12-15-2021 9:30 AM

Short-term vs. Long-term Warming and Nitrogen Treatment Effects on Soil Carbon and Microbial Activity in a Temperate Old Field

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

Abstract

Climate warming and atmospheric nitrogen deposition, two elements of global change, are expected to exert strong effects on northern temperate ecosystems over the next century. I added new nitrogen addition and warming plots to a pre-existing nitrogen and warming field experiment in London, Ontario to compare the short-term (1-2 year; new plots) versus long-term (14-15 year; old plots) treatment effects on soil carbon and microbial activity. I used soil density fractionation and size fractionation to separate soil carbon fractions and analyzed carbon quality using Fourier-transform infrared spectroscopy (FTIR). I used extracellular enzyme assays to assess microbial activities. The soil organic matter free light fraction recovery increased with nitrogen addition in the old plots but decreased in the new plots. Interactions between warming and plot age were significant for some hydrolase enzymes. These results confirm short-term responses of soil carbon and microbial activity differed from long-term responses in this field experiment.

Keywords

Carbon cycle, grassland, extracellular enzymes, nitrogen fertilization, climate warming, soil organic matter, soil carbon, Fourier-transform infrared spectroscopy, density fractionation, global change

Summary for Lay Audience

Since the industrial revolution, the burning of fossil fuels and agricultural intensification have increased globally. These activities have accelerated global change through the release of greenhouse gases, such as carbon dioxide (CO_2) and methane (CH_4), and increased nitrogen pollution. Greenhouse gases trap radiation from the sun in the form of heat, ultimately increasing the Earth's global surface temperatures. In the coming decades, average global surface temperatures are expected to increase around 2 °C, with the greatest warming effect towards the poles. In addition to warming, nitrogen pollution threatens the surrounding environment when excess nitrogen enters a system. Nitrogen-based fertilizers are often applied excessively to agricultural soils as nitrate, ammonia, urea, and/or ammonium, and can have non-specific effects, such as runoff contaminating aquatic environments. Nitrogen released to the atmosphere also can be deposited back into ecosystems through atmospheric nitrogen deposition. I was interested in understanding how soils would respond to warming and nitrogen addition over time. To study this, I conducted a long-term field experiment in a temperate old field to examine the short-term (1-2 year) versus the long-term (14-15 year) responses of soil organic matter (primarily decomposing plant material) and microbial activities to warming and nitrogen treatments. Rather than comparing short-term responses from previous studies with long-term responses, new treatment and control plots were established to control for weather variation over time. Overall, long-term plots exhibited stronger treatment responses than the short-term plots. Nitrogen addition enhanced the accumulation of organic matter in the long-term plots for the free light fraction of organic matter in soil, which is comprised mostly of root fragments. Warming alone had no effect on organic matter accumulation but, when combined with nitrogen addition, organic matter in soil aggregates (i.e. small soil clumps protected from microbial decomposition) was greater. Lastly, both warming, and nitrogen addition treatments affected microbial enzyme activities, but carbon-acquiring enzymes, targeting easily decomposable organic matter, responded more to treatments than the enzymes produced to target more difficult substrates. Results from my thesis suggest that short-term treatment responses cannot be extrapolated to the long-term, and that cumulative responses can occur in the long-term.

Co-Authorship Statement

The research described in this thesis is a result of my contributions and those of my supervisor Dr. Hugh Henry. I wrote the thesis and it was edited by Dr. Henry. We both contributed to the development of the methods. Any manuscripts arising from this thesis will be prepared and submitted for publication by me and Dr. Henry.

Acknowledgments

First, I would like to sincerely thank my supervisor Dr. Hugh Henry for giving me the utmost guidance and respect as a graduate student. Dr. Henry was always there for me when I needed it most. He was a great help for every aspect of my project – giving me rides to the field and editing my work during the writing process, despite things often taking an unexpected turn, and the tight deadlines. I would also like to thank my lab mates, Spencer Heuchan, Samuel Rycroft, Breanna Craig, Benjamin Souriol and Ricky Kong, for all of their support throughout this process, answering all my questions and making me feel so welcomed.

