		
Con Hydro.	**0.79**	**0.80**	0.18	-0.02	0.00	**0.54**	-0.09	-0.02	0.31	**0.76**	**-0.71**	**0.91**	**0.44**	**-0.66**			
Pro.	**-0.46**	**-0.72**	0.21	0.11	0.10	**-0.48**	0.18	0.11	-0.30	**-0.52**	0.25	**-0.61**	0.06				
Amino Sugars	**0.50**	0.14	0.41	0.05	0.04	**0.44**	0.09	0.05	**0.50**	0.36	**-0.82**	**0.56**					
Carb.	**0.85**	**0.63**	0.30	-0.22	-0.20	**0.61**	-0.25	-0.22	**0.47**	**0.65**	**-0.81**						
Lig.	**-0.67**	-0.36	-0.31	0.17	0.17	**-0.41**	0.15	0.18	**-0.72**	**-0.54**							
Tan.	**0.61**	**0.87**	-0.12	0.07	0.08	**0.44**	0.02	0.07	0.01								
Comp. NA	0.33	-0.02	0.18	-0.29	-0.30	0.25	-0.25	-0.29									
NO_3-N	-0.13	0.23	0.04	**1.00**	**1.00**	-0.18	**0.92**										
NH_4-N	-0.16	0.13	0.02	**0.92**	**0.88**	-0.11											
TC	**0.49**	**0.42**	0.19	-0.18	-0.18												
TN	-0.11	0.25	0.11	**1.00**													
DIN	-0.13	0.23	0.04														
DON	0.27	-0.05															

Figure 2.8: Leachate DOM Interaction Plots

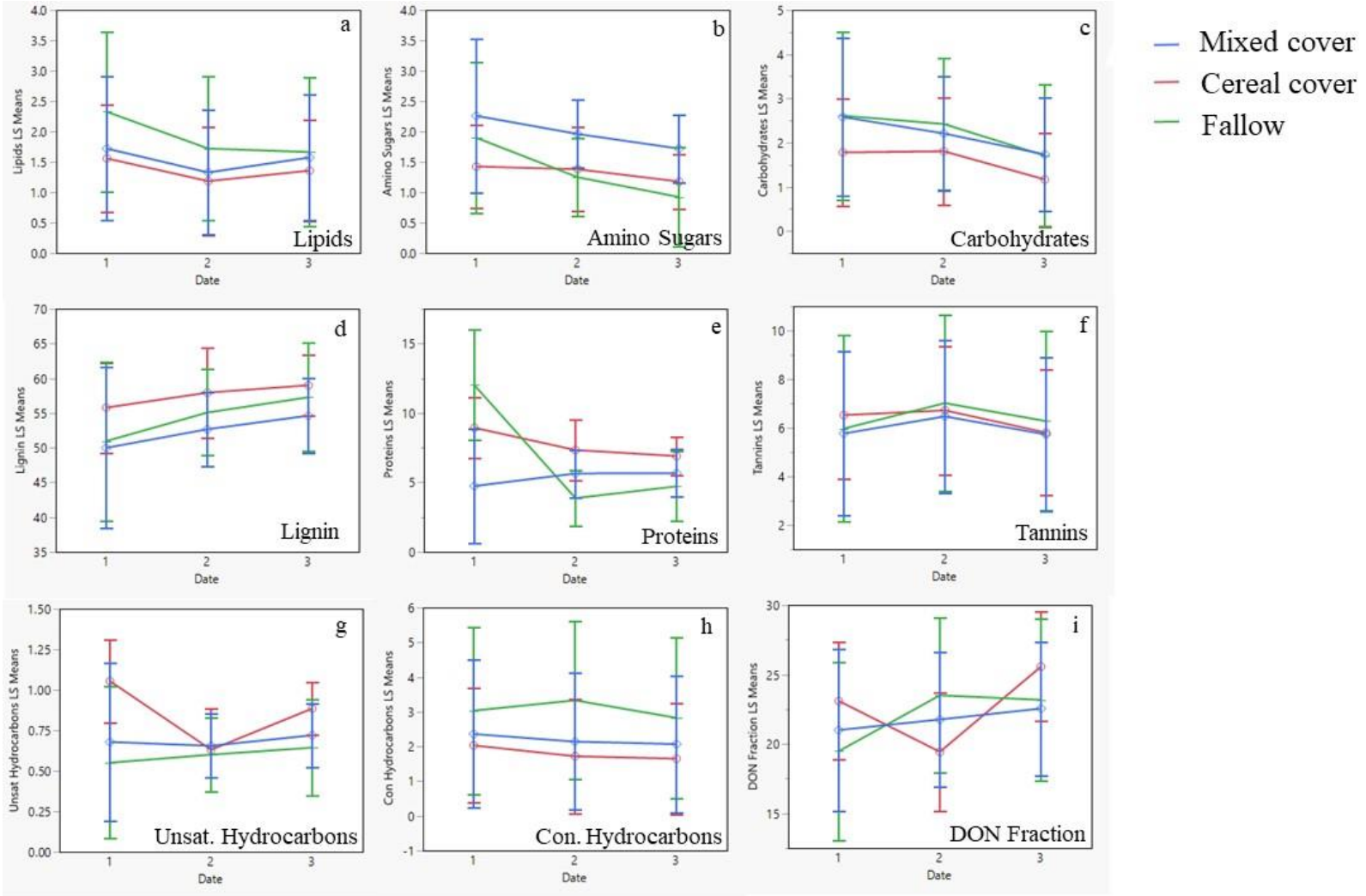

Figure 2.8: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage of each compositional class (lipids, amino sugars, carbohydrates, lignin, proteins, tannins, unsaturated hydrocarbons, condensed hydrocarbons, and the relative percentage of N containing compounds) within the leachate DOM samples.

Protein and tannins comprised the next largest fraction of DOM in almost all the leachate samples; 89% of samples had protein as the 2nd or 3rd largest DOM fraction and 85% of samples had tannins as the 2nd or 3rd largest DOM fraction. In all samples there were compounds that could not be assigned a biochemical compound classification, accounting for 19-29% of compounds within the DOM samples.

Van Krevelan diagrams indicate fewer unique compounds (represented as individual points on the diagrams) in 90cm leachate samples and illustrate a general change of DOM composition with increasing sampling depth (Figure 2.7). A higher proportion of compounds are plotted in the center rectangle (lignin) of the Van Krevelan diagram for the 90cm samples compared to the 60cm and 30cm diagrams. In comparison, fewer compounds were plotted in the two top right rectangles (amino sugars and carbohydrates) with increasing depth. Pearson correlations were conducted to determine the statistical significance of this shift in DOM composition (Table 2.5). There was a significant ($p < 0.05$) increase in the relative percentage of lipids (r=0.76), lignin (r= 0.61), and proteins (r=0.55) with depth, and a decrease in the relative percentage of amino sugars (r=-0.48), condensed hydrocarbons (r=-0.80), carbohydrates (r=-0.72), and tannins (r=-0.88) with increasing sampling depth (Table 2.5). Depth also had a significant negative relationship with TC (r =-0.44), the relative percent of highly photodegradable compounds (AI> 0.67) (r=-0.62), and the average DBE/C of the sample (r=-0.82), indicating that more recalcitrant compounds were observed in leachate samples collected from deeper in the soil profile.

There was a slight shift in the composition of leached DOM throughout the growing season (Table 2.4 and Figure 2.8). Though not statistically significant, there was an observed decrease in the relative percentage of lipids (F=2.539, p=0.138), amino sugars (F=1.546,

p=0.249), proteins (F=3.773, p=0.053), and carbohydrates (F=3.377, p=0.084) (Figure 2.8 a, b, c, & e). There was a slight increase in the relative percentage of lignin (F=1.263, p=0.322) (Figure 2.8d). Van Krevelan diagrams comparing the composition of samples under the different cover cropping strategies appear very similar in spread (Figure 2.7), and Repeated Measure ANOVAs indicate that the values for most of the composition classes were similar between the cover cropping treatments, with exception to amino sugars, proteins, and lignin (Figure 2.8). Though not significant (F=2.758, p=0.125), amino sugars comprised a higher relative percentage of leached DOM in cover cropped plots with the highest values reported in the mixed cover plots (Figure 2.8b). Proteins comprised a higher relative percentage (F=3.362, p=0.106) of leached DOM in fallow plots at the beginning of the sampling season and quickly declined through time (cover*time, F=2.524, p=0.096) (Figure 2.8e). At all sampling dates, the cereal cover plots reported the highest relative percentage of lignin followed by fallow and mixed cover plots. Analysis of the photodegradability metrics also revealed a slight decrease in percentage of highly aromatic compounds over time (F=1.748, p=0.228) (Figure 2.9a).

Data points clustered by sampling depth in PCoA; most of the 30cm samples are loosely clustered (Figure 2.10). Samples also clustered more based upon cover class at lower sampling depths, indicating that cover class may have more influence over composition of leached DOM at lower depths (Figure 2.10).

Leachate DON:

Fourteen to thirty one percent of identified compounds were present in the DON pool. As with the DOM pool, the majority of DON was comprised of lignin (70 - 91% of the N containing compounds across all the leachate samples) (Table 2.6).

Figure 2.9: Leachate Photodegradability Interaction Plots

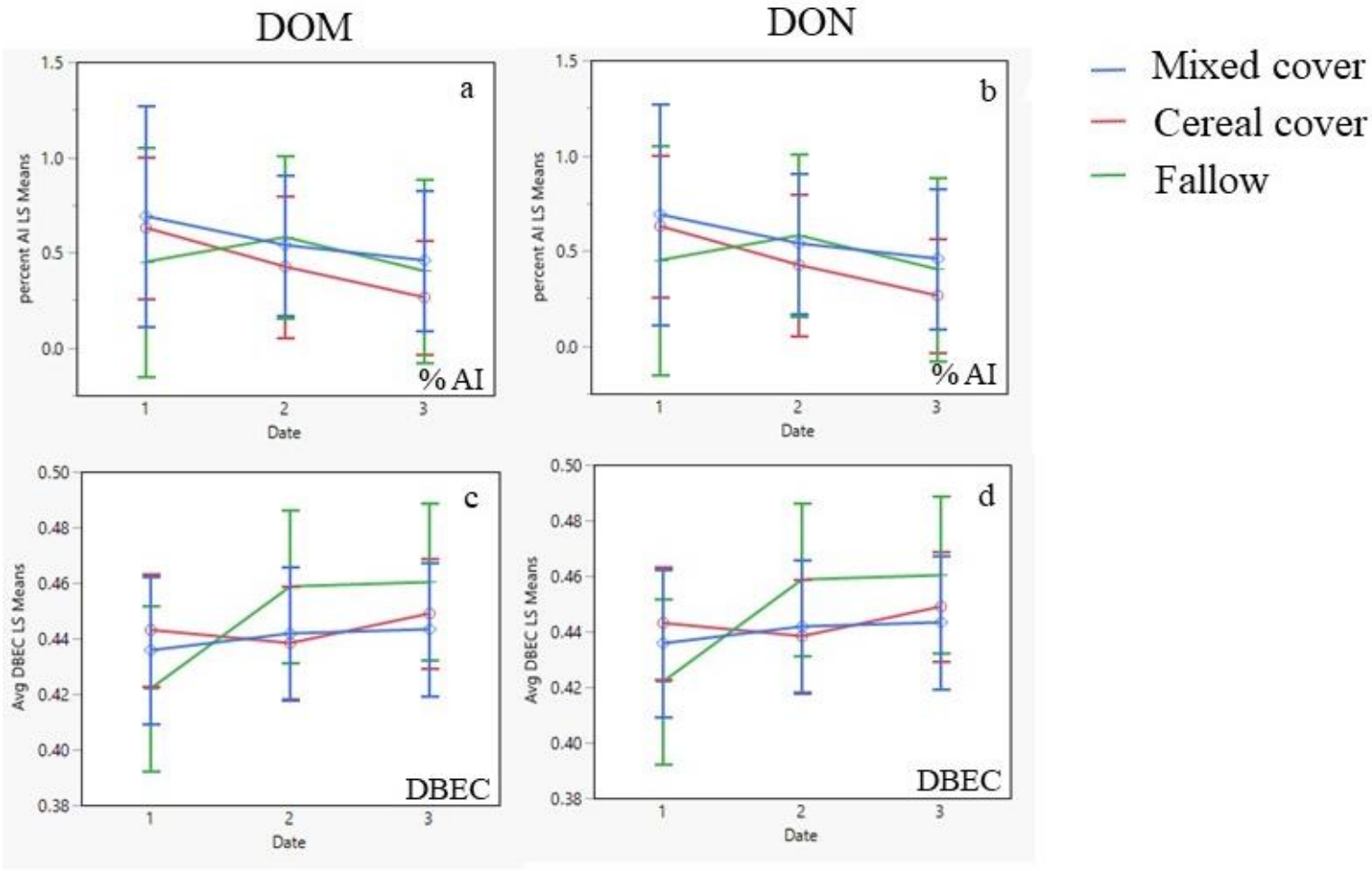

Figure 2.9: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage highly photodegradable compounds (AI>0.67) and the average DBE/C ratio within the leachate DOM and DON samples.

Figure 2.10: Leachate DOM PCoA

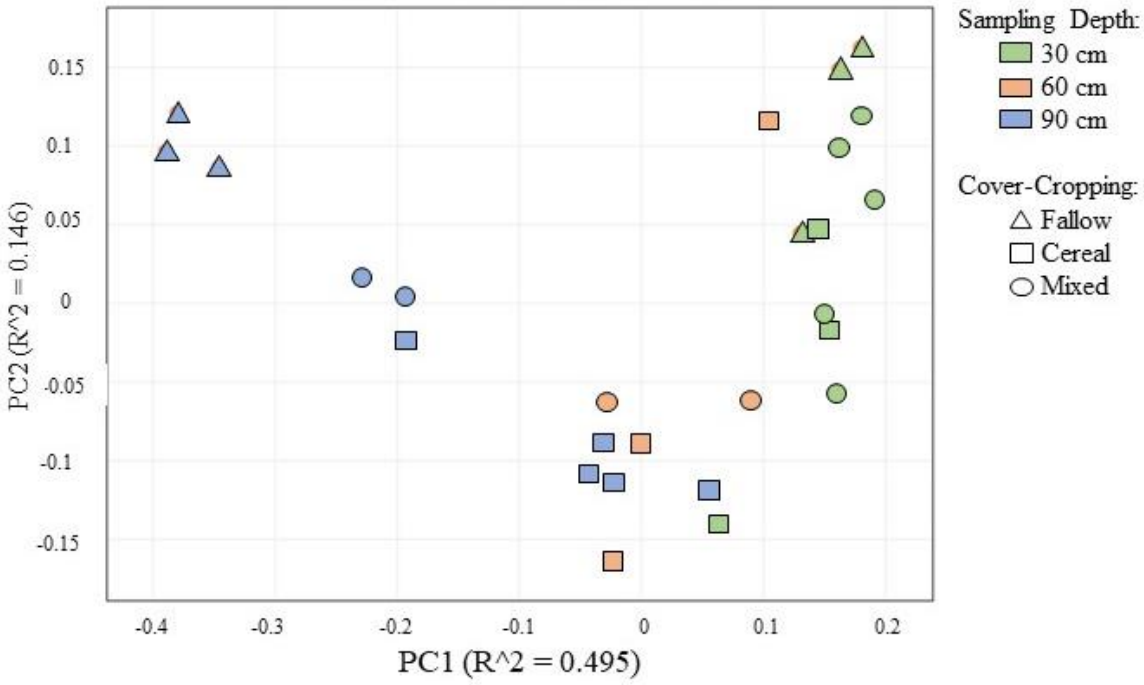

Figure 2.10. The above figure illustrates the results of the Principal Coordinate Analysis conducted on the FT-ICR-MS dataset. The shapes of the data points indicate the cover cropping strategy of the sample plot, and the points are shaded based on the sampling depth of the leachate.

None of the DON samples contained carbohydrates or unsaturated hydrocarbons. There was variability in the other biochemical classes between the leachate samples (Table 2.6).

Van Krevelan diagrams (Figure 2.12) and Pearson correlations (Table 2.7) reveal a similar relationship between sampling depth and the DON composition as was seen in the DOM pool. As sampling depth increased, fewer datapoints clustered in the amino sugar and carbohydrate regions of the Van Krevelan diagrams and a relatively higher proportion of datapoints clustered in the central, lignin region (Figure 2.12). Depth was significantly correlated ($p<0.05$) with a decrease in TC ($r=-0.44$), total peaks ($r=-0.82$), the relative percentage of N containing compounds ($r=-0.80$), and tannins ($r=-0.87$), as well as an increase in the relative percent of amino sugars ($r=-0.46$) (Table 2.7). There was also an increase in the relative percent of lipids ($r=0.56$), lignin ($r=0.36$), and proteins ($r=0.48$) and a decrease in the relative percent of condensed hydrocarbons ($r=-0.77$) with depth (Table 2.7). As with DOM, sampling depth was negatively correlated with photodegradability metrics (average DBEC, $r=-0.82$; percent of compounds with AI> 0.67, $r=-0.62$) (Table 2.7).

There were non-significant decreases in the relative percent of lipids ($F=1.821$, $p=0.206$) and proteins ($F=2.134$, $p=0.171$) (Figure 2.11 a & d) in the N-containing DOM compounds, and an increase in the relative percent of lignin ($F=1.599$, $p=0.249$) (Figure 2.11c). Unlike the overall DOM pool, there was also a decrease in the percentage of condensed hydrocarbons ($F=3.151$, $p=0.097$) (Table 2.6 and Figure 2.11e). Cover cropping did not influence most of the compositional classes, but amino sugars comprised a higher percentage ($F=3.246$, $p=0.083$) of cover cropped plots (Mixed>Cereal>Fallow) (Figure 2.11b). The percentage of highly aromatic, recalcitrant N- containing compounds also decreased over time ($F=1.748$, $p=0.228$) (Figure 2.9b).

Table 2.6: The sample number, sampling date, cover cropping strategy, DOM FT-ICR-MS peaks (indicating the total number of unique compounds), the relative percentage of each compositional class (lignin, condensed hydrocarbons, unclassifiable compounds, lipids, tannins, proteins, and amino sugars), the average DBE/C ratio and the percentage of compounds with an AI> 0.67.

Leachate DON Composition

						Relative Percentage						
#	Depth	Date	Cover	Lig.	Con. Hydro.	Other	Lip.	Tan.	Pro.	Amino Sugars	DBE/C	% AI
15		1	Mixed	74.0	10.3	0.3	0.7	5.8	4.7	4.3	0.5 +/- 0.2	2.5
18			Cereal	77.7	5.7	0.3	1.5	5.4	4.1	5.3	0.5 +/- 0.2	1.8
20			Fallow	77.3	7.5	0.0	1.3	8.0	3.1	2.9	0.5 +/- 0.2	1.8
21		2	Mixed	74.0	9.6	0.1	0.9	6.4	4.2	4.8	0.4 +/- 0.2	2.2
22			Fallow	73.2	9.1	0.5	0.6	7.6	4.5	4.4	0.4 +/- 0.2	2.3
25	30		Mixed	77.7	5.4	0.3	0.8	7.0	4.5	4.3	0.5 +/- 0.2	1.9
28			Cereal	81.1	3.6	0.5	1.0	7.3	4.8	1.7	0.5 +/- 0.1	0.5
31			Mixed	75.1	8.5	0.4	0.8	6.6	3.9	4.7	0.5 +/- 0.2	2.0
32		3	Fallow	80.5	6.7	0.5	1.2	5.5	3.6	2.1	0.5 +/- 0.2	1.9
34			Cereal	82.4	5.4	0.1	0.1	7.1	2.8	2.2	0.5 +/- 0.2	1.1
37			Mixed	77.5	6.1	0.3	1.5	6.5	4.3	3.8	0.5 +/- 0.2	1.5
13		1	Cereal	81.4	2.0	0.7	2.1	4.0	6.6	3.3	0.5 +/- 0.2	0.6
26		2	Mixed	78.0	2.5	0.8	2.3	7.4	3.9	5.2	0.5 +/- 0.2	0.8
29	60		Cereal	87.2	0.8	0.5	0.5	3.4	6.1	1.5	0.5 +/- 0.1	0.4
35		3	Cereal	77.6	6.6	0.4	1.8	5.4	5.2	3.1	0.5 +/- 0.2	1.7
38			Mixed	89.3	0.7	0.2	1.3	4.9	2.6	1.1	0.5 +/- 0.1	0.2
14			Cereal	80.2	2.7	0.7	1.5	3.7	7.1	4.1	0.5 +/- 0.2	0.9
16		1	Fallow	75.8	1.6	3.2	8.6	3.2	6.5	1.1	0.5 +/- 0.2	0.5
17			Cereal	85.2	2.0	0.5	1.0	3.8	5.9	1.8	0.5 +/- 0.2	0.5
19			Cereal	89.5	2.1	0.8	0.0	3.0	2.9	1.7	0.5 +/- 0.1	0.8
23		2	Fallow	81.1	1.3	3.1	6.1	3.1	4.8	0.4	0.5 +/- 0.2	0.4
24	90		Cereal	81.3	2.7	0.2	1.3	4.4	7.8	2.5	0.5 +/- 0.2	1.3
27			Mixed	75.0	2.6	2.0	4.9	2.3	9.1	4.2	0.4 +/- 0.2	0.9
30		3	Cereal	84.3	1.5	0.5	3.8	3.1	5.6	1.2	0.5 +/- 0.1	0.3
33			Fallow	83.5	1.3	2.5	6.8	3.4	2.5	0.0	0.5 +/- 0.2	0.0
36			Cereal	90.9	1.1	0.2	0.7	2.3	3.9	1.0	0.5 +/- 0.1	0.5
39			Mixed	69.8	4.2	2.3	7.6	2.3	9.1	4.9	0.4 +/- 0.2	1.0

Table 2.7: The results of Pearson Correlations comparing the sampling depth, the relative percent of N containing peaks, relative percent of highly photodegradable compounds (AI>0.67), the average DBE/C ratio, the amount of DON, TN, TC, NH_4-N, and NO_3-N, the relative percentage of each compositional class (tannins, lignin, amino sugars, proteins, condensed hydrocarbons, lipids) with the leached DON and the number of N peaks. Bolded values within the chart indicate statistically significant correlations ($p<0.5$).

Leachate DON Pearson Correlations

	DON Frac.	% AI	Avg DBE/C	DON	DIN	TN	TC	NH_4-N	NO_3-N	Tan.	Lig.	Amino Sugars	Pro.	Con Hydro.	Lip.	N peaks
Depth	**-0.80**	**-0.62**	**-0.82**	-0.27	-0.14	-0.15	**-0.44**	-0.10	-0.14	**-0.87**	**0.36**	**-0.46**	**0.48**	**-0.77**	**0.56**	**-0.90**
N peaks	**0.88**	**0.70**	**0.85**	0.14	0.31	0.32	**0.45**	0.24	0.31	**0.83**	-0.30	**0.52**	-0.37	**0.76**	**-0.70**	
Lip.	**-0.68**	**-0.46**	**-0.72**	0.09	-0.24	-0.25	-0.32	-0.18	-0.24	**-0.52**	-0.29	-0.22	**0.41**	-0.37		
Con Hydro.	**0.49**	**0.84**	**0.69**	0.23	-0.03	-0.02	**0.59**	-0.07	-0.03	**0.68**	**-0.67**	**0.63**	-0.22			
Pro.	**-0.47**	-0.14	**-0.70**	-0.03	-0.19	-0.21	-0.25	-0.09	-0.20	**-0.50**	-0.36	0.24				
Amino Sugars	0.14	**0.57**	0.22	0.00	-0.25	-0.26	**0.52**	-0.19	-0.25	**0.42**	**-0.73**					
Lig.	0.02	**-0.50**	-0.04	-0.19	0.24	0.25	-0.34	0.18	0.25	-0.35						
Tan.	**0.74**	**0.57**	**0.80**	-0.01	0.17	0.17	**0.40**	0.16	0.17							
NO_3-N	**0.56**	-0.13	0.23	0.04	**1.00**	**1.00**	-0.18	**0.92**								
NH_4-N	**0.48**	-0.16	0.13	0.02	**0.92**	**0.88**	-0.11									
TC	0.26	**0.49**	**0.42**	0.19	-0.18	-0.18										
TN	**0.57**	-0.11	0.25	0.11	**1.00**											
DIN	**0.56**	-0.13	0.23	0.04												
DON	0.08	0.27	-0.05													
Avg DBEC	**0.83**	**0.62**														
% AI	**0.46**															

Figure 2.11: Leachate DON Interaction Plots

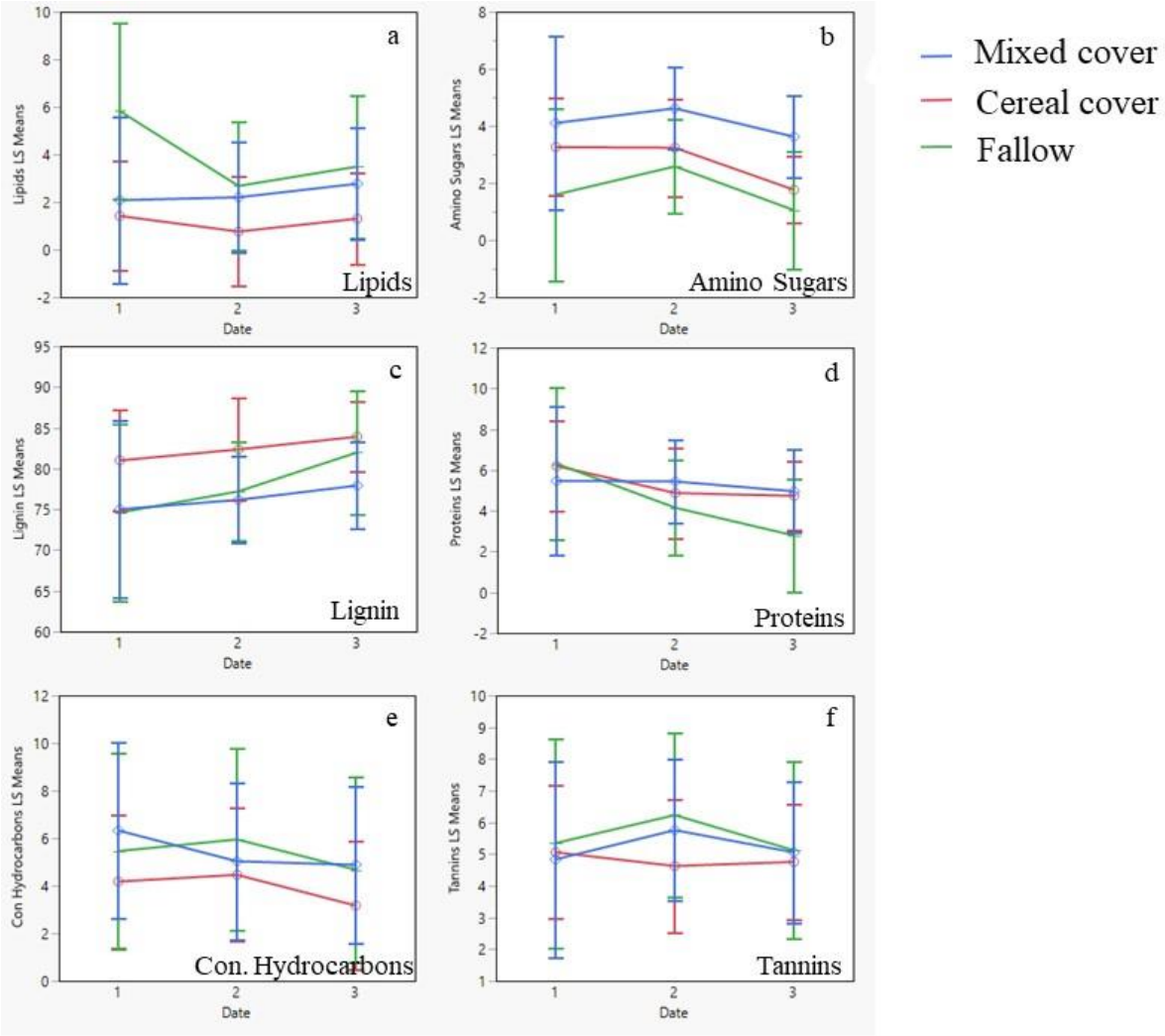

Figure 2.11: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage of each compositional class (lipids, amino sugars, lignin, proteins, condensed hydrocarbons and tannins) within the leachate DON samples.

Figure 2.12: Leachate DON Van Krevelan Diagrams

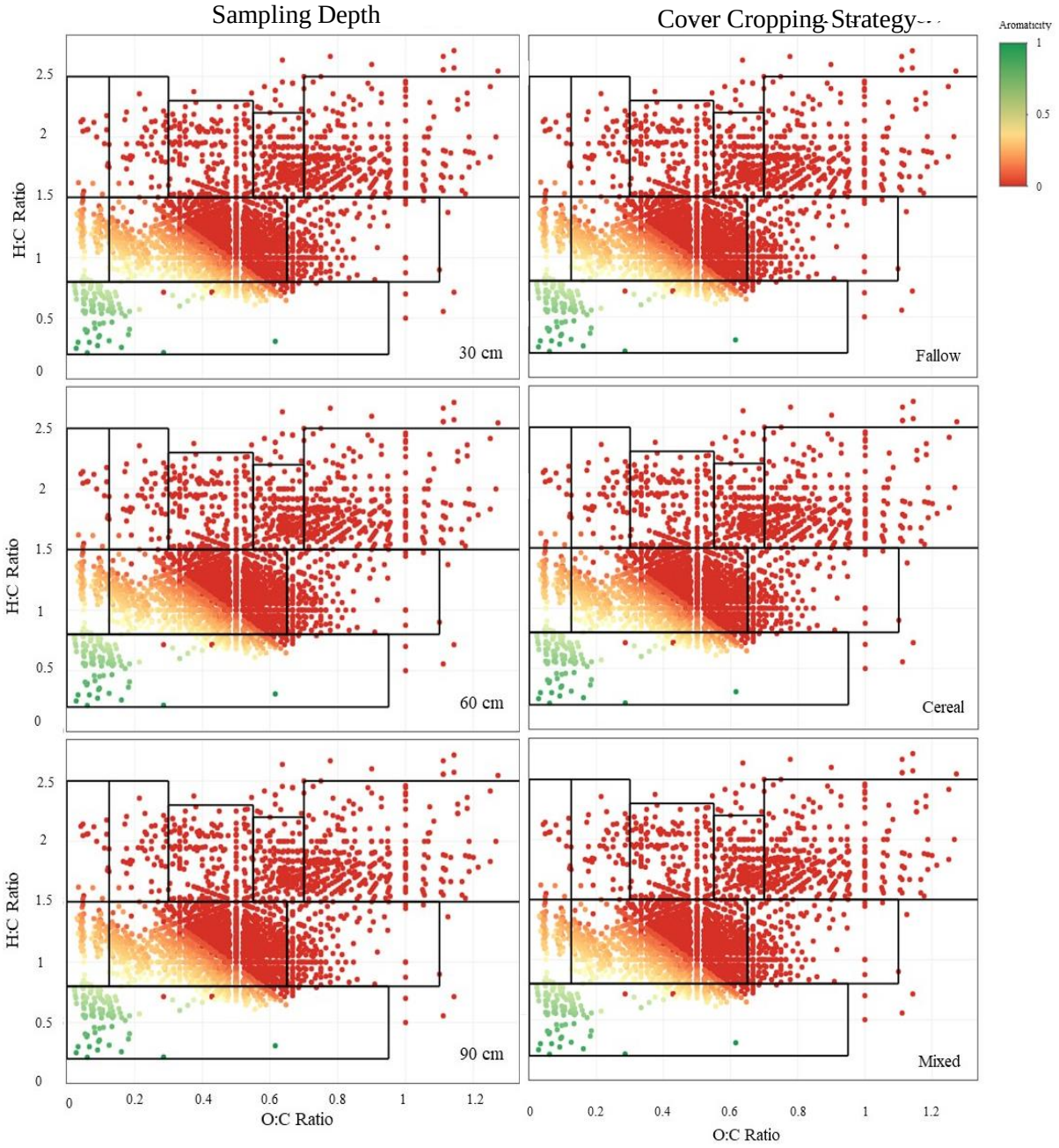

Figure 2.12. The figure is comprised of two sets of Van Krevelan Diagrams illustrating the DON composition of the leachate samples. One separates the DON based on the sampling depth of the leachate, and one separates the samples based upon the cover cropping strategy of the given sample plot. The graphs are also color coded based upon the aromaticity of the compounds (red indicating highly labile and green indicating highly recalcitrant).

Analysis of the DON fraction reveals both a significant temporal effect (F=5.140, p=0.035) and sampling date and cover interaction (F=4.105, p=0.040) (Figure 2.8i).

Soil DOM:

There were differences in DOM composition between the two soil extractions techniques (Table 2.8). Of the classifiable compounds, water extracted DOM was primarily comprised of lignin (11 - 30%), followed by lipids (3-10%), proteins (5 - 22%), tannins (0.6 – 4%), unsaturated hydrocarbons (0.8 – 4%), condensed hydrocarbons (1 - 3%), amino sugars (1 – 3%), and carbohydrates (0.4 – 1%). In comparison, the chloroform extractable DOM was primarily lipids (6 – 29%) followed by proteins (3 – 11%), lignin (2– 9%), unsaturated hydrocarbons (0.3 – 1%), condensed hydrocarbons (0.2 – 2%), amino sugars (0.3 – 1.5%), carbohydrates (0 – 1.6%), and tannins (0 – 1%). A large portion of all samples were not identified in either extraction (water extractable: 39 – 70%; chloroform extractable: 56 – 83%). Comparison via Van Krevelan diagrams visually confirms this difference in DOM composition: the compounds in the water extractable soil DOM are heavily clustered in the center region (lignin) of the plot while the compounds in the chloroform extractable soil DOM are clustered in the upper left region (lipids) of the plot (Figure 2.16).

There was no correlation between microbial biomass N, but microbial biomass C was correlated with changes in DOM composition in both the water and chloroform extractions (Tables 2.9 and 2.10). In water extractable DOM, there was a positive correlation between proteins (r=0.67), amino sugars (r=0.65), and tannins (r=0.65) and a negative correlation with unsaturated hydrocarbons (r=-0.65), carbohydrates (r=-0.65), and photodegradability (% of AI>0.67, r=-0.65) (Table 2.9).

Table 2.8: The sample number, sampling date, plot number, DOM FT-ICR-MS peaks (indicating the total number of unique compounds), extraction method (water or chloroform), cover cropping strategy, the relative percentage of each compositional class (lipids, unsaturated hydrocarbons, condensed hydrocarbons, proteins, amino sugars, carbohydrates, lignin, tannins, and unclassifiable compounds), the average DBE/C ratio and the percentage of compounds with an AI> 0.67.

Soil DOM Composition																
						Relative Percentage										
#	Date	Plot	Peaks	Extract.	Cover	Lip,	UnSat. Hydro.	Con. Hydro.	Pro.	Amino Sugars	Carb.	Lig.	Tan.	Comp. NA	DBE/C	% AI
40		1	4276		Cereal	9.7	3.7	1.9	5.4	0.9	1.2	12.5	0.6	64.1	0.3 +/- 0.2	3.1
41		2	4317		Fallow	7.2	3.7	2.9	5.1	1.0	1.2	14.3	1.5	63.3	0.4 +/- 0.2	4.7
42	1	3	4056		Mixed	5.4	2.3	2.7	4.7	1.2	1.2	11.0	1.5	70.0	0.4 +/- 0.2	5.0
43		4	3127		Fallow	6.5	1.2	1.9	21.5	2.9	0.4	24.9	3.8	37.1	0.4 +/- 0.2	0.4
44		5	3443		Cereal	5.6	0.8	2.3	19.6	2.6	0.6	25.4	4.4	38.8	0.4 +/- 0.2	0.1
45		6	3740	Water	Mixed	5.7	1.8	1.7	19.6	2.9	0.8	23.0	4.0	40.6	0.4 +/- 0.2	0.4
46		1	2771		Cereal	7.2	0.7	1.3	19.7	2.5	0.5	23.9	3.0	41.3	0.3 +/- 0.2	0.7
47		2	2979		Fallow	5.7	1.2	1.9	19.8	2.2	0.4	29.9	4.4	34.5	0.4 +/- 0.20	0.5
48	3	3	9750		Mixed	3.0	0.8	2.3	7.5	1.2	0.7	14.0	2.4	68.0	0.4 +/- 0.2	0.9
49		4	3121		Fallow	5.5	1.2	2.8	19.5	2.7	0.5	29.6	4.2	34.0	0.4 +/- 0.20	0.4
50		5	3228		Cereal	6.8	1.8	1.1	21.6	3.2	0.4	23.4	2.7	39.0	0.3 +/- 0.20	0.3
51		6	3739		Mixed	5.3	1.3	2.5	18.8	2.6	0.6	28.1	4.20	36.7	0.4 +/- 0.20	0.2
40		1	1944		Cereal	5.7	0.8	1.9	3.3	0.6	1.6	2.2	0.6	83.4	0.3 +/- 0.3	3.3
41		2	970		Fallow	23.5	1.1	0.2	5.7	0.3	0.5	5.1	0.1	63.5	0.2 +/- 0.2	0.3
42	1	3	2163		Mixed	18.9	0.9	1.0	10.2	1.5	0.4	6.0	0.8	60.3	0.2 +/- 0.2	1.2
43		4	1987		Fallow	19.8	0.4	0.5	7.6	0.9	0.3	8.0	0.7	62.0	0.2 +/- 0.2	0.8
44		5	1067		Cereal	29.0	0.5	0.3	8.4	1.2	0	3.6	0.4	56.7	0.2 +/- 0.1	0.7
45		6	2090	Chlor.	Mixed	22.9	0.4	1.4	6.3	0.7	0.2	5.6	0.7	62.0	0.2 +/- 0.2	1.7
46		1	758		Cereal	29.8	1.1	0.3	6.6	0.4	0.9	5.4	0	55.5	0.2 +/- 0.2	0.3
47		2	651		Fallow	19.4	0.8	0.2	3.1	0.3	0.3	2.6	0.2	73.3	0.2 +/- 0.2	0.6
48	3	3	1846		Mixed	18.3	0.3	1.8	5.5	0.7	0.2	5.6	1.1	66.4	0.3 +/- 0.2	2.7
49		4	1759		Fallow	20.0	0.4	1.3	7.3	0.9	0.4	5.3	0.6	63.9	0.2 +/- 0.2	2.0
50		5	1163		Cereal	17.1	0.3	1.7	4.8	0.7	0.4	4.7	1.0	69.2	0.3 +/- 0.2	3.3
51		6	1882		Mixed	16.7	1.6	1.5	11.1	1.3	0.5	8.7	0.4	58.2	0.3 +/- 0.2	1.0

Table 2.9: The results of Pearson Correlations comparing the sulfur containing compounds, the relative percentage of each compositional class in the soil water extractions (lipids, unsaturated hydrocarbons, condensed hydrocarbons, proteins, amino sugars, carbohydrates, lignin, tannins, and unknown compounds), the average DBE/C ratio, the relative percent of highly photodegradable compounds (AI>0.67), water extractable NO_3-N, NH_4-N, TC, TN, DIN, and DON, KCl extractable NO_3-N, NH_4-N, PMN, K_2SO_4 TC and TN, and microbial biomass C and N. Bolded values within the chart indicate statistically significant correlations ($p<0.5$).

Soil Water DOM Pearson Correlations

	Micro. Bio. N	Micro. Bio. C	K_2SO_4 TN	K_2SO_4 TC	PMN	KCl NH_4-N	KCl NO_3-N	DON	DIN	Water TN	Water TC	Water NH_4-N	Water NO_3-N	% AI	DBEC
CHOS	-0.30	**-0.74**	-0.49	**0.59**	**0.59**	-0.26	-0.39	-0.40	-0.36	-0.40	0.23	-0.39	-0.36	**0.93**	0.06
CHONS	-0.29	**-0.74**	-0.51	**0.59**	**0.58**	-0.34	-0.40	-0.42	-0.38	-0.43	0.26	-0.41	-0.38	**0.94**	0.10
CHOSP	-0.30	**-0.78**	-0.52	0.58	0.44	-0.35	-0.40	-0.47	-0.37	-0.46	0.18	-0.33	-0.37	**0.85**	0.02
CHONSP	-0.22	**-0.71**	-0.57	**0.58**	**0.58**	-0.41	-0.47	-0.39	-0.46	-0.42	0.29	-0.57	-0.46	**0.93**	0.07
Lip.	-0.24	-0.39	-0.35	0.33	0.23	-0.03	-0.31	-0.22	-0.29	-0.24	-0.09	-0.39	-0.29	0.32	**-0.69**
UnSat. Hydro.	-0.26	**-0.65**	-0.43	0.57	0.52	-0.10	-0.33	-0.41	-0.30	-0.40	0.14	-0.29	-0.30	**0.78**	-0.08
Con. Hydro.	0.07	-0.37	0.01	0.30	0.30	-0.10	0.06	-0.08	0.08	-0.05	0.22	-0.01	0.09	0.47	**0.81**
Pro.	0.27	**0.67**	0.40	-0.44	-0.38	0.48	0.29	0.40	0.27	0.39	-0.31	0.19	0.27	**-0.88**	-0.46
Amino Sugars	0.33	**0.65**	0.43	-0.38	-0.35	0.53	0.34	0.45	0.32	0.43	-0.39	0.15	0.32	**-0.82**	-0.48
Carb.	-0.08	**-0.65**	-0.50	**0.62**	0.49	-0.23	-0.38	-0.42	-0.37	-0.42	0.34	-0.42	-0.36	**0.90**	0.23
Lig.	0.18	0.57	0.47	-0.51	-0.40	0.52	0.35	0.44	0.35	0.43	-0.26	0.33	0.34	**-0.82**	-0.20
Tan.	0.45	**0.66**	0.35	-0.36	-0.29	0.48	0.23	0.28	0.22	0.28	-0.05	0.23	0.22	**-0.82**	0.02
Comp. NA	-0.23	**-0.58**	-0.40	0.42	0.34	-0.54	-0.29	-0.40	-0.28	-0.38	0.29	-0.21	-0.28	**0.82**	0.39
DBEC	-0.03	-0.12	0.14	-0.06	0.06	-0.21	0.15	-0.04	0.16	0.00	0.28	0.29	0.15	0.25	
% AI	-0.24	**-0.69**	-0.47	0.57	**0.66**	-0.26	-0.37	-0.30	-0.36	-0.32	0.35	-0.40	-0.35		
Water NO_3-N	-0.24	0.30	**0.98**	**-0.67**	**-0.65**	0.30	**1.00**	**0.84**	**1.00**	**0.90**	**-0.65**	**0.77**			
Water NH_4-N	-0.46	0.15	**0.77**	**-0.76**	**-0.71**	0.19	**0.76**	0.55	**0.78**	**0.61**	-0.50				
Water TC	0.43	0.13	**-0.62**	0.52	**0.60**	-0.03	**-0.64**	-0.51	**-0.65**	-0.56					
Water TN	-0.22	0.33	**0.92**	**-0.70**	-0.57	0.48	**0.90**	**0.99**	**0.89**						
DIN	-0.25	0.29	**0.98**	**-0.67**	**-0.66**	0.30	**1.00**	**0.84**							
DON	-0.21	0.33	**0.87**	**-0.68**	-0.53	0.51	**0.85**								
KCl NO_3-N	-0.24	0.33	**0.99**	**-0.69**	**-0.66**	0.29									
KCl NH_4-N	0.29	0.31	0.33	-0.13	-0.13										
PMN	0.23	-0.29	**-0.69**	**0.78**											
K_2SO_4 TC	0.50	-0.36	**-0.76**												
K_2SO_4 TN	-0.22	0.42													
Micro. Bio. C	0.47														

Table 2.10: The results of Pearson Correlations comparing the sulfur containing compounds, the relative percentage of each compositional class in the soil chloroform extractions (lipids, unsaturated hydrocarbons, condensed hydrocarbons, proteins, amino sugars, carbohydrates, lignin, tannins, and unknown compounds), the average DBE/C ratio, the relative percent of highly photodegradable compounds (AI>0.67), water extractable NO_3-N, NH_4-N, TC, TN, DIN, and DON, KCl extractable NO_3-N, NH_4-N, PMN, K_2SO_4 TC and TN, and microbial biomass C and N. Bolded values within the chart indicate statistically significant correlations ($p<0.5$).