Next, I would like to thank my Advisory Committee, Dr. Zoë Lindo and Dr. Brian Branfireun, for supporting me and guiding me throughout my degree. They provided me with useful advice and fresh perspectives that really helped shape the overall project. Dr. Branfireun was also gracious enough to offer the use of his lab equipment for my FTIR work. Ericka James, Dr. Branfireun's former graduate student, took time out of her post-graduation life to help me with the FTIR process.

I would also like to give thanks to the many lab groups (the Guglielmo lab, the Staples lab, the Kelly lab, the Cumming lab, and the Timoshenko lab) that let me borrow laboratory equipment that I needed for my project, and to Albert at AAFC for his help during my field seasons.

I would also like to thank my family and friends for all the unwavering support they have given me throughout all of the ups and downs. I couldn't have done it without their support and encouragement throughout this process.

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List of Abbreviations

ANOVA	Analysis of variance
ATR	Attenuated total reflection
C:N	Carbon to nitrogen ratio
DOC	Dissolved organic carbon
dw	Dry weight
EEAs	Extracellular enzyme activities
FLF	Free light fraction
FTIR	Fourier-transform infrared spectroscopy
L-DOPA	L-3,4-dihydroxyphenyl-alanine
MaOM	Mineral-associated organic matter
MUB	Methylumbelliferone
NAG	N-acetyl-glucosaminidase
NaPT	Sodium polytungstate
NPP	Net primary productivity
OLF	Occluded light fraction
POM	Particulate organic matter
SOM	Soil organic matter

Chapter 1

1 Introduction

1.1 Climate Warming

In recent decades, anthropogenic climate change has become an increasing global concern. Global temperatures have risen an average of 1.1 °C from pre-industrial levels (IPCC, 2021), and if future climate predictions are correct, global surface temperatures will increase by another 0.4 °C in the next two decades and by 2100, temperatures could rise up to 4.4 °C from pre-industrial levels, with more intense climate warming expected to occur with increasing latitude (IPCC, 2021). Soils in northern latitudes, including mineral soils in northern temperate regions, are therefore expected to experience large temperature increases with climate warming relative to most other soils. Soils are the largest terrestrial store of carbon, and environmental changes such as warming, could cause soil carbon losses that may encourage positive carbon-climate feedbacks (Bradford et al., 2016; Crowther et al., 2016). Many biogeochemical processes, such as decomposition, are sensitive to temperature, and temperature increases consistent with future projections have the potential to alter global ecosystem functioning in the coming decades (Millennium Ecosystem Assessment, 2005; Stone et al., 2012; Li et al., 2019); however, the extent to which climate warming will affect ecosystem functioning remains unclear.

1.2 Atmospheric Nitrogen Deposition

Nitrogen is an important mineral nutrient for plants; the availability of nitrogen in soil limits plant growth in most terrestrial ecosystems (Lebauer and Treseder, 2008). Nitrogen is extremely abundant in the atmosphere in the form of nitrogen gas (N_2), which makes up approximately 78% of the atmosphere (Galloway et al., 2004). However, N_2 is an inert form of nitrogen, and atmospheric N_2 must be converted into a bioavailable form, such as ammonium (NH_4^+) and/or nitrate (NO_3^-). The first step of this process is nitrogen fixation. Nitrogen fixation can occur naturally when the energy of lightning breaks the bond of N_2 in the atmosphere, or when it is fixed by microbes in soils. Specialized