Soil Chloroform DOM Pearson Correlations

	Micro. Bio. N	Micro. Bio. C	K_2SO_4 TN	K_2SO_4 TC	PMN	KCl NH_4-N	KCl NO_3-N	DON	DIN	Water TN	Water TC	Water NH_4-N	Water NO_3-N	% AI	DBEC
CHOS	**-0.59**	-0.16	0.00	-0.21	-0.05	-0.56	0.05	-0.05	0.02	-0.04	-0.10	0.10	0.02	0.27	0.32
CHONS	0.34	-0.03	0.19	0.30	-0.14	0.22	0.31	0.12	0.33	0.16	-0.12	0.16	0.33	**0.69**	**0.62**
CHOSP	-0.29	-0.49	-0.18	0.13	-0.31	-0.31	-0.08	-0.27	-0.05	-0.23	-0.21	0.12	-0.05	**0.71**	**0.77**
CHONSP	-0.30	-0.13	0.19	-0.11	-0.44	-0.17	0.29	0.06	0.30	0.11	-0.34	0.38	0.30	**0.93**	**0.84**
Lip.	0.46	**0.62**	0.03	-0.08	0.19	0.05	-0.07	0.14	-0.12	0.09	0.32	-0.42	-0.11	**-0.71**	**-0.87**
UnSat. Hydro.	-0.47	**-0.63**	-0.22	-0.08	0.10	-0.04	-0.25	-0.06	-0.23	-0.10	-0.11	-0.16	-0.23	-0.44	-0.04
Cond. Hydro.	-0.17	-0.21	0.34	-0.16	**-0.55**	0.12	0.43	0.17	0.45	0.23	-0.52	0.51	0.45	**0.89**	**0.89**
Pro.	0.26	-0.21	0.04	0.11	0.08	0.13	0.03	0.18	0.02	0.15	-0.19	-0.23	0.03	-0.38	-0.10
Amino Sugars	0.36	-0.32	-0.02	0.27	0.05	0.09	0.02	0.08	0.04	0.07	-0.24	-0.11	0.04	0.01	0.17
Carb.	-0.53	**-0.58**	-0.19	0.04	-0.16	-0.17	-0.13	-0.09	-0.11	-0.10	-0.20	-0.07	-0.11	0.33	0.53
Lig.	-0.11	-0.06	0.19	-0.17	-0.07	0.05	0.17	0.20	0.15	0.20	-0.32	0.03	0.15	-0.26	0.08
Tan.	0.08	0.06	0.28	-0.01	-0.27	-0.06	0.37	0.11	0.38	0.17	-0.28	0.41	0.38	**0.76**	**0.62**
Comp. NA	-0.38	-0.32	-0.10	0.07	-0.10	-0.09	-0.03	-0.23	0.01	-0.19	-0.03	0.35	0.00	**0.64**	0.56
DBEC	-0.38	-0.49	0.14	0.00	-0.38	-0.09	0.25	0.00	0.28	0.06	-0.52	0.38	0.28	**0.84**	
% AI	-0.17	-0.08	0.32	-0.11	-0.48	0.00	0.42	0.13	0.44	0.20	-0.46	0.49	0.44		
Water NO_3-N	-0.24	0.30	**0.98**	**-0.67**	**-0.65**	0.30	**1.00**	**0.84**	**1.00**	**0.90**	**-0.65**	**0.77**			
Water NH_4-N	-0.46	0.15	**0.77**	**-0.76**	**-0.71**	0.19	**0.76**	**0.55**	**0.78**	**0.61**	-0.50				
Water TC	0.43	0.13	**-0.62**	**0.52**	**0.60**	-0.03	**-0.64**	**-0.51**	**-0.65**	-0.56					
Water TN	-0.22	0.33	**0.92**	**-0.70**	-0.57	0.48	**0.90**	**0.99**	**0.89**						
DIN	-0.25	0.29	**0.98**	**-0.67**	**-0.66**	0.30	**1.00**	**0.84**							
DON	-0.21	0.33	**0.87**	**-0.68**	-0.53	0.51	**0.85**								
KCl NO_3-N	-0.24	0.33	**0.99**	**-0.69**	**-0.66**	0.29									
KCl NH_4-N	0.29	0.31	0.33	-0.13	-0.13										
PMN	0.23	-0.29	**-0.69**	**0.78**											
K_2SO_4 TC	0.50	-0.36	**-0.76**												
K_2SO_4 TN	-0.22	0.42													
Micro. Bio. C	0.47														

Figure 2.13: Soil Water DOM Interaction Plots

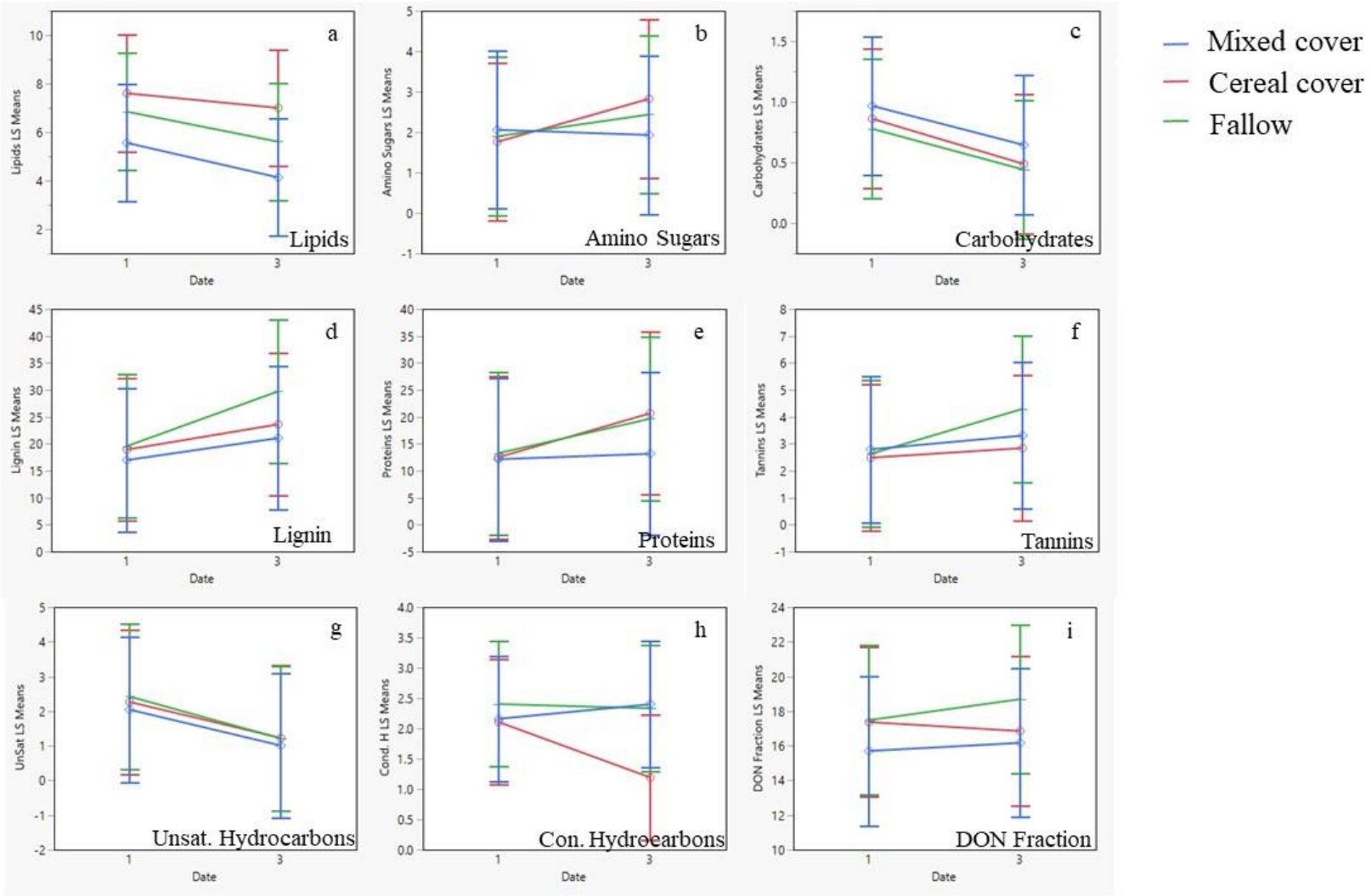

Figure 2.13: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage of each compositional class (lipids, amino sugars, carbohydrates, lignin, proteins, tannins, unsaturated hydrocarbons, condensed hydrocarbons, and the relative percentage of N containing compounds) within the leachate DOM samples.

Figure 2.14: Soil Chloroform DOM Interaction Plots

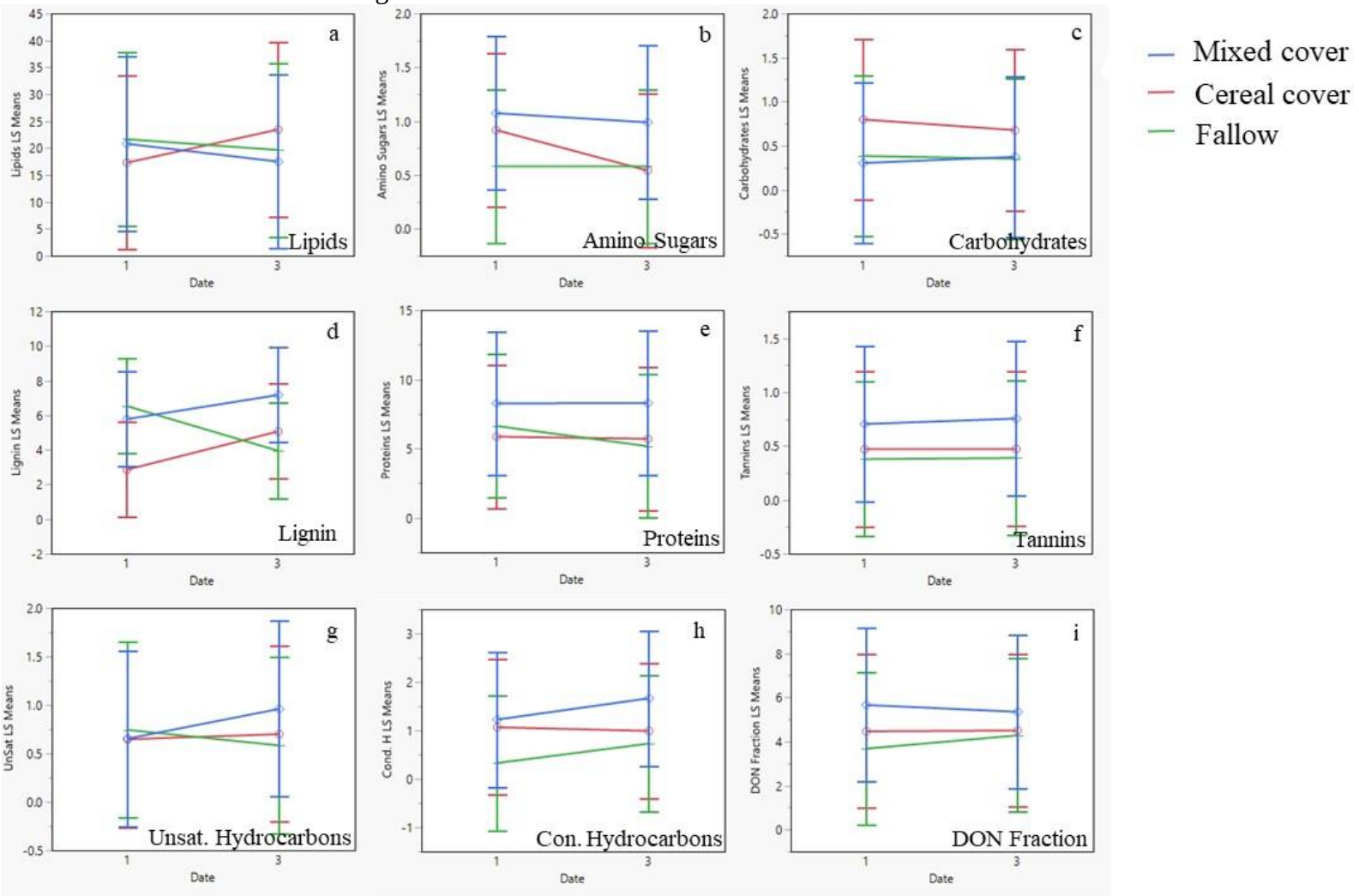

Figure 2.14: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage of each compositional class (lipids, amino sugars, carbohydrates, lignin, proteins, tannins, unsaturated hydrocarbons, condensed hydrocarbons, and the relative percentage of N containing compounds) within the leachate DOM samples.

Figure 2.15: Soil DOM and DON Photodegradability Interaction Plots

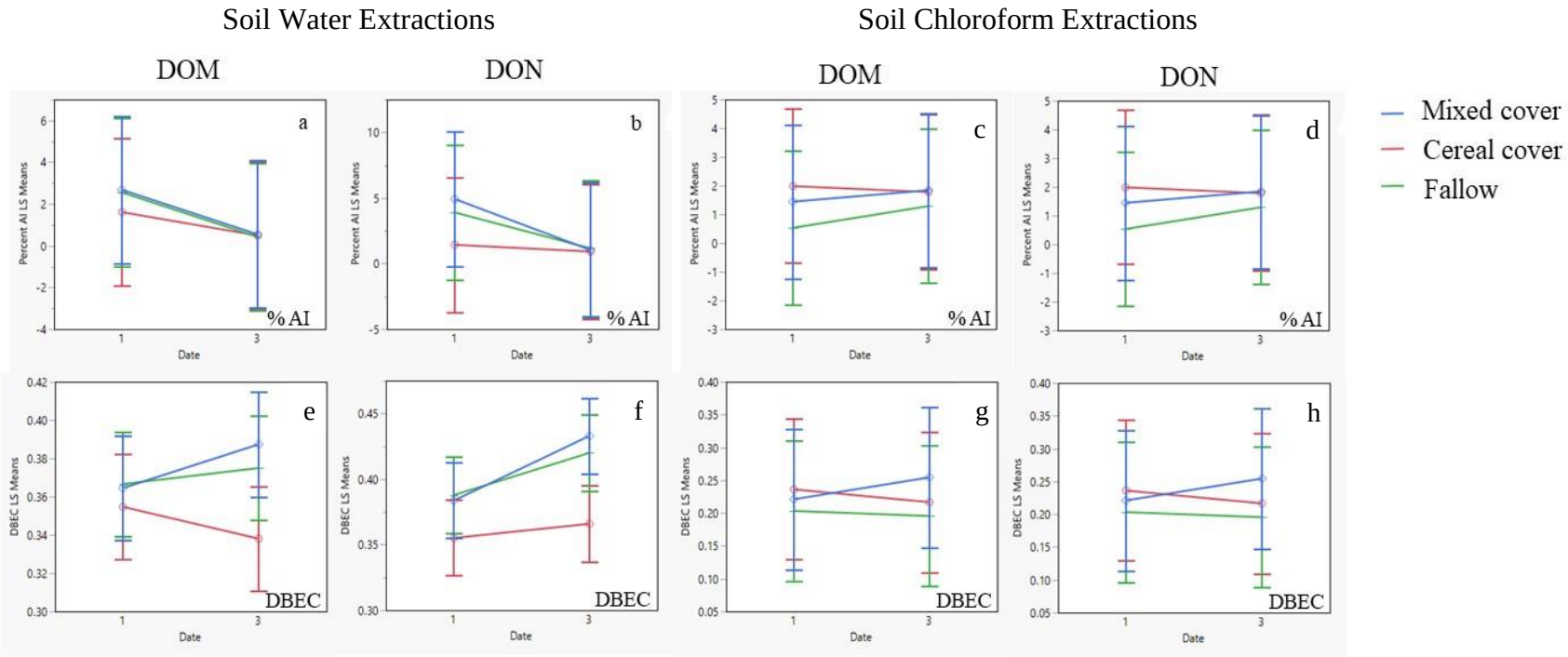

Figure 2.15: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage highly photodegradable compounds (AI>0.67) and the average DBE/C ratio within the Soil Water extractable and Soil Chloroform extractable DOM and DON samples.

Figure 2.16: Soil Extractable Organic Matter Van Krevelan Diagrams
(by Cover Cropping Strategy)

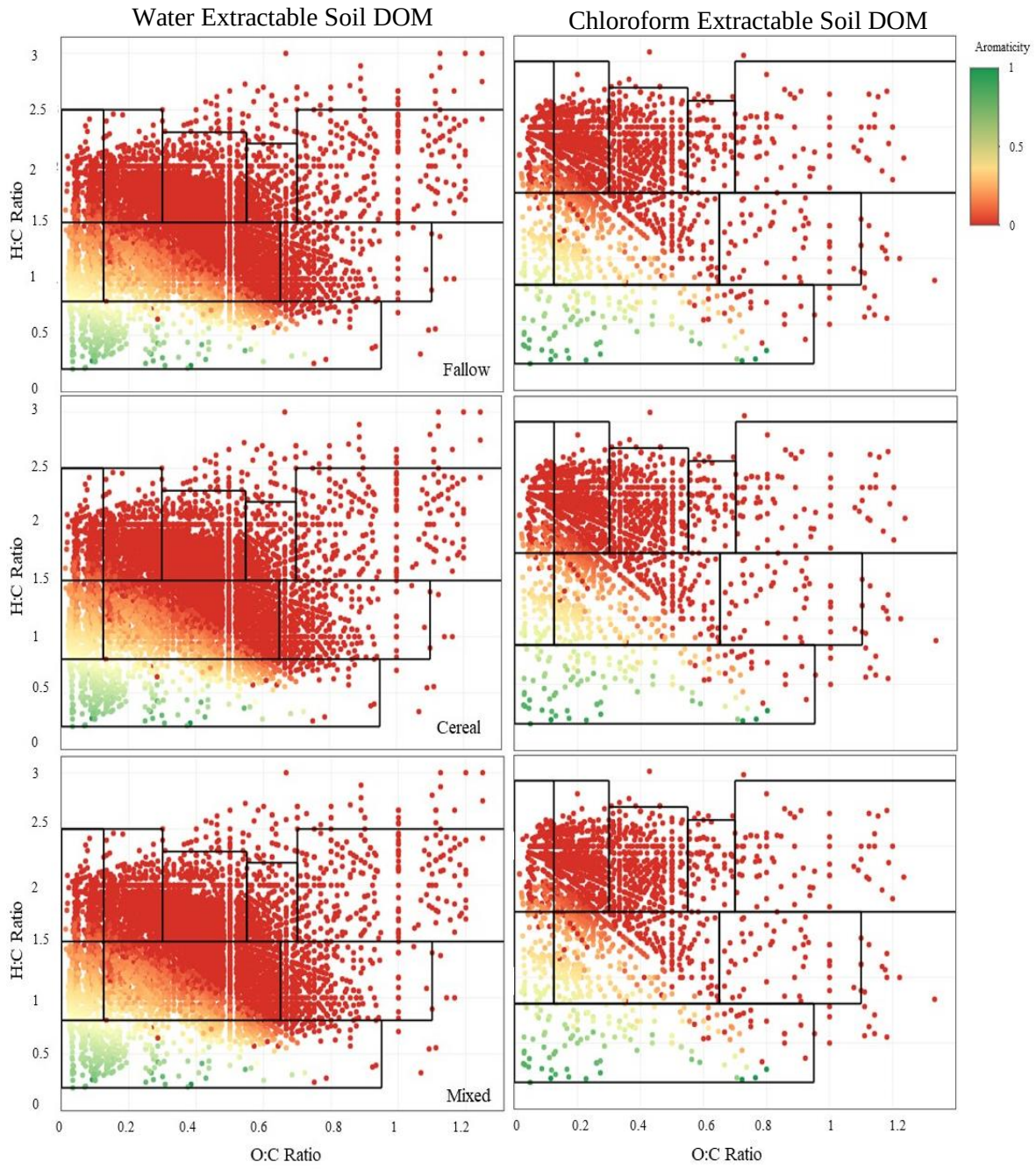

Figure 2.16. The above figure is comprised of two sets of Van Krevelan Diagrams illustrating the composition of the extractable soil organic matter composition of the leachate samples. One set represents the water extractable DOM pool while the other set represents the chloroform extractable DOM pool. The graphs are also color coded based upon the aromaticity of the compounds (red indicating highly labile and green indicating highly recalcitrant).

In the chloroform extractions a similar decrease in unsaturated hydrocarbons (r=-0.63) and carbohydrates (r=0.58) was reported, along with a positive correlation with the relative percentage of lipids (r=0.62) (Table 2.10). Potentially mineralizable N was associated with a shift in composition as well, significantly correlated with an increase in S containing compounds (CHOS, r=0.59; CHONS, r=0.60; CHONSP, r=0.58) as well as with an increase in photodegradability (relative percentage AI>0.67, r=0.66) (Table 2.10).

There was no temporal change in the composition of soil DOM (Figures 2.13 and 2.14) . The only trend visible in the chloroform extractable DOM pool was an increase in the relative percentage of lignin in cover cropped plots, and a decrease in the lignin pool in the fallow plots (sampling date* cover, F=4.645, p=0.121) (Table 2.8 and Figure 2.14d). Though not significant, cover had a slight impact on the relative percentages of chloroform extractable lignin (F=1.940, p=0.288), condensed hydrocarbons (F=5.028, p=0.110), and proteins (F=1.840, p=0.301) (Figure 2.14 d, h, & e). In each of these examples, the cereal and fallow plots had similar values, while the mixed cover plots reported higher relative percentages for the given compound classes. Clearer trends were observed in the water extractable DOM pool (Table 2.8 and Figure 2.13). Though non-significant, there was a slight decrease in the relative percentage of water extractable lipids (F= 2.426, p=0.217), amino sugars (F=2.667, p=0.201), carbohydrates (F=3.282, p=0.168), unsaturated hydrocarbons (F=1.905, p=0.261) (Figure 2.13 a, b, c, & g) and the overall percentage of highly aromatic compounds (F=2.949, p=0.184) (Figure 2.15a). There was also a slight increase in lignin (F=4.741, p=0.118) and proteins (F=2.216, p=0.233) (Figure 2.15 d & e). Cover did not have much impact on the DOM composition, and Van Krevelan diagrams (Figure 2.16) of the DOM under the three cover classes reveal similar compositions among the treatments.

There was considerable variability in PCoA coordinates that represented the June water extractable DOM samples, but the July water extractable DOM coordinates were tightly clustered (Figure 2.17). The influence of the cover cropping on the DOM composition may have lessened throughout the growing season. There was evidence for more clustering by cover cropping in the PCoA of chloroform extractable DOM (Figure 2.18). There coordinates varied more between fallow and cereal covered plots, with less variability in the mixture. This trend is presumably due to the inclusion of legumes in the mixed cover crop.

Soil DON:

There were similar temporal trends in the DON composition as to the overall DOM pool (Table 2.11), but nothing of statistical significance in the chloroform extractable pool (Figure 2.20). In the water extractable pool there was a significant increase in the relative percentage of lignin ($F=11.467$, $p=0.043$) and a slight increase in tannins ($F=7.345$, $p=0.073$) over time (Figure 2.19 c & f). Though not significant, there was a decrease in lipids ($F=4.362$, $p=0.128$) (Figure 2.19a) and the percentage of aromatic compounds ($F=3.420$, $p=0.162$) (Figure 2.15b). Cover class had no clear impact on DON composition.

There was no correlations between either water or chloroform extractable DON and either cover cropping strategy (Figures 2.19 and 2.20, Tables 2.12 and 2.13) but there was significantly more N peaks ($p<0.05$) from plots cover cropped with legumes (Figure 2.14i). There was also a significant relationship between microbial biomass C and water extractable DON composition ($p<0.05$): a decrease in condensed hydrocarbons ($r=-0.59$) and lipids ($r=-0.75$), an increase in in proteins ($r=0.70$) and amino sugars ($r=0.73$) (Table 2.12).

Figure 2.17: Soil Water Extractable DOM PCoA

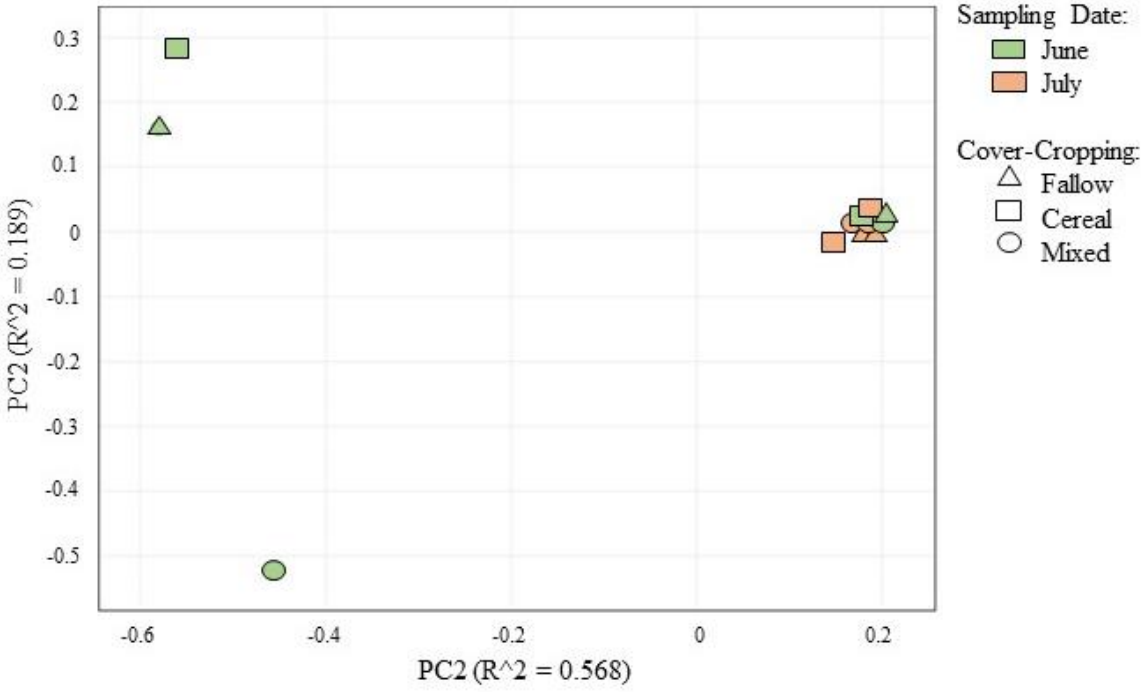

Figure 2.17. The above figure illustrates the results of the Principal Coordinate Analysis conducted on the soil water extractable DOM FT-ICR-MS dataset. The shapes of the data points indicate the cover cropping strategy of the sample plot, and the points are shaded based on the sampling date.

Figure 2.18 Soil Chloroform Extractable DOM PCoA

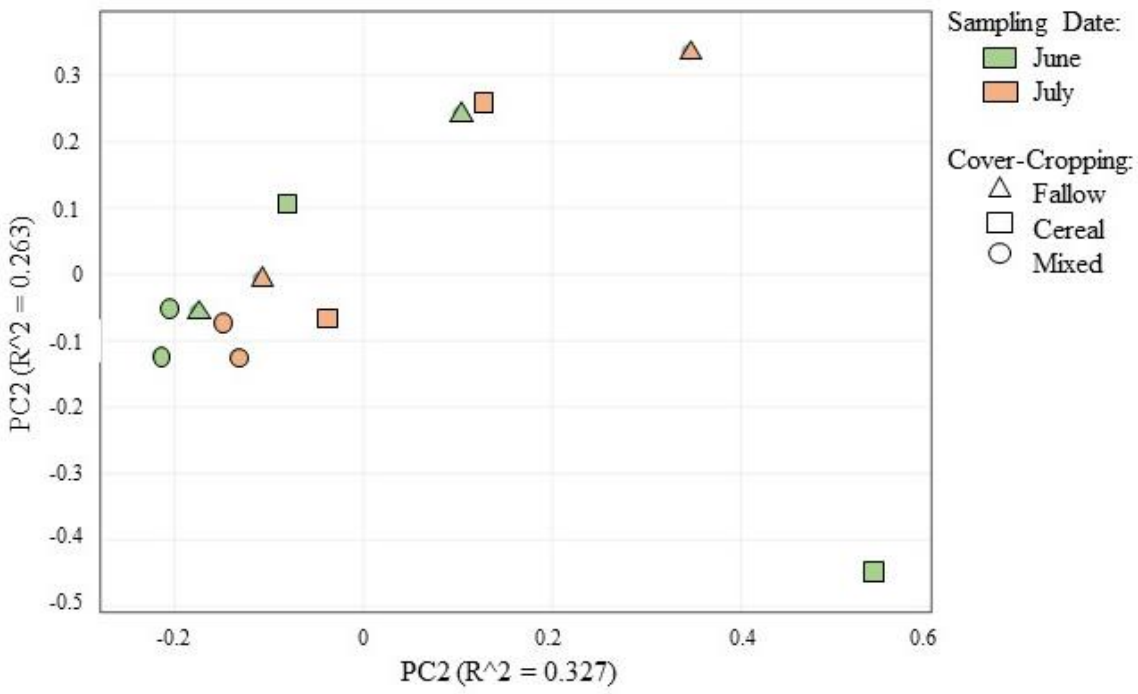

Figure 2.18. The above figure illustrates the results of the Principal Coordinate Analysis conducted on the soil chloroform extractable DOM FT-ICR-MS dataset. The shapes of the data points indicate the cover cropping strategy of the sample plot, and the points are shaded based on the sampling date.

Table 2.11: The sample number, sampling date, plot number, DOM FT-ICR-MS peaks (indicating the total number of unique compounds), extraction method (water or chloroform), cover cropping strategy, the relative percentage of each compositional class (lignin, condensed hydrocarbons, unclassifiable compounds, lipids, proteins, and amino sugars), the average DBE/ ration and the percentage of compounds with an AI> 0.67.

Soil DON Composition

					Relative Percentage								
#	**Date**	**Plot**	**Cover**	**Extract.**	**Lig.**	**Con. Hydro.**	**Other**	**Lip.**	**Tan.**	**Pro.**	**Amino Sugars**	**DBE/C**	**% AI**
40		1	Cereal		24.0	6.0	3.9	28.2	0.5	16.9	3.3	0.3 +/- 0.2	2.7
41		2	Fallow		32.2	11.4	3.7	18.9	1.5	11.8	3.0	0.4 +/- 0.2	6.7
42	1	3	Mixed		30.4	13.1	5.4	19.1	2.0	11.6	4.7	0.4 +/- 0.2	8.7
43		4	Fallow		36.9	3.0	1.0	6.2	2.1	32.9	10.8	0.4 +/- 0.2	1.1
44		5	Cereal		38.6	2.0	1.0	6.5	3.5	32.8	9.3	0.4 +/- 0.2	0.2
45		6	Mixed	Water	33.8	2.5	0.8	5.4	4.7	32.0	9.5	0.4 +/- 0.2	1.1
46		1	Cereal		38.7	3.9	1.1	8.9	1.7	30	9.35	0.4 +/- 0.2	1.3
47		2	Fallow		46.2	3.8	0.8	4.4	3.2	27.1	7.8	0.4 +/- 0.2	1.5
48	3	3	Mixed		45.1	8.4	1.0	7.8	5.1	16.8	6.7	0.4 +/- 0.2	2.0
49		4	Fallow		46.8	5.2	1.0	3.4	4.2	24.4	8.3	0.4 +/- 0.2	0.8
50		5	Cereal		35.1	2.2	0.5	5.6	3.1	32.4	11.4	0.4 +/- 0.2	0.5
51		6	Mixed		47.2	4.6	0.7	4.1	5.3	23.2	7.6	0.4 +/- 0.2	0.1
40		1	Cereal		6.8	18.8	3.4	22.2	9.4	18.0	6.8	0.3 +/- 0.3	3.3
41		2	Fallow		12.5	0	0	37.5	4.2	37.5	4.2	0.2 + /- 0.2	0.3
42	1	3	Mixed		22.0	7.6	3.8	33.3	9.9	9.1	7.6	0.2 +/- 0.2	1.2
43		4	Fallow		25.8	4.1	4.1	36.1	10.3	12.4	4.1	0.2 +/- 0.2	0.8
44		5	Cereal		22.6	0	3.2	45.2	9.7	16.1	3.2	0.2 +/- 0.1	0.7
45		6	Mixed	Chlor.	17.4	9.2	4.6	37.6	11.9	10.0	3.7	0.2 +/- 0.0	1.7
46		1	Cereal		10.0	0	0	30	0	45	0	0.2 +/- 0.2	0.3
47		2	Fallow		13.3	6.7	6.7	26.7	6.7	20	6.7	0.2 +/- 0.2	0.6
48	3	3	Mixed		23.4	12.3	6.1	26.3	14.9	11.4	3.5	0.3 +/- 0.2	2.7
49		4	Fallow		14.6	7.3	6.4	39.1	9.1	13.6	4.6	0.2 +/- 0.2	2.0
50		5	Cereal		21.6	9.5	8.1	28.4	13.5	10.8	2.7	0.3 +/- 0.2	3.3
51		6	Mixed		20.0	4.7	0	29.4	4.7	14.1	11.8	0.3 +/- 0.2	1.0

Table 2.12: The results of Pearson Correlations comparing the number of N peaks, the relative percentage of each compositional class (lignin, condensed hydrocarbons, lipids tannins, proteins, and amino sugars), the average DBE/C ratio, the relative percent of highly photodegradable compounds (AI>0.67), water extractable NO_3-N, NH_4-N, TC, TN, DIN, and DON, KCl extractable NO_3-N, NH_4-N, PMN, K_2SO_4 extractable TC and TN, and microbial biomass C and N. Bolded values within the chart indicate statistically significant correlations ($p<0.5$).

Soil Water DON Pearson Correlations

	DON Frac.	Micro. Bio. N	Micro. Bio. C	K_2SO_4 TN	K_2SO_4 TC	PMN	KCl NH_4-N	KCl NO_3-N	DON	DIN	Water TN	Water TC	Water NH_4-N	Water NO_3-N	% AI	Avg. DBEC
N Peaks	-0.48	-0.20	-0.20	0.07	-0.07	-0.23	-0.52	0.13	-0.27	0.14	-0.19	-0.01	0.34	0.14	0.13	0.53
Lig.	0.19	-0.06	0.46	**0.63**	**-0.74**	-0.55	0.18	0.53	0.48	0.52	0.50	-0.20	**0.63**	0.52	-0.52	**0.75**
Cond. Hydro	-0.32	-0.31	**-0.59**	-0.21	0.30	0.45	-0.29	-0.12	-0.11	-0.12	-0.11	0.30	-0.13	-0.12	**0.92**	0.38
Lip.	-0.18	-0.25	**-0.75**	-0.58	**0.60**	0.44	-0.39	-0.46	-0.47	-0.44	-0.47	0.22	-0.42	-0.44	**0.68**	-0.38
Tan.	0.00	0.26	0.45	0.51	-0.46	-0.52	0.33	0.45	0.28	0.45	0.32	-0.20	0.53	0.44	-0.49	**0.63**
Pro.	0.22	0.41	**0.70**	0.19	-0.25	-0.29	0.32	0.09	0.18	0.07	0.16	-0.20	-0.01	0.07	**-0.80**	-0.38
Amino Sugar	0.07	0.31	**0.73**	0.40	-0.41	-0.37	0.31	0.31	0.39	0.28	0.38	-0.33	0.16	0.28	**-0.72**	-0.17
DBEC	-0.02	-0.21	0.07	0.47	-0.46	-0.23	0.04	0.44	0.31	0.45	0.35	0.01	**0.58**	0.44	0.08	
% AI	-0.29	-0.17	-0.56	-0.42	0.51	**0.71**	-0.17	-0.34	-0.20	-0.34	-0.24	0.44	-0.38	-0.34		
Water NO_3-N	0.24	-0.24	0.30	**0.98**	**-0.67**	**-0.65**	0.30	**1.00**	**0.84**	**1.00**	**0.90**	**-0.65**	**0.77**			
Water NH_4-N	0.07	-0.46	0.15	**0.77**	**-0.76**	**-0.71**	0.19	**0.76**	0.55	**0.78**	**0.61**	-0.50				
Water TC	-0.37	0.43	0.13	**-0.62**	0.52	**0.60**	-0.03	**-0.64**	-0.51	**-0.65**	-0.56					
Water TN	0.27	-0.22	0.33	**0.92**	**-0.70**	-0.57	0.48	**0.90**	**0.99**	**0.89**						
DIN	0.24	-0.25	0.29	**0.98**	**-0.67**	**-0.66**	0.30	**1.00**	**0.84**							
DON	0.27	-0.21	0.33	**0.87**	**-0.68**	-0.53	0.51	**0.85**								
KCl NO_3-N	0.21	-0.24	0.33	**0.99**	**-0.69**	**-0.66**	0.29									
KCl NH_4-N	0.48	0.29	0.31	**0.33**	-0.13	-0.13										
PMN	-0.05	0.23	-0.29	**-0.69**	**0.78**											
K_2SO_4 TC	-0.10	0.50	-0.36	**-0.76**												
K_2SO_4 TN	0.25	-0.22	0.42													
Micro. Bio. C	-0.10	0.47														
Micro. Bio. N	-0.06															

Table 2.13: The results of Pearson Correlations comparing the number of N peaks, the relative percentage of each compositional class (lignin, condensed hydrocarbons, lipids tannins, proteins, and amino sugars), the average DBE/C ratio, the relative percent of highly photodegradable compounds (AI>0.67), water extractable NO_3-N, NH_4-N, TC, TN, DIN, and DON, KCl extractable NO_3-N, NH_4-N, PMN, K_2SO_4 extractable TC and TN, and microbial biomass C and N. Bolded values within the chart indicate statistically significant correlations ($p<0.5$).

Soil Chloroform DON Pearson Correlations

	DON Frac.	Micro. Bio. N	Micro. Bio. C	K_2SO_4 TN	K_2SO_4 TC	PMN	KCl NH_4-N	KCl NO_3-N	DON	DIN	Water TN	Water TC	Water NH_4-N	Water NO_3-N	% AI	DBEC
N Peaks	**0.92**	0.06	-0.29	0.09	0.22	-0.17	0.15	0.20	0.07	0.22	0.10	-0.17	0.21	0.22	**0.62**	**0.76**
Lig.	0.30	0.31	0.18	0.10	0.04	0.06	-0.14	0.10	0.00	0.08	0.02	-0.17	0.07	0.08	-0.01	-0.03
Cond. Hydro	**0.73**	-0.20	-0.29	0.01	0.05	-0.34	0.02	0.11	-0.10	0.14	-0.05	-0.14	0.42	0.14	**0.87**	**0.83**
Other	0.52	0.12	0.42	0.41	-0.21	-0.26	0.26	0.44	0.30	0.45	0.34	-0.09	**0.58**	0.44	**0.59**	0.21
Lip.	-0.24	**0.76**	0.40	0.00	0.40	0.42	0.22	-0.03	0.00	-0.04	-0.01	0.26	-0.47	-0.04	-0.48	-0.57
Tan.	**0.72**	0.30	0.18	0.13	0.16	-0.10	-0.03	0.21	-0.09	0.22	-0.03	-0.08	0.31	0.21	**0.69**	0.45
Pro.	**-0.71**	-0.32	0.02	-0.14	-0.14	0.14	-0.27	-0.19	-0.02	-0.22	-0.06	0.19	-0.36	-0.22	-0.52	-0.46
Amino Sugar	0.16	-0.24	**-0.69**	-0.12	0.08	0.02	0.18	-0.10	-0.12	-0.05	-0.11	-0.18	0.24	-0.06	0.01	0.36
DBEC	**0.82**	-0.38	-0.49	0.14	0.00	-0.38	-0.09	0.25	0.00	0.28	0.06	-0.52	0.38	0.28	**0.84**	
% AI	**0.82**	-0.17	-0.08	0.32	-0.11	-0.48	0.00	0.42	0.13	0.44	0.20	-0.46	0.49	0.44		
Water NO_3-N	0.48	-0.24	0.30	**0.98**	**-0.67**	**-0.65**	0.30	**1.00**	**0.84**	**1.00**	**0.90**	**-0.65**	**0.77**			
Water NH_4-N	0.41	-0.46	0.15	**0.77**	**-0.76**	**-0.71**	0.19	**0.76**	0.55	**0.78**	**0.61**	-0.50				
Water TC	-0.42	0.43	0.13	**-0.62**	0.52	**0.60**	-0.03	**-0.64**	**-0.51**	**-0.65**	**-0.56**					
Water TN	0.33	-0.22	0.33	**0.92**	-0.70	-0.57	0.48	**0.90**	**0.99**	**0.89**						
DIN	0.48	-0.25	0.29	**0.98**	**-0.67**	**-0.66**	0.30	**1.00**	**0.84**							
DON	0.29	-0.21	0.33	**0.87**	**-0.68**	-0.53	0.51	**0.85**								
KCl NO_3-N	0.46	-0.24	0.33	**0.99**	**-0.69**	**-0.66**	0.29									
KCl NH_4-N	0.15	0.29	0.31	0.33	-0.13	-0.13										
PMN	-0.34	0.23	-0.29	**-0.69**	**0.78**											
K_2SO_4 TC	0.00	0.50	-0.36	**-0.76**												
K_2SO_4 TN	0.35	-0.22	0.42													
Micro. Bio. C	-0.16	0.47														
Micro. Bio. N	-0.05															

Figure 2.19: Soil Water DON Interaction Plots

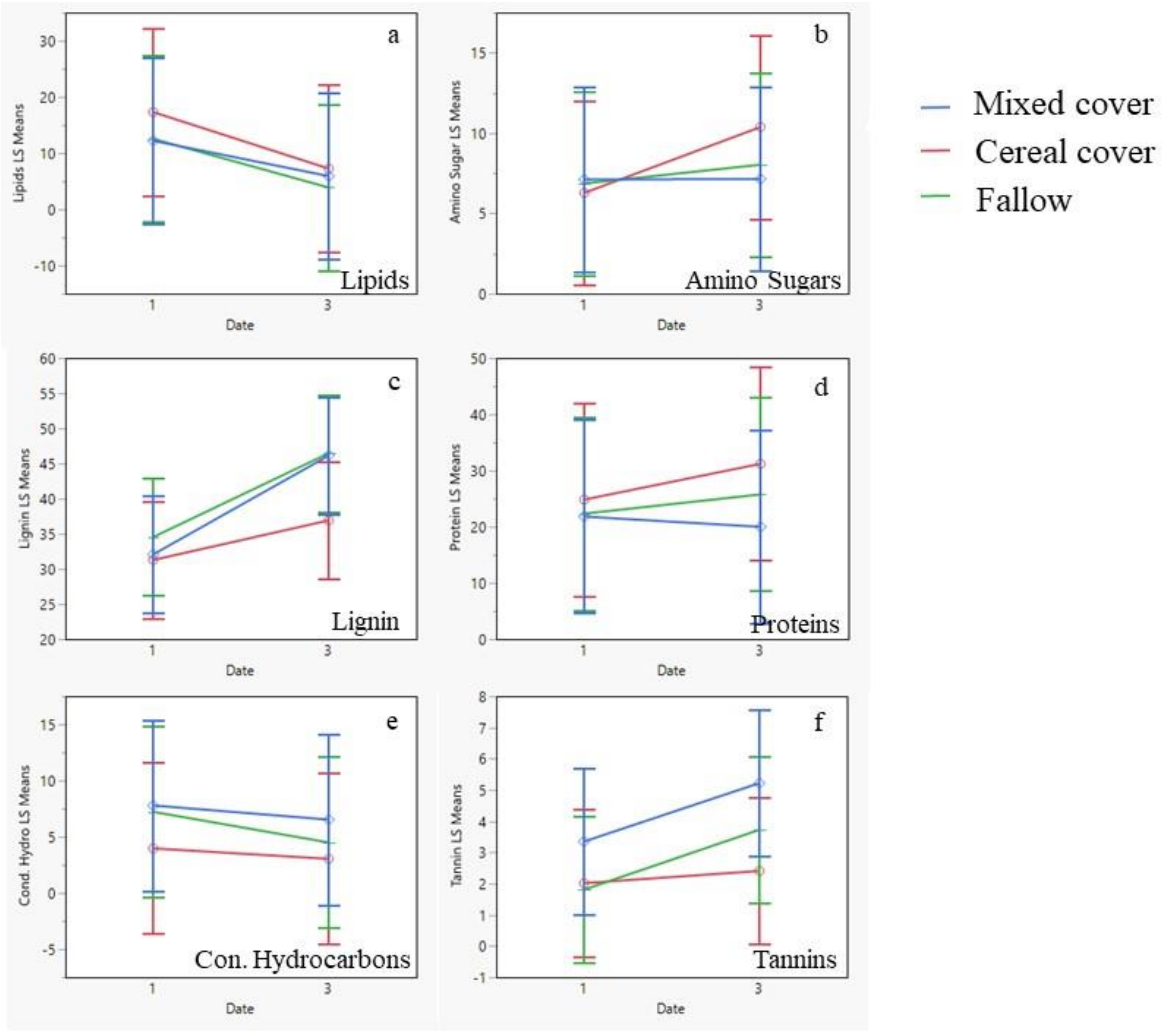

Table 2.19: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage of each compositional class (lipids, amino sugars, lignin, proteins, condensed hydrocarbons, and tannins) within the soil water extractable DON samples.

Figure 2.20: Soil Chloroform DON Interaction Plots

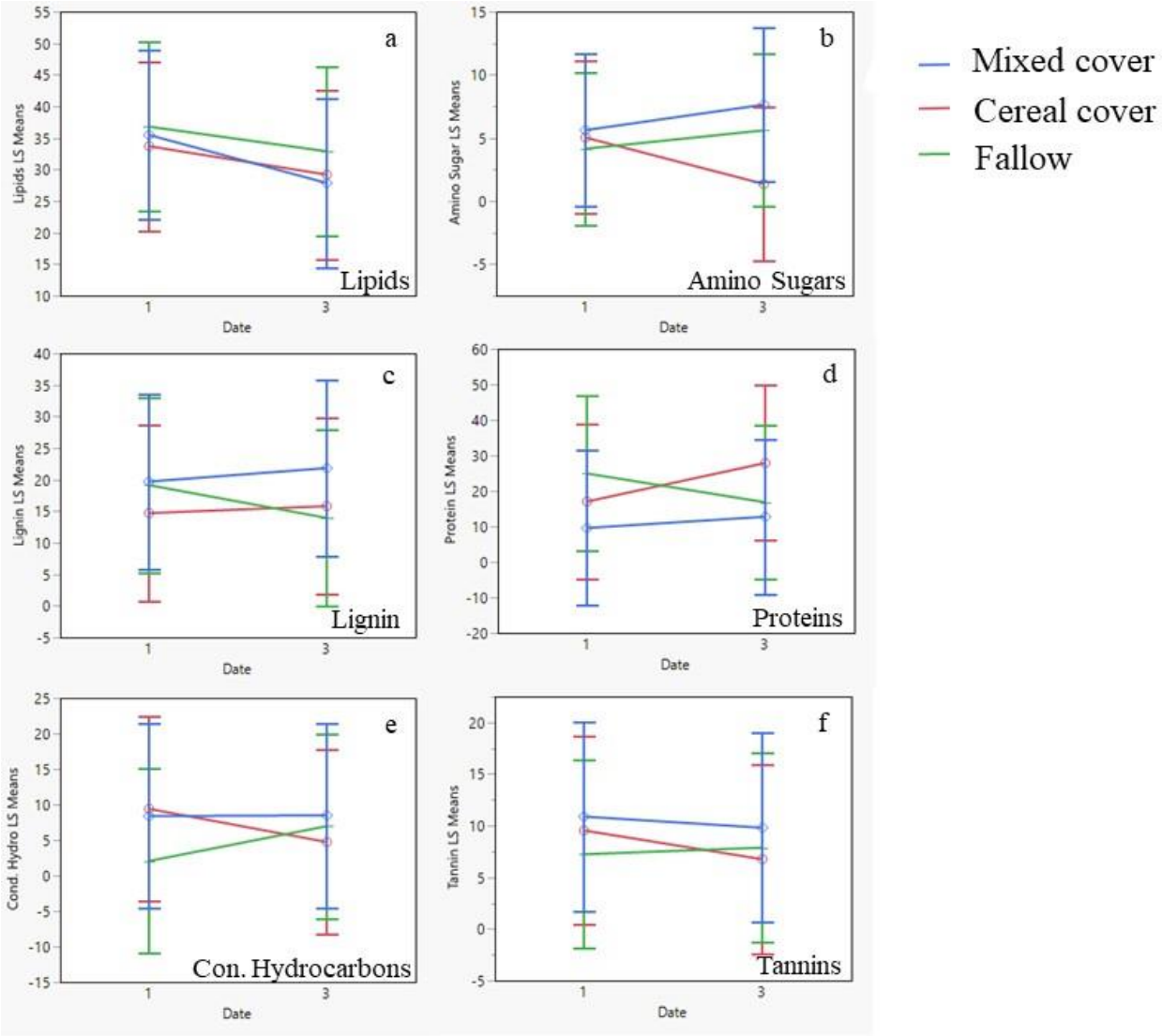

Figure 2.20: Interaction plots illustrating the comparisons made with the Repeated Measure ANOVAs to investigate the impact of sampling date and cover class on the relative percentage of each compositional class (lipids, amino sugars, lignin, proteins, condensed hydrocarbons, and tannins) within the soil chloroform extractable DON samples.

Potentially mineralizable N was again correlated with an increase in the relative percent of recalcitrant compounds (r=0.71).

Discussion:

Leachate DOM and DON Composition:

Although the composition of DOM and DON varied between samples, lignin represented the largest fraction of the organic pool in every leachate sample (Table 2.4). This is consistent with many previous studies of DOM (Heinz et al., 2015; Li et al., 2018; Wagner et al., 2015). A highly recalcitrant compound class, lignin is more resistant to microbial degradation than other DOM fractions and has also long been utilized as a tracer for terrestrial DOM (Nebbioso & Piccolo, 2013). Previous studies of porewater and soil-derived DOM report high relative percentages of aromatic lignin-like or lignin-derived compounds, and one study reporting that it accounted for 61% of DON in agricultural runoff (Jiang et al., 2017; Li et al., 2018; Nebbioso & Piccolo, 2013). It is therefore unsurprising that lignin accounted for at least 48.4% of leached DOM and 69.8% of leached DON in our study. Tannins also comprised large fractions of DOM in almost all of the leachate samples. Derived from vascular and non-vascular plants, tannins are another highly aromatic DOM fraction and, after lignin, are a major source of biologically produced polyphenols (Arbenz & Avérous, 2015). Though tannins have been increasingly studied as an alternative source of macromolecular architectures for chemical development, they have been understudied in agricultural soils (Arbenz & Avérous, 2015) .