nitrogen-fixing bacteria free-living in the soil, or with symbiotic relationships with plants, produce nitrogenase enzymes to break the nitrogen bonds (Chapin et al., 2002; Galloway et al., 2004). The resulting product of nitrogen fixation is NH_4^+, which can then be taken up by plants. Nitrogen mineralization by microbes, the process describing the conversion of organic nitrogen (nitrogen originating from living material) into inorganic forms of ammonium (NH_4^+) and/or nitrate (NO_3^-), which are the two dominant forms of inorganic nitrogen in soil available for plant uptake, occurs when microbes break down detritus after organisms die, and release it into the soil (Galloway et al., 2004). Microbes can use this organic nitrogen to produce biomass and excrete nitrogen-containing compounds, which can then be used by plants. Nitrogen can also enter soil systems through fertilizer application to increase crop productivity (typically produced via industrial nitrogen fixation), or natural processes, including atmospheric deposition, or other inputs from decaying plant and animal matter (Galloway et al., 2004). Nitrogen may leave soil when NH_4^+ undergoes nitrification to become NO_3^-, which due to its negative charge is highly mobile in soil and susceptible to leaching, or nitrogen may leave under anaerobic conditions through denitrification, when NO_3^- is converted to nitrous oxide (N_2O) or N_2 gas (Phoenix et al., 2012). Other nitrogen-based gases, nitrogen oxides (NO_x), are reactive, toxic gases produced by the combustion of fossil fuels, most notably from automobile engines (Kanakidou et al., 2016). Deposits of reactive nitrogen in terrestrial ecosystems can occur when NO_x gases fall as wet or dry atmospheric nitrogen deposition; wet deposition occurs through precipitation, and dry deposition occurs through processes such as sedimentation. Oversaturation of soils with nitrogen due to natural and anthropogenic causes can lead to nitrogen runoff into surrounding environments causing eutrophication, groundwater contamination, as well as detrimental changes in the nitrogen and carbon cycles (Smith and Schindler, 2009; Phoenix et al., 2012).

Atmospheric nitrogen deposition is a natural process, but over the last century, human activities have become the main source of reactive nitrogen deposition in terrestrial ecosystems (Galloway et al., 2008). Regional differences in atmospheric nitrogen deposition occurs between remote, undeveloped areas, and highly developed areas that feature intense industrial and agricultural production. Areas with intense agricultural production currently receive high levels of nitrogen deposition, and future nitrogen

deposition is expected to continue to increase in these areas (Galloway et al., 2004; Kanakidou et al., 2016). Because bioavailable forms of nitrogen (NH_4^+ and NO_3^-) are limited in terrestrial environments, intense atmospheric nitrogen deposition can alter plant productivity and nutrient cycling in terrestrial systems, and enhance global change (Phoenix et al., 2012).

1.3 Soil Organic Matter and Carbon Fractions

Soils contain the majority of all carbon in terrestrial ecosystems and are therefore an integral component of carbon storage on Earth (Crowther et al., 2016). Carbon enters the terrestrial system when plants photosynthesize and fix CO_2 into plant aboveground and belowground biomass. Symbiotic relationships with mycorrhizal fungi can form when plants provide carbon as an energy source to fungi through plant roots, while the fungi provide plants with essential nutrients, such as nitrogen and phosphorus (Allison and Treseder, 2008). Carbon is lost from soil systems when plants and soil microbes respire, or when dissolved organic carbon (DOC) leaches out of the system (Barker et al., 2003).

Soil carbon is largely composed of organic carbon, and mineral carbon. Mineral carbon describes the inorganic carbon, such as mineral carbonates, in soils released from weathering of rocks (Barker et al., 2003). Organic carbon describes carbon originating from living organisms, and organic carbon inputs can enter from aboveground plant material, as well as recycled organic carbon from soil organism waste and dead cells, which can enter the soil food web. Soil organic carbon is strongly related to soil organic matter (SOM), a carbon-rich, heterogenous mixture of organic material in various stages of decomposition, from recently decomposed to highly decomposed organic matter, originating from plant and soil organisms and their wastes (Barker et al., 2003). Soil organic matter contributes to soil functioning by facilitating nutrient and moisture retention, carbon turnover and sequestration, as well as influencing soil structure (Wander, 2004). SOM can be divided into distinct fractions with different carbon structure profiles and residence times. Labile organic matter is sometimes referred to as particulate organic matter (POM) and is generally composed of highly desirable carbon compounds for microbial degradation, such as soluble saccharides, which are quickly degraded (Wander, 2004). Recalcitrant carbon or mineral-associated organic matter

(MaOM) tends to be less desirable for microbes, due to the high energetic costs of breaking down the complex compounds, such as lignin or phenol, and has slow carbon turnover (Wander, 2004; Song et al., 2012).