Though a large percentage of the leached DOM and DON were composed of recalcitrant compounds, proteins made up 12% of leached DOM compounds (Table 2.4). Proteins and other high H/C and low O/C compounds are considered more bioavailable and can serve as a readily

available nutrient pool for microbes and plants (D'Andrilli et al., 2015). The number of unique N containing compounds (r =-0.90) and the lability of the DOM decreased with sampling depth; the relative percentage of highly recalcitrant, lignin-like compounds had a significant ($p<0.05$) positive relationship with sampling depth (Table 2.5). This may indicate that more of the bioavailable DOM fraction does not leach through the soil profile and is retained in the soil closer to the surface. Additionally, this may indicate that labile DOM is being degraded at soil depths closer to the surface. Studies have shown that soil microbial activity decreases with increasing depth, with the majority of activity confined to the top few centimeters of the soil profile (Tate, 1979). This activity, in turn, in a known control on soil OM degradation and chemical composition, as soil microbes preferentially use bioavailable organic compounds in the soil solution as a source of N (Ros et al., 2010). Therefore, it may be that the labile fraction of DOM and DON present in our plots is being taken up by microbes before it has the opportunity to leach from the soil profile, leading to a higher relative proportion of recalcitrant compounds in the 90cm leachate samples. This finding supports one of our initial hypotheses: that leachate samples would be primarily composed of lignin, but that we would observe a shift in composition with increasing sampling depth.

Although less abundant in the 90cm samples, labile DOM still accounted for an average of 17 ± 5% of leached organic compounds. Previous studies of leached OM have observed similar trends. In DOM from citrus grove runoff approximately 14% of DON compounds were biolabile (Li et al., 2018). While there is still need for further research into the impact of leached agricultural DON, studies of DON and non-purgeable organic carbon leached from landfills and soils amended from manure indicate that leached DON could contribute significant amounts of labile N to downstream aquatic systems, increase the risk of eutrophication in those waters

(Bolyard & Reinhart, 2017; Jensen et al., 2000). Studies show that photodegradation of more recalcitrant leached compounds may also have an important role to play in the transformation of DOM and the leaching of nutrients from agricultural fields (Li et al., 2018). In contrast, our dataset indicates <1% of DOM compounds in the leachate samples are highly photodegradable, and as depth increased, the percentage of highly photodegradable DOM decreased.

Soil DOM and DON Composition:

Though not one of our hypotheses, there was a distinct difference in DOM and DON composition between the two extraction methods. Lipids comprised the most classifiable compounds in all of the chloroform extracted samples. In comparison, the composition of the water extractable DOM was more similar to that of the leachate samples: with lignin as the most abundant compound in the water extract. Regardless of extraction methodology, protein was either the 2nd or 3rd largest functional group (Table 2.4). This difference is widely supported by past literature; studies have shown that extraction methodology and extraction solution can impact the organic compounds that one is able to capture during FT-ICR-MS analysis (Tfaily et al., 2015). Chloroform has been used in previous studies as a tool to investigate the lipid fraction of DOM, as it preferentially extracts non-polar compounds (McKee & Hatcher, 2015). In comparison, water extracts a wider range of compounds and is slightly selective for carbohydrates and compounds with high O/C ratios (Tfaily et al., 2015).

Although not significant for all the compositional classes, sampling date had an impact on the overall composition of soil DOM. Over time, there was a slight decrease in the relative percentage of the labile, readily available classes (i.e.: proteins, amino sugars, lipids) and an increase in the relative percentage of more complex, recalcitrant compounds (i.e.: lignin). As

plant material decomposes, the more labile fraction is used preferentially by plants and microbes as a source of C and N (Healey et al., 2004; Lehmann & Kleber, 2015). Previous studies have correlated the abundance of lipids, and small, labile compounds with the increased microbial activity and the biodegradation of SOM (Hanson et al., 2018). It is likely that the smaller organic compounds in our plots were utilized during the growing season resulting in shifts in DOM composition. The shift may also explain the increase in highly photodegradable compounds, as recalcitrant compounds (such as lignin and both classes of hydrocarbons) are more susceptible to photodegradation (Li et al., 2018). As DOM composition shifted and the relative percentage of lignin increased, the percentage of highly photodegradable compounds increased as well. This indicates that we can accept our second hypothesis – that the chemical composition of OM would shift throughout the growing as the corn plants utilized the labile N fraction as a source of N. However, the trends we observed were not statistically significant, and further sampling dates may provide more clarity on these trends. Previous seasonal studies of DOM observed temporal changes in arable soils, with the largest differences occurring between spring and fall (Rosa & Debska, 2018). The post-harvest sample set might have exhibited a more drastic, statistically significant DOM shift, but we were unable to process these data due to COVID restriction.

Cover Cropping:

Cover cropping did not have much impact on the composition of soil or leached DOM. We are therefore unable to accept our first hypothesis with any statistical significance: that the inclusion of a leguminous cover crop will increase the relative proportion of labile in DON and DON. The presence of legumes may partially be responsible for a subtle shift in DOM composition between the plots (Figures 2.10 and 2.14). The cereal and fallow plots had similar soil DOM compositions but differed from mixed cover plots. The mixed plots had more N peaks

(indicating a higher number of N containing compounds) and higher relative percentages of lignin, condensed hydrocarbons, and proteins. Legume cover crops have been shown to increase to overall and available C and N (Wu et al., 2017). The increase of N peaks may, therefore, be explained by the increase in SOM under leguminous cover (Wei et al., 2018). Though this does not account for the higher percentage of recalcitrant compounds, this would also explain the higher relative percentage of proteins in the plots. Additionally, leached DOM from plots that included a leguminous cover had a higher relative percentage of amino sugars. The inclusion of legumes in cover cropping strategies can lead to the enrichment of amino sugar and amino acid N in agricultural soils (Praveen-Kumar et al., 2002). Since the legumes in the mixed cover could lead to a higher concentration of amino sugars in the soil, leachate from the mixed cover plots would contain a higher relative percentage of amino sugars compared to the other cover treatments.

However, the potential influence of legumes in our study was subtle. This could be due in part to the fertilization regime of our study. The entire field received a combined 831 lbs of N from UAN regardless of cover cropping treatment. Past studies have shown that additional N fertilizer can inhibit N fixation and nodulation in legumes (Tanner & Anderson, 1964), one study reporting a 23% decrease in N fixation in soybeans when in the presence of a 0.01M NO_3^- solution (Arrese-Igor et al., 1997). It is possible that the abundance of supplemental N sources in our plots reduced the potential effect of the leguminous cover crop. Additionally, it is worth noting that, though the plots used in our study were part of an intended long-term study, they were sampled in the first year the plots were established. While studies have noted positive short term effects of cover-cropping – decreasing soil pH and slightly increasing soil organic C – many studies indicate that it can take years of continued management to observe significant

effects of cover-cropping on soil fertility properties (Basche et al., 2016; Mukherjee & Lal, 2015). Therefore, it may be that we would have observed more distinct differences in DOM and DOM composition due to cover cropping if we were able to sample once the plots had been established for several more years.

Conclusion:

While FT-ICR-MS analysis revealed several trends in DOM and DON composition of our samples, we were not able to report any significant temporal shifts or any significant impact made from cover-cropping. Our first hypothesis postulated that the presence of N-fixing, leguminous cover crops would lead to a higher proportion of labile N – containing compounds in the mixed cover crop plots. Our analysis indicates that the inclusion of red-clover in the cover crop had a slight effect on DOM composition, increasing the number of N peaks (indicating a higher proportion of N containing organic compounds within the samples) and increasing the relative percentages of lignin, condensed hydrocarbons, and proteins. However, this shift was not statistically significant and further study in is needed to determine if the effect would be more pronounced after multiple years of cover-cropping management.

Our second hypothesis anticipated a temporal shift in the composition of soil OM within our plots. As a corn plant grows, it utilizes N from the surrounding soil to fulfill its nutrient requirements. We then hypothesized that the OM present in the soil would act as a source of nutrients, and that we would observe a decrease in the relative proportion of labile organic N throughout the growing season. This was somewhat supported by our study, as we observed a slight decrease in the relative percentage of proteins, amino sugars, and lipids, and a slight increase in the relative percentage of recalcitrant, lignin-like compounds. However, this shift was

not statistically significant, and additional sampling dates would be needed to determine the significance of the decrease in the percentage of labile DOM throughout the growing season.

Our third and final hypothesis predicted that, as previous studies have indicated that lignin is a major component of leachate, leached DOM and DON will be primarily composed of lignin. Furthermore, we hypothesized that the composition of organic compounds within the leachate samples would shift with increasing depth, and that we would observe a higher proportion of recalcitrant, lignin-like compounds at lower sampling depths. This was supported by our study, as lignin accounted for at least 48.4% of leached DOM and 69.8% of leached DON in our study. We also observed the expected composition shift with increasing sampling depth, and the relative percentage of lignin ($r=0.61$) had a significant ($p<0.05$) positive correlation with increasing sampling depth. We are therefore able to accept our final hypothesis as sampling depth had a significant impact on the composition of leached DOM.

Additional research is needed to confirm the trends we have observed, this study indicates that DOM and DON composition may vary over the growing season and with depth. This could have potential impacts on how we understand organic N cycling, and better inform fertilizer recommendations and field management practices. Current N fertilizer recommendations are often based on yield goals; until 2005 the commonly accepted method of estimating the fertilizer needed for a field was developed in 1973 and suggested that farmers apply 1.2lbs of fertilizer for every expected bushel of corn (Morris et al., 2018). Since then, additional methods have been developed: farmers utilize soil N tests to inform N recommendations, can use regression models to determine the maximum economic return of various N application rates, and can use plant measurements such as chlorophyll content and corn stalk nitrate content to inform fertilizer application. However, many of these metrics may be

underestimating the contribution of organic N as a source of nutrients in agricultural fields (Morris et al., 2018). While more research is needed, the slight temporal shifts in DOM and DON composition indicate that plants and microbes are utilizing this organic pool of nutrients throughout the growing season. Therefore, while we are not prepared to make any recommendations at this time, further investigation into the line of research may help to improve N fertilizer recommendations and prevent over-fertilization.

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Chapter III

Title: Agricultural management impacts nitrification and denitrification gene abundance

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Abstract:

Nitrous oxide (N_2O) is a powerful greenhouse gas, accounting for 7% of total emissions in the United States. Agricultural contributes the largest percentage, accounting for ~75% of emissions in the United States. These emissions are inextricably linked to soil N cycling as N_2O is created as an intermediate in nitrification and denitrification. Past research has focused primarily on denitrification, as it was thought to be the major contributor of atmospheric N_2O. However, recent studies indicate that nitrification might have a greater role to play in N_2O emissions and indicate that there could be microbes present in agricultural fields that are capable of completely oxidizing ammonia (NH_4^+). This project quantified functional genes associated with nitrification (*nxrA* and *amoA AOB*) and dentification (*nirS* and *nirK*) to investigate impact of agricultural management on alternative N cycling pathways. Soil samples were collected from three of the University of Minnesota's Research Farms and from the Farming System's Project in Beltsville, MD. Samples were collected from experimental plots under a range of systems to compare the impact of tillage and fertilization (fertilized v. not fertilized, inorganic v. organic fertilizer, and tilled v. no-till plots). Comparison of the average gene abundance of *16S* rRNA

and the selected functional genes revealed variation among the sites. Analysis of the nitrifier community indicates that management had limited impact. Nitrification gene abundance was lower in non-fertilized plots when compared to fertilized plots. Analysis of the denitrification community yielded mixed results. However, *nirK* and *nirS* abundances were higher in no-till plots than conventionally tilled plots, and the *nirS:nirK* ratio was higher in the Beltsville sites than in the Minnesota plots.

Introduction:

Nitrous oxide (N_2O) is a powerful greenhouse gas, with 300x the warming potential of CO_2 and accounts for approximately 7% of total greenhouse gas emissions in the United States (Billings & Tiemann, 2014; US EPA, 2021a). Additionally, stratospheric N_2O leads to the production of nitrogen oxide which subsequently reacts with ozone, contributing to ozone depletion (Stolarski et al., 2015). There has been limited success in curbing N_2O emissions; vehicle emission regulations and the Clean Air Act of 1990 led to a small decrease in atmospheric N_2O from energy production. However, recent publications by the EPA show little change in total N_2O emissions, reporting 466.81 MMT CO_2 equivalent of N_2O emission in 2019 (US EPA, 2021b).

Nitrous oxide is a common by-product of wastewater treatment, fuel combustion, and plastic production. However, agriculture is responsible for the majority (75%) of modern N_2O emissions, as N_2O production is a key step in soil N cycling (US EPA, 2021a). N_2O is created as an intermediate step in nitrification and denitrification and has repeatedly been shown to be influenced by a variety of biotic and abiotic environmental factors. Both nitrification and denitrification are microbially mediated processes and microbial abundance and community composition can have an impact on N_2O emissions (Žurovec et al., 2021). Abiotic factors such as

temperature, soil moisture, soil pH, and mineral N availability have been shown to influence microbial N-cycling and N_2O emissions. Increases in temperature and soil moisture can lead to increases in soil organic N mineralization and nitrification which can lead to a subsequent increase in N_2O (Aliyu et al., 2021; Daly & Hernandez-Ramirez, 2020). Results also indicate that soil moisture may be a strong determinant of the roles of nitrification and denitrification as N_2O sources as nitrification requires an aerobic soil environment, while denitrification requires an anerobic soil environment. Studies have shown that in soils with greater than 60% of water filled pore spaces (WFPS) denitrification can account for up to 80% of N_2O production, and pulse N_2O emission events are often triggered by weather events in which soil moisture passes this 60% WFPS threshold (Aliyu et al., 2021). Lower soil pH has also been correlated with increased N_2O emissions because pH shifts in the microbial community and hinders the synthesis of enzymes crucial to N_2O reduction (Žurovec et al., 2021). Additionally, the use of fertilizer has been repeatedly reported to increase N_2O emissions (Daly & Hernandez-Ramirez, 2020; Ding et al., 2013). One study of long term (18 year) plots showed an 106% increase of background N_2O emissions in fields with a history of compost application and a 46-76% increase of background N_2O emissions in a history of inorganic fertilizer application when comparted to non-fertilized control plots (Ding et al., 2013). Another study indicated that by adding organic amendments to non-fertilized plots the activity of overall soil microbial community can increase from an initial 0.1-2% activity to 40% activity within minutes, leading to increases in N_2O emissions from nitrification (Benckiser et al., 2015). However, it is worth noting that several studies have indicated wide variation in the spatial distribution of N-cycling microbial communities in arable and wetland soils, which can lead to location specific N_2O fluxes (Correa-Galeote et al., 2013; Philippot et al., 2009). The uneven application of organic amendments, urea, and nitrification

inhibitors can lead to uneven nutrient availability and have been shown to enhance potential spot-wise nitrification and N_2O emissions (Benckiser et al., 2015).

Recent studies have begun to apply molecular techniques, quantifying microbial functional genes to gain a better understanding of dynamics within and the influence of abiotic factors on the nitrifying and denitrifying microbial communities (Garbeva et al., 2007). By targeting genes that encode for specific enzymes, researchers can better focus on pertinent members of the microbial community and investigate links between functional gene abundance, process rates, and N_2O emissions. Most studies of agricultural N_2O have focused on denitrification as it was thought to be the more important N_2O production pathway compared to nitrification (Bakken et al., 2012). However, recent functional gene studies have suggested that nitrification could play an overlooked role in N_2O emissions (Wei et al., 2014). One Minnesota study reported that the ratio of two nitrification genes *nxrA* and *amoA AOB* could explain 78% of variance in cumulative NO_2^- and 79% of the variance in N_2O emissions (Breuillin-Sessoms et al., 2017). Other studies have investigated the impact of agricultural management strategies on microbial populations, several reporting that N additions can have a significant influence on microbial community composition.

Inorganic N fertilizer has been shown to increase the abundance of both nitrifying (*amoA AOB*) and denitrifying (*nirS, nirK,* and *nosZ*) genes (Kong et al., 2021). Studies of organic N fertilizer have reported similar increases in denitrifying gene abundance (*nosZ*) but have also reported substantial decreases in potential ammonia oxidizing bacteria activity and decreases in N_2O emissions up to 14% (Kong et al., 2021). Tillage has also been noted as a factor that can influence N-cycling microbes with no-till (NT) treatments shifting the dominant nitrite oxidizing bacteria (NOB) populations from *nxrA* (*Nitrobacter sp*.) to *nxrB* (*Nitrospira sp*.) (Breuillin-

Sessoms et al., 2017). Furthermore, studies of *Nitrospira sp*. (traditionally thought of as a nitrification species) have identified a novel subset of the genus capable of complete ammonia oxidation, harboring all the enzymes needed to convert ammonia (NH_4^+) to nitrite (NO_2^-) and NO_2^- to nitrate (NO_3^-) (Van Kessel et al., 2015). Originally isolated from aquaculture filters in 2015 (Van Kessel et al., 2015), comammox *Nitrospira* have been detected in soil. *Nitrospira* could play an important role in nutrient limited, oligotrophic soils (Kits et al., 2017; Li et al., 2019) but might be outcompeted by traditional nitrifying communities in nutrient rich environments (Xu et al., 2020). However, there is still much unknown about the role of *Nitrospira* and its potential impact on N_2O emissions.

Our study aims to target nitrification and denitrification functional genes to investigate the impact of management strategies on these novel N-cycling pathways. We had several hypotheses:

1) Previous studies have indicated that long-term management strategies (e.g. tillage and fertilization regime) (Breuillin-Sessoms et al., 2017; Kong et al., 2021) can impact the composition of the bacterial population in agricultural soils. We hypothesize that as organic, no-till systems often manipulate the landscape less than conventionally fertilized and tilled fields, the abundance of nitrite-oxidizing and denitrification genes will increase under no-till, organic management.
2) Comammaox *Nitrospira* has been increasingly observed in agricultural and forest soils, and we anticipate that comammox bacteria will be present in all the soil samples, though they will be in lower abundance compared to the traditional nitrifiers.

3) Fertilizer additions are known to increase nitrification activity within soils (Kong et al., 2021) and we hypothesize that the inclusion of either manure or inorganic fertilizer will increase the overall abundance of nitrification genes (*nxrA* and *amoA AOB*).

Experimental Procedures:

Study Site and Sample Collection:

In collaboration with the USDA and the University of Minnesota, composite 0-20 cm soil samples were collected via push probe in late Fall 2019 from plots at the USDA's Farming Systems Project in Beltsville, MD (sampled 10/29), the University of Minnesota's Research and Outreach Centers in St. Paul (sampled 11/6) and Rosemount (sampled 10/28), MN, and the Sand Plain Research Farm in Becker, MN (sampled 11/1).

All the plots were part of long-term agricultural research projects with histories of N_2O research and all were under corn (*Zea mays* L.) production during the 2019 growing season. An additional archival sample from the Becker, MN site was included that was taken on 10/1/2018 in plots under potato production. There was variation in the management strategies at each site and among several of the Beltsville plots (Tables 3.1 and 3.2). The Rosemount plots were silt loam Waukegan (fine-silty over skeletal mixed, superactive, mesic Typic Hapludoll) and received 120 kg N ha^{-1} of urea-based fertilizer prior to the start of the growing season. A series of 6 plots – 3 under no-till (NT) and 3 under conventional tillage (CT) – were used for this study. The St. Paul site is also classified as Weaukegan silt loam soils (fine-silty over skeletal mixed, superactive, mesic Typic Hapludoll), and 3 CT, non-fertilized (NF) plots were used for the study. The soils at the Becker site differed from the other Minnesota site and are classified as loamy sand Hubbard soils (sandy, mixed, frigid, Entic Hapludolls). At the Becker site, a subset of NF,

CT plots were selected for our study (samples were taken from 4 plots in 2019 and 1 plot in 2018). The source plot for the 2018 Becker sample was tilled (on 5/4/2018) and had herbicide applied (on 5/23/2018) before planting. We were unable to obtain the records for the exact tillage, fertilizer application, and herbicide application dates at the Minnesota sites for 2019.

The Beltsville plots were located on silt loam soils (Christiana (fine, kaolinitic, mesic Typic Paleudults), Matapeake (fine-silty, mixed, semiactive, mesic, Typic Hapludults), Keyport (fine, mixed, semiactive, mesic Aquic Hapludults), and Mattapex (fine-silty, mixed, active, mesic Aquic Hapludults)). A subset of 9 plots were selected for our study: 3 CT plots and 3 NT plots that received inorganic NPK fertilizer and 3 organic plots. For the context of this study, the organic plots were determined as soils that relied on organic fertilizers (manure) and utilized cultural weed management (instead of herbicides). We were unable to obtain the records for the exact tillage, fertilizer application, and herbicide application dates at the Beltsville sites for 2019.

After collection, soils were immediately stored at -40^{o}C for later analysis, with exception to the archival sample. The 2018 Becker sample was air dried, sieved, and stored before being shipped to University of Maryland in December of 2019 for further analysis.

Sample Preparation:

DNA was extracted from the soil samples using a Qiagen DNeasy PowerMax soil kit (Qiagen, Hilden, Germany). Following the manufacturer's directions, 0.25g of defrosted soil was added to PowerBead tubes and shaken with a series of proprietary solutions using an MP FastPrep-24 bead beating grinder and lysis system for 45 seconds at 5.5 ms^{-1} and an Eppendorf Centrifuge 5430 at 10,000g for 3 minutes (in order to collect the supernatant after each solution

Table 3.1. Study site information about the Maryland sampling site. Includes: treatment, location, soil type, number of plots sampled, crop rotation, tillage strategy, weed control strategy, and fertilizer applied in 2019.

Maryland Study Site Information

Treatment	Location	Soil Type	# of plots	Crop Rotation	Primary Tillage	Weed Control	Fertilizer	Source
FSP – NT FSP – CT	Beltsville, MD	Silt loam • Christiana (fine, kaolinitic, mesic Typic Paleudults) • Matapeake (fine-silty, mixed, semiactive, mesic, Typic Hapludults) • Keyport (fine, mixed, semiactive, mesic Aquic Hapludults)	3 3	Corn (*Zea mays* L.)/ Rye cover (*Secale cereale* L.)/ Soybean (*Glycine max* L.) / Winter Wheat (*Triticum aestivum* L.)/ Soybean (*Glycine max* L.)	None Chisel Till	Herbicides	Inorganic NPK	(Cavigelli et al., 2008)
FSP – Org3		• Mattapex (fine-silty, mixed, active, mesic Aquic Hapludults)	3	Corn (*Zea mays* L.) / Rye cover (*Secale cereale* L.)/ Soybean (*Glycine max* L.) / Winter Wheat (*Triticum aestivum* L.)/Vetch cover (*Vicia villosa* L.)	Disk/ Moldboard plow/ Chisel plow	Cultural (Rotary Hoe/ Between Row Cultivator)	Manure, K_2SO_4	

Table 3.2. Study site information about the Minnesota sampling sites. Includes: treatment, location, soil type, number of plots sampled, crop rotation, tillage strategy, weed control strategy, and fertilizer applied in 2019.

Minnesota Study Site Information

Treatment	Location	Soil Type	# of plots	Crop Rotation	Primary Tillage	Weed Control	Fertilizer	Source
Sand Plain – 2018 Sand Plain – 2019	Becker, MN	Loamy sand • Hubbard (sandy, mixed, frigid, Entic Hapludolls)	1 4	Rye (*Secale cereale* L.)/ Potato (*Solanum tuberosum* L.)/Soybean (*Glycine max* L.) & Wheat (*Triticum aestivum* L.)	Chisel & disc plow	Herbicides (0.5#/ac SencorDF + 1.0pt/ac Linex + 1.5pt/ac Prowl H_2O) (applied pre-planting)	None	(Breuillin-Sessoms et al., 2017)
St. Paul	St. Paul, MN	Silt loam • Waukegan/ (fine-silty over sandy or sandy-skeletal, mixed, superactive, mesic Typic Hapludolls)	3	Continuous corn (*Zea mays* L.)	Conventionally Tilled	Herbicides (Glyphosphate) (applied pre-planting)	None	(Breuillin-Sessoms et al., 2017)
Rosemount – NT Rosemount – CT	Rosemount, MN	Silt loam • Waukegan (fine-silty over skeletal mixed, superactive, mesic Typic Hapludoll)	3 3	Corn (*Zea mays* L.)/Soybean (*Glycine max* L.)	None Moldboard (after corn), chisel till (after soy)	Herbicides (Glyphosphate) (applied pre-planting)	Urea (120 kg N ha^{-1} applied via sidedress application when corn was approx.. 20cm high.	(Breuillin-Sessoms et al., 2017; Venterea et al., 2016)

Table 3.3. A series of commonly studied functional genes associated with N cycling microbes. For each gene, the table lists the process associated with the gene, the enzyme the gene encodes, common microbes that harbor the gene, the corresponding primer set and sequence we used in this study, the amplicon size, the needed thermocycling conditions, and the source of information.

Gene	Process	Enzyme	Microbe	Primer	Sequence	Amp size (bp)	Thermocycling Conditions	Source
nxrA	Nitrite Oxidation	Nitrite Oxido-reductase alpha subunit	Nitrite oxidizing bacteria (*Nitrobacter sp.*)	F1norA / R2norA	5'-CAG ACC GAC GTG TGC GAA AG-3' / 5' -TCC ACA AGG AAC GGA AGG TC-3'	322	Initialization: 94C (3 min.) Cycling: 94C (30s), 55C (45s), 72C (45s) for 35 cycles. Elongation: 72C (5 min.)	(Breuillin-Sessoms et al., 2017; Wertz et al., 2008)
nxrB			Nitrite oxidizing bacteria (*Nitrospira sp.)*	nxrB169f / nxrb638r	5' - TAC ATG TGG TGG AAC A - 3' / 5' - CGG TTC TGG TCR ATC A - 3'	485	Initialization: 95C (5 min.) Cycling: 95C (40s), 56.2C (40s), 72C (90s) for 35 cycles. Elongation: 72C (10 min.)	(Breuillin-Sessoms et al., 2017; Pester et al., 2014)
nirK	Nitrification & de-nitrification	Nitrite reductase (copper form)	Nitrifying & denitrifying bacteria	nirK876 / nirK1040	5' -ATY GGC GGV AYG GCG A - 3' / 5'- GCC TCG ATC AGR TTR TGG TT-3'	165	Initialization: 95C (5 min.) Cycling: Two step touchdown. 95C (15s) annealing temperature started at 63C (60s) and lowered 1C each cycle for the first 6 cycles, 72C (30s). A total of 46 cycles. Elongation: 72C (10 min.)	(Henry et al., 2004)
nirS		Nitrite reductase (heme form)		nirSCD3aF / nirSR3cd	5' – AAC GYS AAG GAR ACS GG - 3' / 5'- GAS TTC GGR TGS GTC TTS AYG AA- 3'	400		(Hristova & Six, 2006)
16S	Protein Synthesis	30S ribosomal subunit	Wide range of bacteria and archaea	Eub 338F / Eub518R	5' - ACT CCT ACG GGA GGC AGC AG - 3' / 5' -ATT ACC GCG GCT GCT GG - 3'	200	Initialization: 95C (5 min.) Cycling: 95C (5s), 55C (15s), 72C (10s) for 40 cycles.	(Fierer et al., 2005)
amoa (AOA)	Ammonia Oxidation	Ammonia mono-oxygenase	Ammonia oxidizing archaea	CrenAmoAQ-F / CrenAmoMo dR	5′-GCA RGT MGG WAA RTT CTA YAA - 3' / 5′-AAG CGG CCA TCC ATC TGT A – 3’	124		(Mincer et al., 2007)
amoa (AOB)			Ammonia oxidizing bacteria	amoA-1F / amoA-2R	5'-GGG GTT TCT ACT GGT GGT-3' / 5'-CCC CTC KGS AAA GCC TTC TTC-3'	491	Initialization: 94C (5 min.) Cycling: 60C (90s), 72C (90s), 94C (60s) for 42 cycles. Elongation: 72C (10 min.)	(Aoi et al., 2004)
amoa (Comammox)	Complete Ammonia Oxidation	Ammonia mono-oxygenase	Comammox Nitrospira Clades A & B	Comaa or Comab 244F / 659R	Clade A: 5’ -ATY AAY TGG GTS AAY TA -3’/ 5’ – ARA TCA TSG TGC TRT G – 3’ Clade B: 5’ – TAY TTC TGG ACR TTY TA – 3’/ 5’ – ARA TCC ARA CDG TGT G – 3’	15	Initialization: 94C (5 min.) Cycling: 94C (30s), 52C (45s), 72C (60s) for 25 cycles. Elongation: 72C (10 min.)	(Klotz et al., 2017; Lin et al., 2020)

addition). DNA was quantified using a Quibit 2.0 Flurometer and stored at -20^{o}C for later analysis.

Q-PCR:

Functional genes related to N cycling were identified and selected for further analysis. These nitrification and denitrification genes, the enzymes they encode, and the primers and thermocycling conditions commonly used to amplify them, are listed in Table 3.3. Primers were tested for each functional genes by conducting a conventional PCR with a subset of the samples of extracted DNA: 10uL of GoTaq Green Master Mix 2x (Promega, Madison, WI, USA), 5uL of water, 1uL of forward primer, 1uL of reverse primer, 1uL of 1000ug uL^{-1} bovine serum albumin (BSA), and 2uL of extracted DNA into each well. Successful amplification was checked via gel electrophoresis using an Amersham Pharmacia Biotech Rig set at 120v, 70mA, and run for 40 minutes. Invitrogen SYBR Safe DNA gel stain (Invitrogen, Waltham, MA, USA) was used in conjunction with the Green Master Mix to stain the DNA during the gel electrophoresis, and UVP BioDoc-It Imaging System was used to take pictures of the resulting gels under UV light. Of the initial list of functional genes, *nxrA, nirK, nirS, amoA AOB,* and *16S* rRNA were selected for further study.

E. coli Q-PCR standards were created using a TOPO TA Cloning Kit (Invitrogen, Waltham, MA, USA) with a pCR 2.1-TOPO Vector following the manufacturer's guidelines. Briefly, a subset of DNA extracts were PCR amplified using the following mix: 9.3uL water, 5uL of 5x Colorless GoTaq Reaction Buffer, 1.8uL of 25mM $MgCl_2$, 1.3uL of Forward and Reverse Primer, 0.5uL of 2.5mM dNTP Mix, 4uL of 0.4% BSA, 0.1uL of Taq DNA Polymerase, and 2uL of 2.5ng uL^{-1} DNA in each well. Next, 4ulLof amplified PCR product for each of the

functional genes was added to a 0.65mL tube along with 1uL of salt solution and 1uL of TOPO Vector. The reaction was allowed to incubate at room temperature (22-23^{o}C) for 5 minutes while a tube of OneShot TOP10 (Invitrogen, Waltham, MA, USA) chemically competent *E.coli* cells was allowed to thaw on ice. The ligation reaction (2uL) was added to the *E.coli*, flipped to mix, and incubated on ice for 5 additional minutes. The *E. coli* cells were then heat shocked in a 42^{o}C water bath for 30 seconds, after which 250uL of room temperature SOC was added to the shocked cells. The resulting cell suspension was capped and incubated at 37^{o}C for 1 hour while shaking. For each of the cell suspensions, 2 Luria broth plates with ampicillin were treated with 40ul of 40 mg mL^{-1} X-gal 1 hour before plating and allowed to warm at 37^{o}C for 30 minutes before plating. The resulting transformed cells were plated on the prepared growth media; for each cell suspension 50uL the mixture was added to one plate while 100uL of the mixture was added to a second plate. All plates were incubated overnight at 37^{o}C.

The following day, the plates were checked for growth, and a subset of white, successfully transformed colonies were re-streaked onto new Luria broth plates with ampicillin. These were allowed to incubate overnight at 37^{o}C. The resulting growth was tested for successful transformation by PCR amplification for each colony in question, using a standard reaction mix.

A subset of the clones that had successfully amplified were regrown in tubes containing 6mL of Luria broth with ampicillin. These were incubated overnight at 37^{o}C. The following day, 1mL of overnight culture was combined with 400uL of glycerol and preserved at -80^{o}C in a cryotube. Plasmids were extracted from the remaining 5mL of overnight culture using the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The resulting plasmids were quantified using a Quibit 2.0 Flurometer, linearized using FastDigest Eco321(Thermoscientific,

Waltham, MA, USA), cleaned with a Qiagen QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and re-quantified using a Quibit 2.0 Flurometer. The resulting plasmids were diluted to 2.5ng ul^{-1}, which was in turn used to create a serial dilution ($2.5x10^{-1}$ ng ul^{-1}, $2.5x10^{-2}$ ng ul^{-1}, $2.5x10^{-3}$ ng ul^{-1}, $2.5x10^{-4}$ ng ul^{-1}, and $2.5x10^{-5}$ ng ul^{-1}) that would serve as a potential standard set for the Q-PCR analysis.

Each of the standards were tested and optimized for Q-PCR using a mastermix that contained: 10uL KicqStart SYBR Green qPCR ReadyMix with ROX (Sigma-Aldrich, St. Louis, MO, USA), 6uL of water, 1uL of each primer, and 2uL of sample. An Applied Biosystems StepOnePlus Real-Time PCR System was used for all Q-PCR assays and each sample was run in triplicate for genes: *nxrA, nirK, nirS, amoA AOB,* and *16S* rRNA.

Data Analysis:

JMP Pro 15 was used for multivariate analysis (Pearson Correlations) and one-way ANOVAs to explore the impact of management strategy and sampling location on the abundance of functional genes. Analysis was conducted on the raw gene abundance data as well as on several ratios (*nxrA:amoA AOB*, *nirS:nirK*) to better understand the dynamics among the populations. The abundance of each functional gene was also divided by *16S* rRNA gene abundance to determine the relative proportion of each functional gene to overall bacterial abundance, effectively normalizing the dataset and allowing comparison among sampling sites.

For each functional gene or gene ratio, we conducted multiple One-Way ANOVAs to compare the abundances or ratios:

1) between the Maryland and Minnesota soils (MD v. MN)
2) among all the sampling sites (Site Diff (all))
3) among the Minnesota sampling sites (Site Diff (MN))

4) between all no-till (NT) and conventionally tilled (CT) plots across all the sampling sites (NT v. CT (all))
5) between the NT and CT plots across all the Minnesota sampling sites (NT v. CT (MN))
6) between the NT and CT plots at the Rosemount, MN site (NT v. CT (Rosemount))
7) between the NT and CT plots at the Beltsville, MD site (NT v. CT (Beltsville))
8) between the fertilized (F) and non-fertilized (NF) plots across all the sample sites (F v. NF (all))
9) between the F and NF plots across the Minnesota sample sites (F v. NF (MN))
10) between the organic and inorganically fertilized plots at the Beltsville, MD site (Organic v. Inorganic (Beltsville)).

Pearson correlations were conducted to determine the significance of the shifting microbial community and any potential relationships between the nitrifiers and denitrifiers we were able to amplify, comparing the raw (*16S, nxrA, amoA AOB, nirS,* and *nirK*) and normalized (*nxrA:16S, amoA AOB:16S, nirS:16S,* and *nirK:16S*) gene abundances. For all the One-Way ANOVA and Pearson Correlation Analyses each data point represented the gene abundance or gene ratio for one of the sampled plots at a site (instead of a mean value across the treatments at a given site).

Results:

Microbial Abundance:

Overall, *16S* rRNA gene abundance was significantly lower in the Beltsville samples compared to the group of Minnesota samples ($F= 16.375$, $p=0.0006$), indicating a relatively

smaller bacterial community at the Maryland site (Table 3.4 & Figure 3.2a). *16S* rRNA gene abundance varied across the Minnesota sites, but most sites had similar *16S* rRNA gene quantities. One exception was the Becker 2018 sample, which was the lowest of the Minnesota samples and much lower than the Becker sample from the following year (Figure 3.1a). Though not significant, it is also worth noting that the no-till plots had a greater *16S* rRNA gene abundance than the corresponding conventionally tilled plots at both the Rosemount and Beltsville sites (Figures 3.1a and 3.2a and Table 3.4)

Nitrification Functional Genes:

Although five different nitrification genes were planned for this study, we had difficulty cloning standards in order to quantify *amoA AOA* (ammonia oxidizing archaea) and *nxrB* (nitrite oxidizing *Nitrospira sp*.). Furthermore, we were unable to amplify Clades A&B of Comammox *Nitrospira* from the soil samples. Our analysis focuses on *amoA AOB* (ammonia oxidizing bacteria) and nxrB (nitrite oxidizing *Nitrobacter sp*). Site location had a significant effect on the abundance of *nxrA* and *amoA AOB* (*nxrA*, $F=6.4514$, $p=0.0021$; *amoA AOB*, $F=4.6975$, $p=0.009$) (Table 3.4). The Beltsville samples had significantly lower *nxrA* ($F=15.261$, $p=0.0008$) and *amoA AOB* ($F=8.054$, $p=0.010$) gene abundances than the Minnesota samples (Figures 3.1 b & c, 3.2 b & c, Table 3.4). When normalized to account for the *16S* rRNA gene at the sites, there were no significant differences in nitrification genes by site or between the Maryland and Minnesota samples (Table 3.5). Although not significant ($p<0.05$), lower normalized nitrification gene counts were observed at the non-fertilized Minnesota sites when compared to fertilized plots (Figure 3.3 a & b).

Figure 3.1: Microbial Abundance in Minnesota Plots

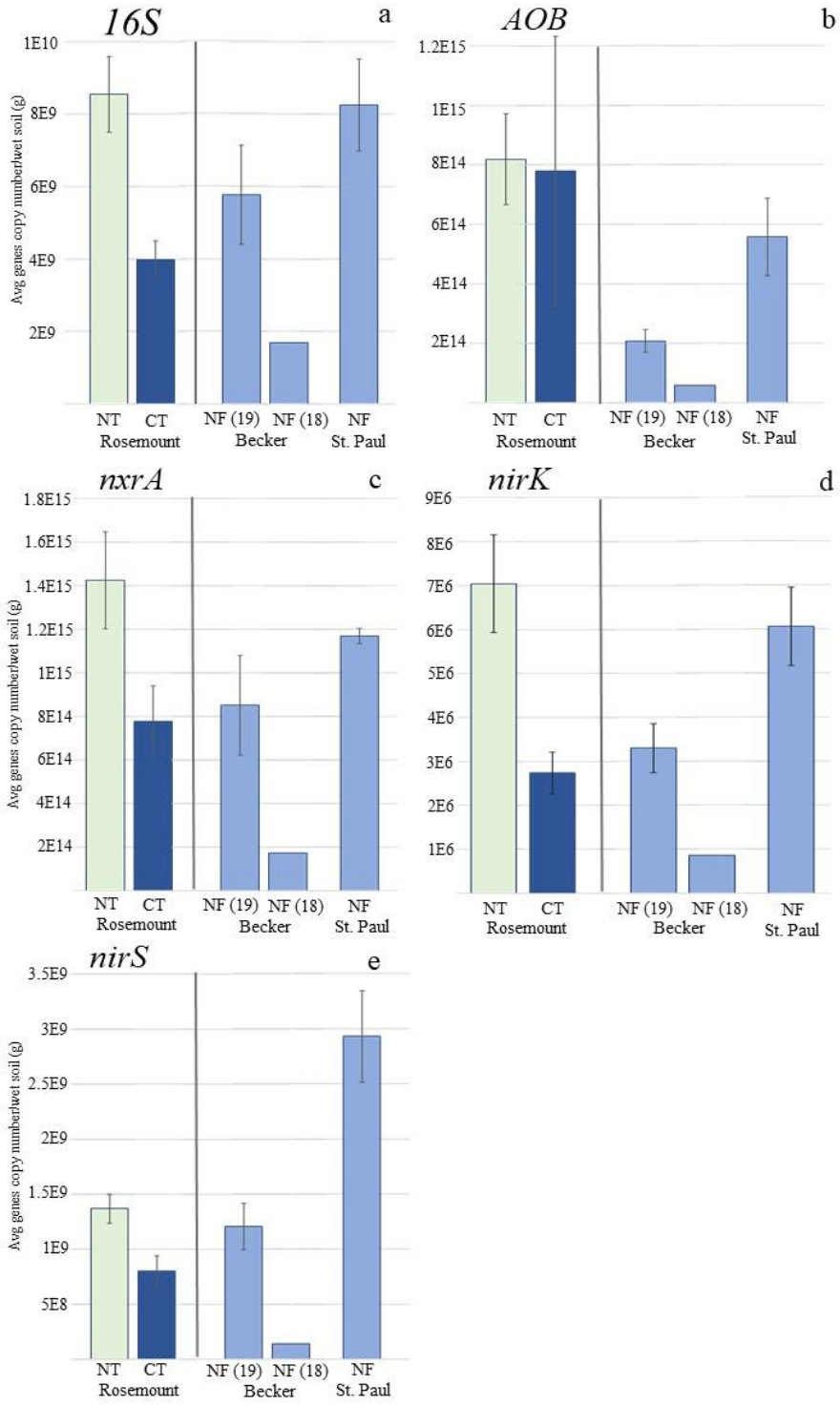

Figure 3.1. Average *16S* rRNA *gene, amoa AOB, nxrA, nirK,* and *nirS* gene abundance (average gene copy number per gram of wet soil) for each of the treatments in the Minnesota sites.

Figure 3.2: Microbial Abundance in Beltsville Plots

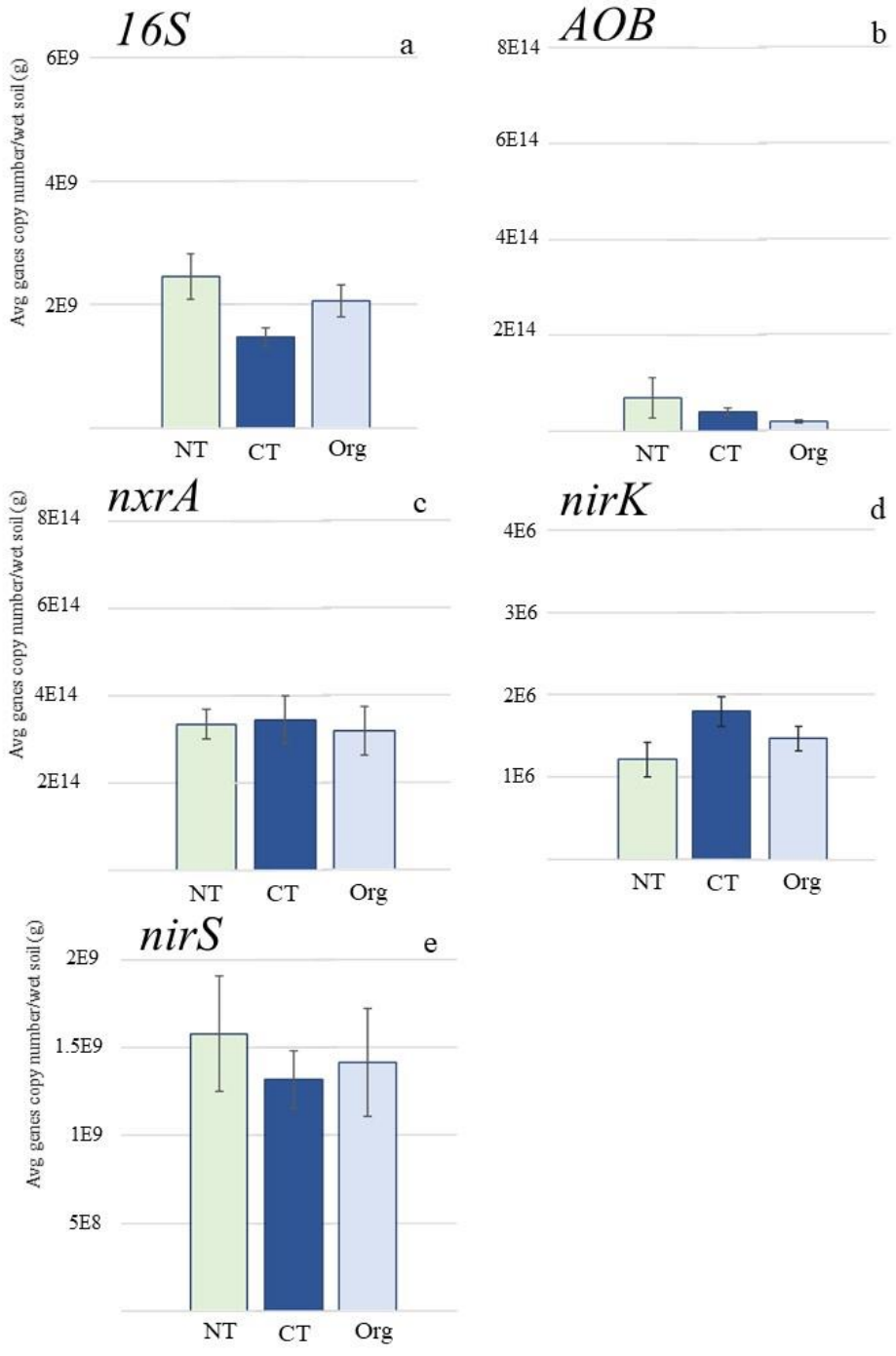

Figure 3.2. Average *16S* rRNA, *amoA AOB, nxrA, nirK,* and *nirS* microbial abundances (average gene copy number per gram of wet soil) for each of the Beltsville treatments.

Table 3.4. Summary table of One-Way ANOVA values for *16S* rRNA, *nxrA, amoa AOB, nirS,* and *nirK* gene abundance (average gene copy number per gram of wet soil). Bolded values indicate significant ($p<0.05$) differences.

Functional Gene Abundance One-Way ANOVA Summary Table															
	16S			***nxrA***			***AOB***			***nirS***			***nirK***		
	F	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**
MD v. MN	16.375	1, 21	**0.001**	15.261	1,21	**0.001**	8.054	1,21	**0.010**	0.001	1, 21	0.974	11.923	1,21	**0.002**
Site Diff. (all)	6.473	4, 18	**0.002**	6.451	4,18	**0.002**	4.698	4,18	**0.009**	8.037	4,18	**0.001**	7.328	3,19	**0.002**
Site Diff (MN)	1.118	2, 11	0.361	1.181	2,11	0.343	2.585	2,11	0.120	11.608	2,11	**0.002**	2.117	2,11	0.167
NT v. CT (all)	2.000	1, 21	0.172	0.884	1,21	0.358	0.203	1,21	0.657	1.889	1, 21	0.184	1.407	1,21	0.249
NT v. CT (MN)	2.239	1, 12	0.160	0.633	1,12	0.442	0.969	1,12	0.344	1.788	1, 12	0.206	1.817	1,12	0.203
NT v. CT (Rosemount)	10.298	1, 4	**0.033**	3.647	1,4	0.129	0.004	1,4	0.950	5.768	1,4	0.074	8.500	1,4	**0.043**
NT v. CT (Beltsville)	0.040	1, 7	0.848	0.075	1,7	0.792	0.007	1,7	0.937	0.209	1,7	0.661	2.983	1,7	0.128
F. v. NF (all)	5.562	1, 22	**0.028**	2.623	1,21	0.120	0.001	1,21	0.972	3.034	1, 21	0.096	2.328	1,21	0.142
F v. NF (MN)	0.358	1, 12	0.561	0.003	1,12	0.954	2.275	1,12	0.157	3.031	1, 12	0.107	0.000	1,12	0.987
Organic v. Inorganic (Beltsville)	2.760	1, 8	0.135	0.520	1,8	0.492	1.092	1,8	0.327	0.014	1,8	0.909	0.352	1,8	0.569

Although also not significant, higher *nxrA:16S* rRNA was observed in the CT plots when compared to the corresponding NT plots at Rosemount and Beltsville (Figures 3.3b and 3.4b). Regarding the effect of tillage on the nitrification community, there were no clear trends in the *AOB:16S* rRNA sample set. The Rosemount NT plots had a lower average *AOB:16S* rRNA that the Rosemount CT plots (Figure 3.3a), however the wide range of the Rosemount CT values led to a large SE for the sample plots and there was not statistically significant difference between the Beltsville NT and CT averages. Though not significant, largest treatment difference at the Beltsville site was between the NT and organic plots (Table 3.5, Figure 3.4); the organic plots had relatively higher average *nxrA:16S* rRNA and slightly lower average *AOB:16S* rRNA (Figure 3.4 a& b) that the NT plots. The *nxrA:amoA AOB* gene ratio was higher at the Beltsville sites ($F=11.751$, $p=0.003$) when compared to the Minnesota samples (Table 3.5). There was less variation in the ratio across Minnesota samples (0.6-9.0), compared to Beltsville (1.7 – 51.8) (Figures 3.3e and 3.4e,).

Denitrification Functional Genes:

Similar to the *16S* rRNA and other functional gene abundance, sampling site ($F=6.059$, $p=0.003$) and state ($F=11.923$, $p=0.002$) had a significant impact on the abundance of *nirK* gene copy numbers but did not impact *nirS* gene copy number (Table 3.4). Though not significant, the NT plots in both Beltsville and Rosemount had higher average *nirS* and *nirK* abundance than the corresponding CT plots at the respective sites (Table 3.4, Figures 3.1 d &e and 3.2 d & e). When normalized using *16S* rRNA gene abundance additional trends emerged. There was significantly higher *nirS:16S* rRNA ratios ($F=46.754$, $p=<0.0001$) in Beltsville plots compared to the Minnesota samples.

Figure 3.3: Microbial Abundance Ratios in Minnesota Plots

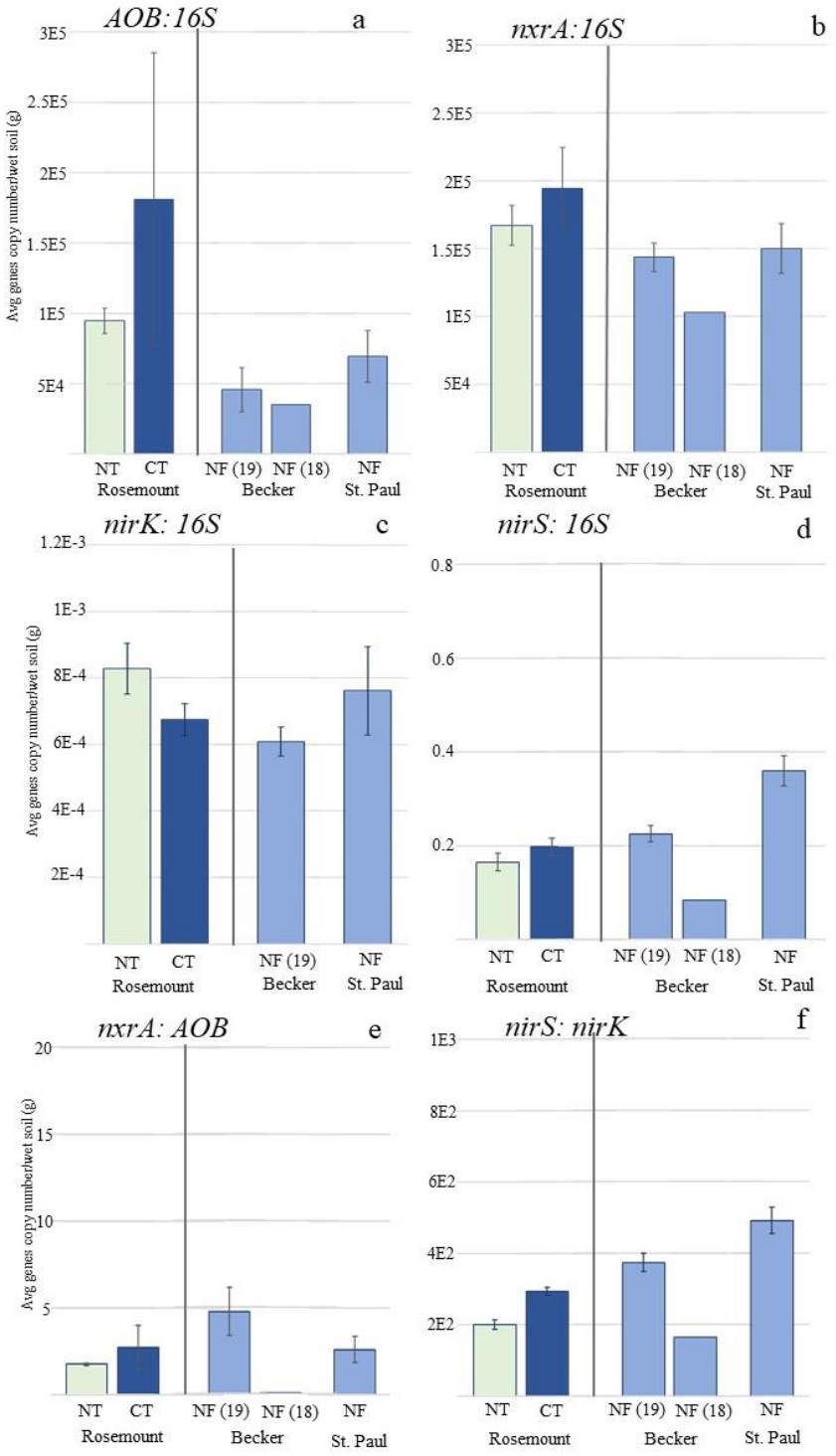

Figure 3.3. Ratios of *amoA AOB: 16S* rRNA, *nxrA:16S* rRNA, *nirK:16S* rRNA, *nirS:16S* rRNA, *nxrA: amoA AOB*, and *nirS:nirK* gene abundances for each of the treatments at the Minnesota study sites.

Figure 3.4: Microbial Abundance Ratios in Beltsville Plots

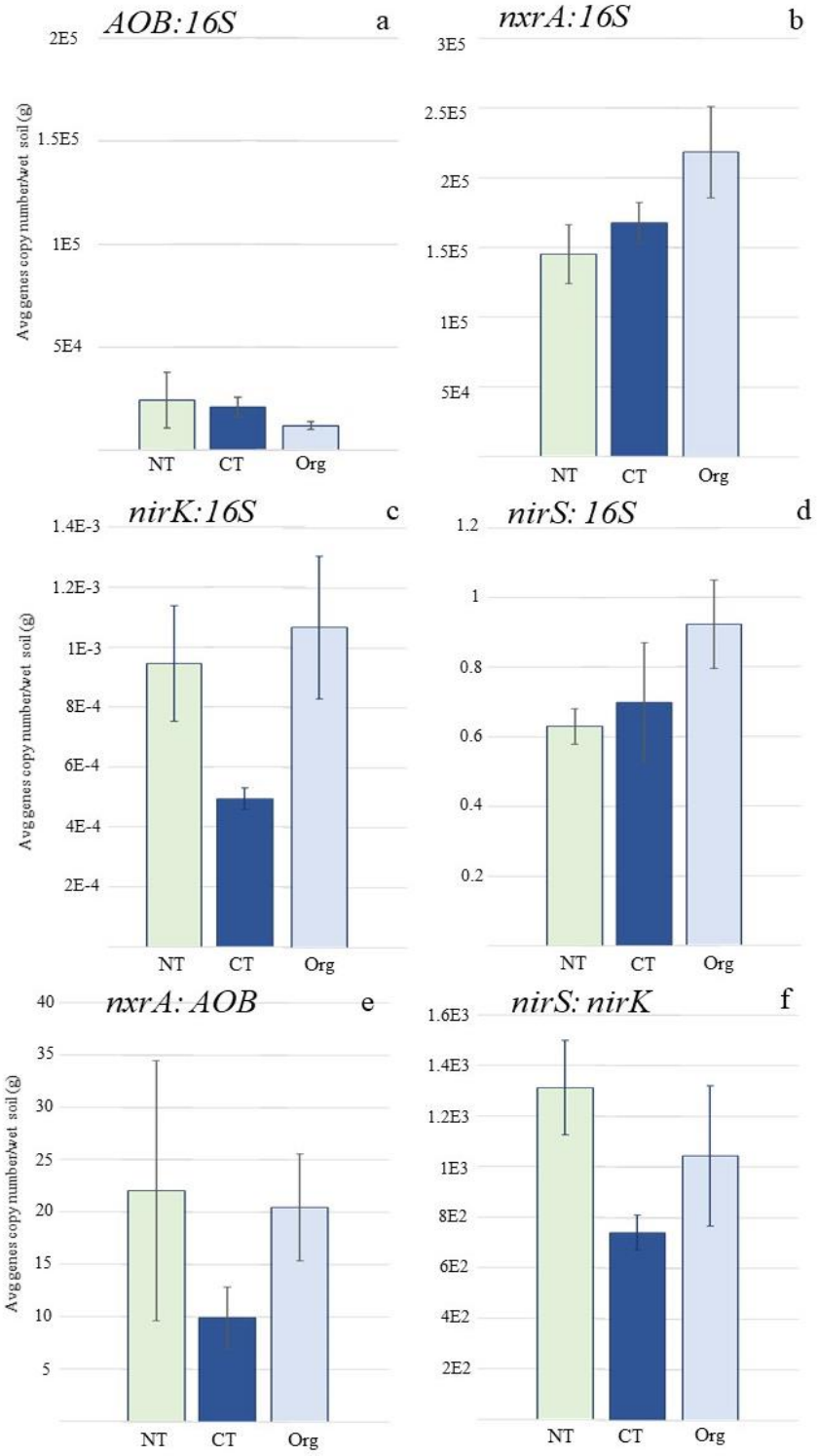

Figure 3.4. Ratios of *amoA AOB: 16S* rRNA, *nxrA:16S* rRNA, *nirK:16S* rRNA, *nirS:16S* rRNA, *nxrA: amoA AOB*, and *nirS:nirK* gene abundances for the Beltsville treatments.

Additionally, though not statically significant the Beltsville NT plots also had lower *nirS:16S* rRNA ratios averages than the CT plots (Figure 3.4d). The ratios of *nirS:16S* rRNA and *nirK:16S* rRNA were higher in organic plots compared to conventional and no-till (Table 3.5). The *nirS: nirK* ratio differed between sampling site (F=8.203, p=0.0006) and state (F=32.959, p= <0.0001), with the highest ratios observed in the Beltsville plots (Figure 3.4f). Though not significant, higher *nirS:nirK* values were observed in the NF Minnesota plots when compared to fertilized plots from the Minnesota sites (Figure 3.3f). A comparison of tillage treatments indicates conflicting trends, there was a slightly higher but not statistically significant difference in *nirS: nirK* ratio in NT plots at Beltsville (F=2.326, p=0.17), but a higher *nirS:nirK* ratio in the CT plots at Rosemount (F=19.291, p=0.012) (Figures 3.3f and 3.4f).

Overall Microbial Community Composition:

PCoA coordinates clustered based on sample site location (Figure 3.5), consistent with the One-Way ANOVAs on individual gene abundances (Table 3.4). There was also weak clustering based on management strategy: the non-fertilized plots loosely clustered in the center and top right quadrant and the no-till data points clustered loosely in the two left hand quadrants. However, sampling site seems to have the strongest influence on clustering, as the points are more strongly grouped by sampling location. Pearson correlations to investigate the composition of the bacterial populations show that *16S* rRNA gene abundance was significantly correlated to increases in all the functional genes (p<0.05) (*nxrA*, r=0.91; *nirS*, r= 0.54; *amoA AOB*, r=0.55) (Table 3.6). The abundance of nitrification genes *nxrA* and *amoA AOB* were strongly correlated to one another (r=0.71, p=0.0001), while *nxrA* was weakly positively correlated to the abundance of *nirS* (r=0.42, p=0.05) (Table 3.6). When normalized with the *16S* rRNA gene abundance, there no significant correlations between the functional genes.

Table 3.5. Summary table of One-Way ANOVA values for *16S* rRNA normalized *nxrA, amoa AOB, nirS,* and *nirK* gene abundance as well as for the *nxrA:amoA AOB* and *nirS:nirK* ratios. Bolded values indicate significance ($p<0.05$).

Normalized Functional Gene Abundance and Gene Ratios
One-Way ANOVA Summary Table

	nxrA:16S			***AOB:16S***			***nirS:16S***			***nirK:16S***		
	F	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**
MD v. MN	0.894	1,21	0.355	3.949	1,21	0.060	46.754	1,21	**<0.0001**	1.266	1,21	0.273
Site Diff. (all)	1.033	4,18	0.417	2.013	4,18	0.136	12.111	4,18	**<0.0001**	0.730	3,19	0.547
Site Diff (MN)	1.837	2,11	0.205	1.217	2,11	0.333	10.243	2,11	0.003	1.604	2,11	0.245
NT v. CT (all)	0.877	1,21	0.360	1.631	1,21	0.216	0.025	1,21	0.876	0.323	1,21	0.576
NT v. CT (MN)	3.332	1,12	0.093	3.476	1,12	0.087	0.325	1,12	0.579	0.050	1,12	0.826
NT v. CT (Rosemount)	0.440	1,4	0.543	0.455	1,4	0.537	1.060	1,4	0.362	1.950	1,4	0.235
NT v. CT (Beltsville)	0.118	1,7	0.741	0.053	1,7	0.824	0.147	1,7	0.713	0.279	1,7	0.614
F. v. NF (all)	2.300	1,21	0.144	0.294	1,12	0.593	3.474	1,21	0.076	1.025	1,21	0.323
F v. NF (MN)	1.729	1,12	0.213	2.653	1,12	0.129	1.573	1,12	0.234	0.683	1,12	0.425
Organic v. Inorganic (Beltsville)	4.149	1,8	0.076	1.263	1,8	0.294	1.071	1,8	0.331	1.813	1,8	0.215

	nxrA:AOB			***nirS:nirK***		
	F	**ndf, ddf**	**p-value**	**F**	**ndf, ddf**	**p-value**
MD v. MN	1.266	2,21	0.243	32.959	1,21	**<0.001**
Site Diff. (all)	0.730	3,19	0.547	11.158	2,19	**0.000**
Site Diff (MN)	1.344	2,11	0.301	8.999	2,11	**0.005**
NT v. CT (all)	0.309	1,21	0.584	0.299	1,21	0.590
NT v. CT (MN)	0.088	1,12	0.771	0.325	1,12	0.579
NT v. CT (Rosemount)	0.389	1,4	0.567	19.291	1,4	**0.012**
NT v. CT (Beltsville)	1.046	1,7	0.341	2.326	1,7	0.171
F. v. NF (all)	1.547	1,21	0.227	2.584	1,21	0.123
F v. NF (MN)	3.010	1,12	0.108	3.425	1,12	0.089
Organic v. Inorganic (Beltsville)	0.340	1,8	0.576	0.132	1,8	0.726

Figure 3.5: PCoA of Microbial Community Composition

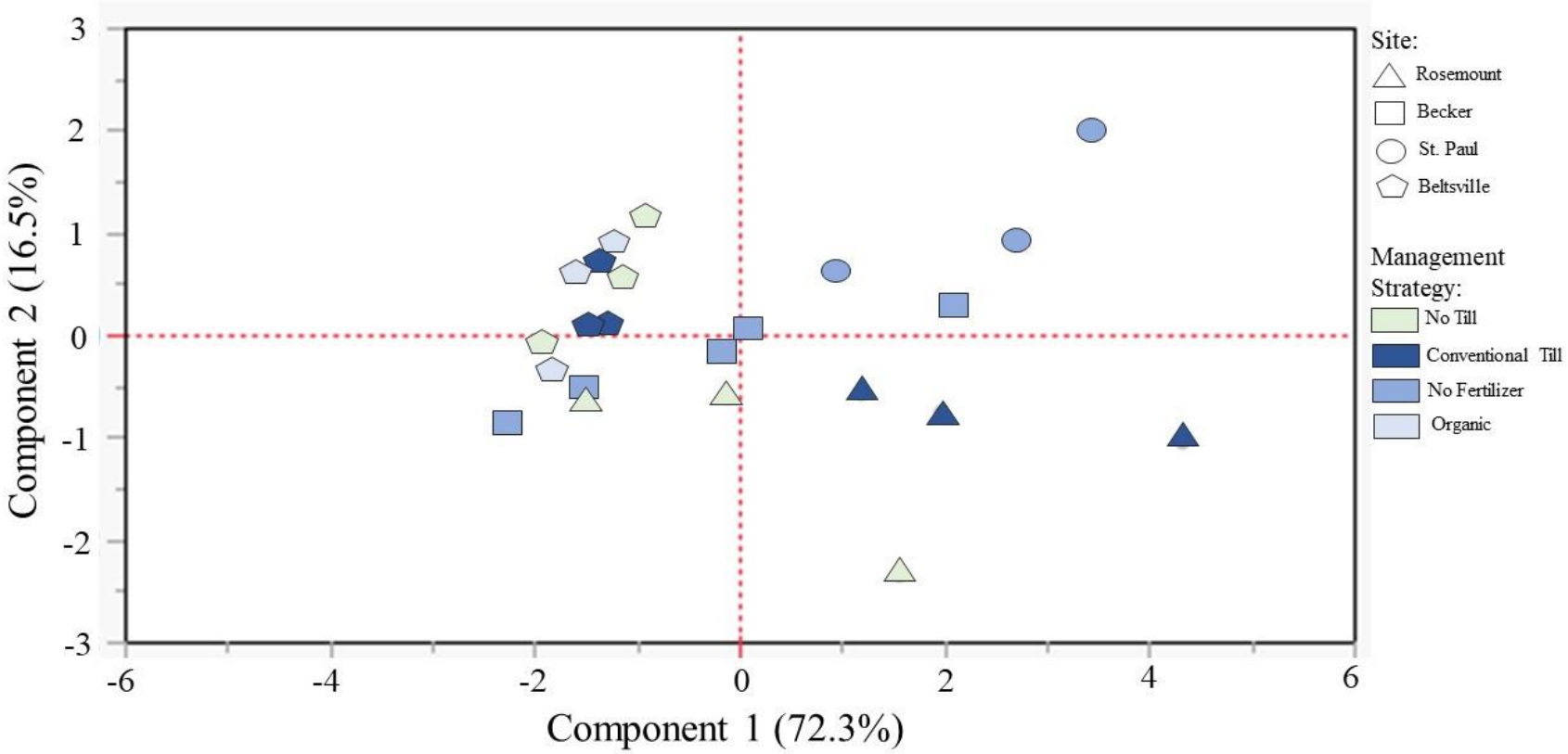

Figure 3.5. The above figure illustrates the results of the Principal Coordinate Analysis conducted on the entire dataset to explore trends in the overall community composition of each soil sample. Shapes are used to differentiate the sample site location of each plot, and the points are shaded to indicate the combination of management strategies.

Table 3.6. Pearson Correlations comparing the *16S* rRNA, *nxrA, amoa AOB, nirS,* and *nirK* gene abundance (average gene copy number per gram of wet soil) as well as *16S* rRNA normalized *nxrA, amoa AOB, nirS,* and *nirK* gene abundances. Bolded values indicate significance ($p<0.05$)

Functional Gene Pearson Correlations								
	nirK: 16S	*nirS: 16S*	*AOB: 16S*	*nxrA: 16S*	*nirK*	*nirS*	*AOB*	*nxrA*
16S	**-0.45**	**-0.54**	0.20	-0.21	**0.89**	**0.54**	**0.55**	**0.91**
nxrA	**-0.50**	**-0.52**	0.39	0.11	**0.91**	**0.42**	**0.71**	
AOB	**-0.41**	-0.41	**0.90**	0.28	**0.65**	0.21		
nirS	0.29	0.26	-0.01	-0.12	**0.51**			
nirK	**-0.51**	**-0.44**	0.29	-0.02				
nxrA:16S	0.02	0.29	0.34					
AOB:16S	-0.30	-0.30						
nirS:16S	**0.81**							

Table 3.7. Historical N_2O emission data for the Minnesota sample sites, under different fertilization regimes. Data adapted from (Breuillin-Sessoms et al., 2017).

	Historical N_2O Emissions ($\mu g\ N\ g^{-1}$soil) in Minnesota Sample Sites				
	Initial urea-N concentration ($\mu g\ N\ g^{-1}$)				
	0	100	250	500	1000
Becker, MN	1.21	1.37	14.3	37.9	12.1
Rosemount - CT	1.05	2.23	2.48	2.49	13.2
Rosemount-NT	1.1	1.39	1.18	1.42	30.5
St. Paul	0.95	1.09	1.07	8.66	21.5

There was a weak relationship between *nxrA:16S* rRNA and the other normalized functional genes: *nirS:16S* (r=0.29 p= 0.18) and *AOB:16S* (r=0.34, p=0.11) (Table 3.6). Though not significant, *nirS:16S* was also weakly negatively correlated with *AOB:16S* (r=-0.30, p=0.17) (Table 3.6).

Discussion:

N_2O Emissions:

Though N_2O data were not collected from all plots during the 2019 sampling season, all sampling sites have a long-term N_2O emissions data. Values reported from the Minnesota sites in 2017 indicate a range of N_2O emissions (Table 3.7) among the study sites and among the various management strategies (Breuillin-Sessoms et al., 2017). Generally, the historical data indicates that N_2O emissions increased with higher N fertilizer application rates. At the Rosemount site N_2O emissions were slightly higher in CT plots than NT plots when receiving 100-500 μg N g^{-1}. Nitrous oxide emissions also varied by site, the Becker site reported the highest N_2O emissions at most fertilization levels, followed by the Rosemount and St. Paul sampling sites. Values reported from the Beltsville site show that the highest emissions were recorded in the organic plots followed by the chisel and no-till plot. Although this difference was statistically significant on some sampling days, cumulative emission records indicate no statistically significant differences in N2O emissions among the treatments (Djurickovic, 2010).

Microbial Abundance:

Abundance of *16S* rRNA genes per gram of soil varied greatly between sampling sites and between the Maryland and Minnesota samples. This variation could be due to a wide variety

of environmental factors including: soil type, rainfall, temperature, and soil pH (Cao et al., 2016). The Becker 2018 sample had lower *16S* rRNA and functional gene abundances, which is likely due to the sample having been previously sieved and dried, and storage conditions are known to effect microbial community analysis (Rubin et al., 2013). *16S* abundance was also correlated to tillage regime, matching what has been observed in the literature and reporting higher relative abundances (RAs) in NT plots. Tillage is known to homogenize soils, and previous studies have indicated that long-term no-till management can increase fungal and bacterial abundance and bacterial richness (Sengupta & Dick, 2015; Sun et al., 2016). Though it was not observed in our study, organic farming practices have also been known to have a positive effect on soil microbial biomass (C and N) as well as increase total phospholipid fatty acids (PLFAs) (Lori et al., 2017).

Nitrification Functional Genes:

Though comammox *Nitrospira* have been observed in agricultural soil samples, we were not able to amplify the corresponding functional genes from any of our samples. We must therefore reject our second hypothesis: that we would observe comammox *Nitrospira* in all of the soil samples, though they would be in lower abundance than traditional nitrifiers. This could indicate that comammox *Nitrospira* was not present in any of our plots. However, this could also be due to our choice of primer sets. A recent study evaluated the effectiveness of the two most common primer sets used to amplify comammox *Nitrospira* and found that the set that we used (comA/B- 244f/659r) was effective in amplifying samples with high relative abundances but that it was not sensitive to clades with a lower RA (Lin et al., 2020). Previous studies that have successfully isolated comammox *Nitrospira* from agricultural soils have indicated lower RAs

compared to more traditional nitrifiers, and the comammox *Nitrospira* might be outcompeted in high nutrient (NH_3^+) environments (Xu et al., 2020).

Analysis of the successfully amplified nitrification functional genes reveal some differences in the nitrifying community in relation to management strategy. These differences were not significant but are supported by similar findings from the literature. Long-term fertilization has repeatedly been shown to increase nitrifiers, especially ammonia-oxidizing bacteria (Aliyu et al., 2021; Sun et al., 2015). The non-fertilized plots in our study had slightly lower *nxrA:16S* rRNA ratio and much lower *amoA AOB:16S* rRNA ratio when compared to the other fertilized Minnesota plots. While not significant, this supports our third hypothesis: that fertilizer applications (either manure-based or inorganic fertilizer) will increase the overall abundance of nitrification genes (*amoA AOB* and *nxrA*).

The potential impact of tillage was mixed, and conflicts with trends reported in previous studies. Though not significant, *nxrA:16S* rRNA was higher in the CT plots than in comparable NT plots at both Beltsville and Rosemount, indicating that tillage may have increased the relative proportion of nitrifiers (Figures 3.3 b &c, 3.4 b & c). Past studies have reported the opposite trend, and this does not support our initial hypothesis (that greater *nxrA* gene abundance would be observed in the no-till plots). One study of long-term experimental plots in France found that no-till soils had greater *nxrA* gene abundance and potential nitrite oxidation (Attard et al., 2010). Furthermore, the ratio between *nxrA* and *amoA AOB* has been used as an indicator for nitrification activity, one previous study reported that it explained 79% of variance in cumulative N_2O emissions (Breuillin-Sessoms et al., 2017). Though not significant, no-till plots in Beltsville did have higher *nxrA: amoA AOB* than the Beltsville CT plots (Figure 3.4e). This indicates that there might be tighter coupling between the communities of ammonia oxidizers and nitrite

oxidizers. Nitrous oxide emissions due to nitrification in agricultural soils has often been referred to metaphorically as a leaky pipe. In this scenario total N_2O emissions are proportional to N-cycling, and nitrification is mediated by two distinct microbial populations: ammonia oxidizers (*amoA*) and nitrite oxidizers (*nxr*) (Xing et al., 2011). Leaks (referring to additional N_2O emissions) can occur when these two populations of nitrifiers within the soil system are not coupled (Breuillin-Sessoms et al., 2017). For example, if there are more ammonia oxidizers present in the soil than nitrite oxidizers, a leak is created and the imbalance of microbial activity can lead to a build-up of nitrite, and as a by-product of increased ammonia oxidation can increase N_2O emissions. Our data therefore suggests that, while not statistically significant, the nitrifying populations are more tightly coupled at the Beltsville NT plots compared to the CT plots. This is also supported by historical N_2O emission data from the Beltsville plots. While not statistically significant, the N_2O emissions at these sample sites reported lower N_2O emissions in the NT sites compared to the CT sites (Breuillin-Sessoms et al., 2017; Djurickovic, 2010). However, the same trend was not observed between the CT and NT treatments in Rosemount (Figure 3.3e). Further research is needed, but this indicates that while there may not have been a substantial difference in the size of the overall nitrifying community between tillage practices, no-till management may still increase potential nitrite oxidation and subsequently decrease overall N_2O emissions.

Denitrification Functional Genes:

In our study, we measured *nirS* and *nirK* functional genes that each correspond to one of the two common forms of nitrate reductase within denitrifying communities. Unlike nitrification, the full pathway for denitrification can be found in one organism, with common denitrifiers

containing all the requisite enzymes for complete denitrification (Jones et al., 2008). As such, each sample's *nirS* and *nirK* abundances can act as a proxy to represent the abundance of general denitrifiers with *nirS* and denitrifiers with *nirK* within each plot.

There were no significant differences between the denitrification gene abundances. However, several of the trends that were observed are supported by past studies. The ratio of *nirK:16S* rRNA was consistently higher in no-till plots comparted to tilled plots at both Rosemount and Beltsville, and previous studies have repeatedly indicated that no-till management can increase soil denitrification activity and gene abundance (Wang & Zou, 2020). This in turn has been correlated with an increase in N_2O emissions in multiple studies (Billings & Tiemann, 2014; Kong et al., 2021; Wang & Zou, 2020). Analysis of *nirS* gene abundance at our study sites, however, yielded contradictory results; CT plots had slightly higher *nirS:16S* rRNA abundance than NT plots at both Rosemount and Beltsville (Figures 3.3d and 3.4d). However, comparing the Beltsville samples to historical N_2O data it is worth noting that the *nirS:16S* rRNA ratio follows the same trend as N_2O past N_2O emissions in the plot. Though not significantly different, the organic plots had the highest *nirS:16S* rRNA ratio, followed by the CT plots, and NT plots. Similarly, though not statistically significant, the historical N_2O data indicates that the highest emissions were recorded in the organic plots followed by the CT and NT plots. Further investigation is needed to explore this relationship.

Comparison of the two denitrifying genes, indicates much greater *nirS* abundance in comparison to *nirK,* and higher *nirS: nirK* in all the Beltsville plots when compared to the Minnesota plots. *NirS* encodes the more common heme form of *nir* (Alvarez et al., 2014); therefore, *nirS* would have a greater abundance in all our plots. The difference in the ratio of *nirS: nirK* could potentially be explained by differences in field conditions and environmental

factors. The niches of *nirS* and *nirK* have been studied, and both have been noted to be influenced differentially by soil pH, soil water content, total soil carbon, and soil development (Bowen et al., 2018; Herold et al., 2018; Szukics et al., 2010). The Beltsville and Minnesota sites have very different climates: Beltsville receives and average of 1110mm of precipitation and has an average temperature of 12.8^{o}C, while the Minnesota sites are drier on average (879mm of precipitation) and colder (6.4^{o}C). This difference can in turn influence soil formation, available water in the soil, and a wide range of other soil properties (Doula & Sarris, 2016). Additionally, this could indicate that the Minnesota sites have a lower pH, as loss of *nirS* abundance has been linked to decreases in pH and is more sensitive to pH change than *nirK* (Herold et al., 2018).

Conclusion:

The results of this study were mixed, and we are unable to accept our hypotheses with any statistical significance. Our first hypothesis postulated that, as previous studies have shown that long-term management can have impact on soil microbial populations, we would expect a greater abundance of nitrite oxidizing and denitrification genes in less manipulated (no-till and organic) plots. Comparison of the *nxrA* and *amoA AOB* gene abundance under different tillage regimes in our plots yielded mixed results. Treatment had no clear difference among the *16S* rRNA normalized *amoA AOB* values in the Beltsville plots. Furthermore, while it is not statistically significant, the *amoA AOB:16S* rRNA at the Rosemount, MN site and the *nxrA:16S* rRNA ratios at both Beltsville and Rosemount were higher in conventionally tilled plots than non-tilled plots. This contradicts what have been observed in past literature. Additional research is needed to understand this relationship further.

We also must reject our second hypothesis: that we would expect to observe comammox *Nitrospira* in all of our soil samples. This could indicate that it may not be as ubiquitous in soils

as other studies indicate, or that successful amplification requires alternative primers to amplify comammox bacteria in environments where it would have lower relative abundances. Further study in needed, as other studies have begun to isolate comammox *Nitrospira* in agricultural soils, and it may yet prove to have an important role to play in agricultural N cycling.

Although we are unable to accept our third and final hypothesis with any statistical significance, the data from our study does support our prediction. As fertilizer application is known to increase nitrification activity within soils, we anticipated that additional N (from manure or inorganic fertilizer) would increase the abundance of nitrification genes (*nxrA* and *amoA AOB*). While not significant, the non-fertilized Minnesota plots reported both lower *amoA AOB:16S* rRNA and *nxrA:16S* rRNA ratios. While gene and microbial abundance is not a proxy for microbial activity, this would indicate smaller nitrifying communities in non-fertilized plots (compared to fertilized plots).

Throughout our analysis, we found that sampling site had the largest impact on overall functional gene abundance. Comparing the Maryland samples to the Minnesota samples, there were statistically significant differences in *16S* rRNA, *nxrA, amoA AOB,* and *nirK* abundance (Table 3.4). Comparing all 5 independent sites, there were significant differences in the abundance of *16S* rRNA and all the studied functional genes (Table 3.4). While there were less significant differences among the sites once normalized with *16S* rRNA abundance, there was still wide variation in the microbial community composition of the various sites. This indicates that, while more research is needed, the site-specific environmental factors may have more control over soil microbial communities and N_2O emissions than management techniques. Current management recommendations to limit N_2O emissions are broad: extension agencies suggest that farmers use less fertilizer, using split application of fertilizer to increase the plant

use efficiency, incorporating N-fixing legumes into crop rotations, minimizing tillage, and preventing fields from becoming water-logged (Sudmeyer et al., 2014). Additionally, farmers have increasingly used nitrification inhibitors such as 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) to decrease N_2O emissions by targeting ammonia-oxidizing bacteria and archaea and decreasing a soil's net nitrification rate (Chen et al., 2015). While this research does not discount these approaches, it emphasizes the heterogenous nature of field work and indicates that farm managers may want to take a more targeted, site-specific approach to limit agricultural N_2O emissions.

References – Ch. III

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Appendix

Table S1. One- Way ANOVA Table for *16S* rRNA abundance.

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
MD	1	9.76E+19	9.76E+19	16.375	0.0006
Error	21	1.25E+20	5.96E+180		
C. Total	22	2.23E+20			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.91E+19	1.91E+19	2.240	0.1604
Error	12	1.03E+20	8.54E+18		
C. Total	13	1.22E+20			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
Site	4	1.31E+20	3.29E+19	6.473	0.002
Error	18	9.14E+19	5.18E+18		
C. Total	22	2.23E+20			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	3.14E+19	3.14E+19	10.298	0.0326
Error	4	1.22E+19	3.05E+18		
C. Total	5	4.36E+19			

Site Diff (MN)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
Site	2	2.06E+19	1.03E+19	1.118	0.361
Error	11	1.01E+20	9.19E+18		
C. Total	13	1.22E+20			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.98E+16	1.98E+16	0.040	0.8477
Error	7	3.49E+18	4.99E+17		
C. Total	8	3.51E+18			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.94E+19	1.94E+19	2.000	0.172
Error	21	2.03E+20	9.69E+18		
C. Total	22	2.23E+20			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	4.66E+19	4.66E+19	5.562	0.0281
Error	21	1.76E+20	8.39E+18		
C. Total	22	2.23E+20			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	3.52E+18	3.52E+18	0.358	0.561
Error	12	1.18E+20	9.84E+18		
C. Total	13	1.22E+20			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	2.80E+18	2.80E+18	2.760	0.1352
Error	8	8.13E+18	1.02E+18		
C. Total	9	1.09E+19			

Table S2. One- Way ANOVA Table for *nxrA* gene abundance.

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
MD	1	2.28E+30	2.28E+30	15.261	0.0008
Error	21	3.14E+30	1.49E+29		
C. Total	22	5.42E+30			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.54E+29	1.54E+29	0.6329	0.4418
Error	12	2.92E+30	2.43E+29		
C. Total	13	3.07E+30			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
Site	4	3.19E+30	7.98E+29	6.4514	0.002
Error	18	2.23E+30	1.24E+29		
C. Total	22	5.42E+30			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	6.28E+29	6.28E+29	3.6465	0.1288
Error	4	6.89E+29	1.72E+29		
C. Total	5	1.32E+30			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	5.43E+29	2.71E+29	1.181	0.343
Error	11	2.53E+30	2.30E+29		
C. Total	13	3.07E+30			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	7.07E+26	7.07E+26	0.0751	0.7919
Error	7	6.59E+28	9.41E+27		
C. Total	8	6.66E+28			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	2.19E+29	2.19E+29	0.884	0.358
Error	21	5.20E+30	2.48E+29		
C. Total	22	5.42E+30			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	6.01E+29	6.01E+29	2.6234	0.1202
Error	21	4.81E+30	2.29E+29		
C. Total	22	5.42E+30			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	8.80E+26	8.80E+26	0.003	0.954
Error	12	3.07E+30	2.56E+29		
C. Total	13	3.07E+30			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	1.19E+28	1.20E+28	0.5196	0.4915
Error	8	1.84E+29	2.30E+28		
C. Total	9	1.96E+29			

Table S3. One- Way ANOVA Table for *amoA AOB* gene abundance.

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	1.28E+30	1.28E+30	8.054	0.010
Error	21	3.33E+30	1.58E+29		
C. Total	22	4.60E+30			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	2.47E+29	2.47E+29	0.969	0.344
Error	12	3.06E+30	2.55E+29		
C. Total	13	3.31E+30			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
Site	4	2.35E+30	5.88E+29	4.698	0.009
Error	18	2.25E+30	1.25E+29		
C. Total	22	4.60E+30			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	2.28E+27	2.28E+27	0.004	0.950
Error	4	2.05E+30	5.13E+29		
C. Total	5	2.05E+30			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	1.06E+30	5.29E+29	2.585	0.120
Error	11	2.25E+30	2.04E+29		
C. Total	13	3.31E+30			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	2.01E+25	2.01E+25	0.007	0.937
Error	7	2.07E+28	2.95E+27		
C. Total	8	2.07E+28			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	4.40E+28	4.40E+28	0.203	0.657
Error	21	4.56E+30	2.17E+29		
C. Total	22	4.60E+30			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	2.68E+26	2.68E+26	0.001	0.972
Error	21	4.60E+30	2.19E+29		
C. Total	22	4.60E+30			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	5.27E+29	5.27E+29	2.275	0.157
Error	12	2.78E+30	2.32E+29		
C. Total	13	3.31E+30			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	1.50E+28	1.50E+28	1.092	0.327
Error	8	1.10E+29	1.38E+28		
C. Total	9	1.25E+29			

Table S4. One- Way ANOVA Table for *nirS* gene abundance.

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
MD	1	7.54E+14	7.54E+14	0.001	0.974
Error	21	1.45E+19	6.92E+17		
C. Total	22	1.45E+19			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.61E+18	1.61E+18	1.788	0.206
Error	12	1.08E+19	8.98E+17		
C. Total	13	1.24E+19			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
Site	4	9.31E+18	2.24E+18	8.037	0.0007
Error	18	5.21E+18	2.90E+17		
C. Total	22	1.45E+19			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	4.88E+17	4.88E+17	5.768	0.074
Error	4	3.38E+17	8.46E+16		
C. Total	5	8.26E+17			

Site Diff (MN)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
Site	2	8.40E+18	4.20E+18	11.608	0.002
Error	11	3.98E+18	3.62E+17		
C. Total	13	1.24E+19			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	6.22E+16	6.22E+16	0.209	0.661
Error	7	2.08E+18	2.97E+17		
C. Total	8	2.14E+18			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.20E+18	1.20E+18	1.889	0.184
Error	21	1.33E+19	6.35E+17		
C. Total	22	1.45E+19			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	1.83E+18	1.83E+18	3.034	0.096
Error	21	1.27E+19	6.05E+17		
C. Total	22	1.45E+19			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	2.50E+18	2.50E+18	3.031	0.107
Error	12	9.89E+18	8.24E+17		
C. Total	13	1.24E+19			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	4.19E+15	4.19E+15	1.092	0.327
Error	8	2.41E+18	3.02E+17		
C. Total	9	2.42E+18			

Table S5. One- Way ANOVA Table for *nirK* gene abundance.

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	4.63E+13	4.63E+13	11.923	0.002
Error	21	8.15E+13	3.88E+12		
C. Total	22	1.28E+14			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.05E+13	1.05E+13	1.817	0.203
Error	12	6.96E+13	5.80E+12		
C. Total	13	8.01E+13			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	3	6.85E+13	2.29E+13	7.328	0.002
Error	19	5.92E+13	3.12E+12		
C. Total	22	1.28E+14			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	2.78E+13	2.78E+13	8.500	0.043
Error	4	1.31E+13	3.27E+12		
C. Total	5	4.09E+13			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	2.23E+13	1.11E+13	2.117	0.167
Error	11	5.79E+13	5.26E+12		
C. Total	13	8.01E+13			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	4.17E+11	4.17E+11	2.983	0.128
Error	7	9.78E+11	1.40E+11		
C. Total	8	1.39E+12			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	8.03E+12	8.03E+12	1.407	0.249
Error	21	1.20E+14	5.70E+12		
C. Total	22	1.28E+14			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	1.28E+13	1.28E+13	2.328	0.142
Error	21	1.15E+14	5.48E+12		
C. Total	22	1.28E+14			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	1.87E+09	1.87E+09	0.000	0.987
Error	12	8.01E+13	6.68E+12		
C. Total	13	8.01E+13			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	1.86E+11	1.86E+11	0.352	0.569
Error	8	4.23E+12	5.28E+11		
C. Total	9	4.41E+12			

Table S6. One- Way ANOVA Table for *amoA AOB:16S* rRNA

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	2.72E+10	2.72E+10	3.949	0.060
Error	21	1.45E+11	6.88E+09		
C. Total	22	1.78E+11			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	3.20E+10	3.20E+10	3.4755	0.0869
Error	12	1.10E+11	9.20E+09		
C. Total	13	1.42E+11			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
Site	4	5.31E+10	1.33E+10	2.013	0.136
Error	18	1.19E+11	6.59E+09		
C. Total	22	1.72E+11			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.12E+10	1.12E+10	0.4549	0.537
Error	4	9.83E+10	2.46E+10		
C. Total	5	1.09E+11			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	2.58E+10	1.29E+10	1.217	0.333
Error	11	1.17E+11	1.06E+10		
C. Total	13	1.42E+11			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	16004363	16004362	0.0532	0.8242
Error	7	2.11E+09	3.01E+08		
C. Total	8	2.12E+09			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.24E+10	1.24E+10	1.631	0.216
Error	21	1.59E+11	7.59E+09		
C. Total	22	1.72E+11			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	2.37E+09	2.37E+09	0.2941	0.5933
Error	21	1.69E+11	8.06E+09		
C. Total	22	1.72E+11			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	2.58E+10	2.58E+10	2.653	0.129
Error	12	1.17E+11	9.72E+09		
C. Total	13	1.42E+11			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	7.29E+08	7.29E+08	1.2626	0.2937
Error	8	4.62E+09	5.77E+08		
C. Total	9	5.35E+09			

Table S7. One- Way ANOVA Table for *nirS:16S* rRNA..

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
MD	1	1.510362	1.51036	46.754	<0.0001
Error	21	0.67839	0.0323		
C. Total	22	2.188752			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	0.002816	0.002816	0.324	0.579
Error	12	0.104127	0.008677		
C. Total	13	0.106943			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob>F
Site	4	1.60E+00	3.99E-01	12.111	<0.0001
Error	18	5.93E-01	3.29E-02		
C. Total	22	2.19E+00			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	0.001621	0.001621	1.060	0.362
Error	4	0.00612	0.00153		
C. Total	5	0.007741			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	0.06958	0.03479	10.243	0.003
Error	11	0.037363	0.003397		
C. Total	13	0.106943			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	0.011763	0.011763	0.147	0.713
Error	7	0.559683	0.079955		
C. Total	8	0.571446			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	0.00261	0.00261	0.025	0.876
Error	21	2.186141	0.104102		
C. Total	22	2.188752			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	0.310668	0.310668	3.474	0.076
Error	21	1.878083	0.089433		
C. Total	22	2.188752			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	0.012394	0.012394	1.573	0.234
Error	12	0.094549	0.007879		
C. Total	13	0.106943			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	0.101749	0.101749	1.071	0.331
Error	8	0.760025	0.095003		
C. Total	9	0.861774			

Table S8. One- Way ANOVA Table for *nxrA:16S* rRNA..

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	2.01E+09	2.01E+09	0.894	0.355
Error	21	4.72E+10	2.25E+09		
C. Total	22	4.92E+10			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	5.06E+09	5.06E+09	3.3321	0.0929
Error	12	1.82E+10	1.52E+09		
C. Total	13	2.33E+10			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
Site	4	9.18E+09	2.29E+09	1.033	0.417
Error	18	4.00E+10	2.22E+09		
C. Total	22	4.92E+10			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.12E+09	1.12E+09	0.4399	0.5434
Error	4	1.02E+10	2.54E+09		
C. Total	5	1.13E+10			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	5.83E+09	2.91E+09	1.837	0.205
Error	11	1.74E+10	1.59E+09		
C. Total	13	2.33E+10			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	3.96E+08	3.96E+08	0.1181	0.7412
Error	7	2.35E+10	3.36E+09		
C. Total	8	2.39E+10			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.97E+09	1.97E+09	0.877	0.356
Error	21	4.72E+10	2.25E+09		
C. Total	22	4.92E+10			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	4.85E+09	4.85E+09	2.2997	0.1443
Error	21	4.43E+10	2.11E+09		
C. Total	22	4.92E+10			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	2.93E+09	2.93E+09	1.729	0.213
Error	12	2.03E+10	1.69E+09		
C. Total	13	2.33E+10			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	8.45E+09	8.45E+09	4.1493	0.076
Error	8	1.63E+10	2.04E+09		
C. Total	9	2.47E+10			

Table S9. One- Way ANOVA Table for *nirK:16S* rRNA..

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	1.09E-07	1.09E-07	1.266	0.273
Error	21	1.80E-06	8.58E-08		
C. Total	22	1.91E-06			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.64E-09	1.64E-09	0.050	0.826
Error	12	3.91E-07	3.26E-08		
C. Total	13	3.93E-07			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
Site	3	1.97E-07	6.58E-08	0.730	0.547
Error	19	1.71E-06	9.01E-08		
C. Total	22	1.91E-06			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	3.51E-08	3.51E-08	1.950	0.235
Error	4	7.19E-08	1.80E-08		
C. Total	5	1.07E-07			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	8.87E-08	4.44E-08	1.604	0.245
Error	11	3.04E-07	2.77E-08		
C. Total	13	3.93E-07			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	5.40E-08	5.40E-08	0.279	0.614
Error	7	1.35E-06	1.93E-07		
C. Total	8	1.41E-06			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	2.89E-08	2.89E-08	0.323	0.576
Error	21	1.88E-06	8.96E-08		
C. Total	22	1.91E-06			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	8.89E-08	8.89E-08	1.025	0.323
Error	21	1.82E-06	8.67E-08		
C. Total	22	1.91E-06			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	2.12E-08	2.12E-08	0.683	0.425
Error	12	3.72E-07	3.10E-08		
C. Total	13	3.93E-07			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	2.64E-07	2.64E-07	1.813	0.215
Error	8	1.17E-06	1.46E-07		
C. Total	9	1.43E-06			

Table S10. One- Way ANOVA Table for *nirS:nirK* rRNA..

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	2707746	2707746	32.959	<0.001
Error	21	1725269	82156		
C. Total	22	4433015			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	5108.31	5108.3	0.325	0.579
Error	12	188413.2	15701.1		
C. Total	13	193521.5			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
Site	2	2827859	942620	11.158	0.0002
Error	19	1605155	84482		
C. Total	22	4433015			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	12963.25	12963.2	19.290	0.012
Error	4	2687.986	672		
C. Total	5	15651.23			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	120113.6	60056.8	8.999	0.005
Error	11	73407.85	6673.4		
C. Total	13	193521.5			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	382071.1	382071	2.326	0.171
Error	7	1149676	164239		
C. Total	8	1531748			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	62240	62240	0.299	0.590
Error	21	4370775	208132		
C. Total	22	4433015			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	485712.8	485713	2.584	0.123
Error	21	3947302	187967		
C. Total	22	4433015			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	42969.49	42969.5	3.425	0.089
Error	12	150552	12546		
C. Total	13	193521.5			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	33385.9	33386	0.132	0.726
Error	8	2025867	253233		
C. Total	9	2059252			

Table S11. One- Way ANOVA Table for *nxrA:amoA AOB* rRNA..

MD v. MN

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
MD	1	1.09E-07	1.09E-07	1.266	0.243
Error	21	1.80E-06	8.58E-08		
C. Total	22	1.91E-06			

NT v. CT (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	0.49753	0.49753	0.088	0.771
Error	12	67.59068	5.63256		
C. Total	13	68.08821			

Site Diff. (all)

Source	DF	Sum of Sq	Mea Sq	F Ratio	Prob> F
Site	3	1.97E-07	6.58E-08	0.730	0.547
Error	19	1.71E-06	9.01E-08		
C. Total	22	1.91E-06			

NT v. CT (Rosemount)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	1.388016	1.38802	0.389	0.567
Error	4	14.25986	3.56497		
C. Total	5	15.64788			

Site Diff (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Site	2	13.36908	6.68454	1.344	0.301
Error	11	54.71913	4.97447		
C. Total	13	68.08821			

NT v. CT (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	254.3303	254.33	1.046	0.341
Error	7	1702.53	243.219		
C. Total	8	1956.86			

NT v. CT (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Till	1	45.8348	45.835	0.309	0.584
Error	21	3112.172	148.199		
C. Total	22	3158.006			

F. v. NF (all)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	216.6422	216.642	1.547	0.227
Error	21	2941.364	140.065		
C. Total	22	3158.006			

F v. NF (MN)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Fert	1	13.65338	13.6534	3.010	0.108
Error	12	54.43483	4.5362		
C. Total	13	68.08821			

Organic v. Inorganic (Beltsville)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Org	1	88.7565	88.756	0.340	0.576
Error	8	2087.702	260.963		
C. Total	9	2176.458			

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