Due to the high background levels of carbon in soil, small changes in soil carbon with experimental treatments can be difficult to detect in bulk soil. However, fractionation techniques can give more insight into changes in distinct organic matter fractions (Song et al., 2012), some of which are disproportionately sensitive to environmental change. Fractionation can be done physically, by separating fractions by particle size or density, and/or chemically, or sometimes using a combination of methods, such as separating by particle size and density (von Lützow et al., 2007). SOM can be divided based on density into two distinct fractions, the heavy fraction and the light fraction. The heavy fraction of soil is composed of MaOM, with long residence times in soils from years to centuries (Golchin et al., 1994; Wander, 2004; Song et al., 2012). The light fraction of soil is made up of POM, which has a shorter residence time in soils than the heavy fraction (Sequeira et al., 2011). The light fraction can be further divided into the free light fraction (FLF), and the occluded light fraction (OLF) (Golchin et al., 1994; Sequeira et al., 2011). The FLF is readily available for breakdown by microbes with a turnover period of a few days to a few years (Wander, 2004). The OLF, on the other hand, is sometimes considered the slow/intermediate organic matter because although it is partly composed of labile organic matter similar to the FLF, this carbon is protected in soil aggregates, and it requires dispersal of the aggregates before it can be broken down by microbes; it therefore has a longer carbon turnover rate than the FLF (Wander, 2004; Cerli et al., 2012). It is important to understand SOM functioning in soils, not only for the intrinsic value of minimizing soil carbon losses, but because the sequestration of carbon from the atmosphere (CO_2 in particular) by soils could have an important role in slowing climate change (Thornton et al., 2009).

The carbon quality of organic matter describes the quality of the carbon as a substrate for microbial degradation. High quality carbon includes carbon compounds that are preferred by microbes as a carbon source while low quality carbon describes carbon substrates that are less desirable due to their high energetic costs for degradation. Carbon quality can be

assessed via analytical techniques such as Fourier-transform infrared spectroscopy (FTIR). FTIR provides spectral data (absorbance) for a variety of solids, liquids, and/or gases by passing infrared light through a sample (Linker et al., 2005). Depending on the chemical composition of the sample, the infrared radiation will be absorbed to various degrees, and a spectrum is produced (Linker et al., 2005). The spectrum for each sample displays the intensity of absorbance for different wavenumbers, with the latter corresponding to the vibrations of specific bonds in different chemical classification groups, such as polysaccharides, lignins, and carboxylic acids (Artz et al., 2008), providing qualitative information regarding the chemical composition of the sample.

1.4 Potential Extracellular Enzyme Activity

As described above, microbes are an extremely important component of ecosystem functioning and they are responsible for regulating many aspects of the carbon and nitrogen cycles through processes such as decomposition and nutrient mineralization. Extracellular enzymes, synthesized by microbes and released into soil, are produced primarily to break down complex chemical structures, releasing nutrients such as carbon and nitrogen, that can then be taken up by microbes or plant roots (Wallenstein et al., 2010; Henry, 2012). Enzymes are released into their surrounding environment either by live cells actively producing them in response to nutrient demand, or in some cases by dead cells through cell lysis (Sinsabaugh, 2010). There are two main classes of enzymes produced by microbes that facilitate ecosystem functioning: hydrolase enzymes and oxidase enzymes. Hydrolase enzymes are C-acquiring enzymes produced by microbes to break down labile saccharides, such as cellulose and hemicellulose (Koyama et al., 2013). They can also be P- and N-acquiring in the case of phosphatase, which releases phosphorus, and N-acetyl-glucosaminidase which breaks down chitin, a compound containing nitrogen (Koyama et al., 2013). Oxidase enzymes, including phenol oxidase and peroxidase, are C- and N-acquiring enzymes produced by microbes, to break down recalcitrant organic matter, especially lignin and phenolic compounds, and these enzymes are typically less stable in the environment than hydrolases (Sinsabaugh, 2010).

Activities of extracellular enzymes are measured using enzyme assays, and information provided by assays can be used to assess soil microbial responses to environmental

change (Henry, 2012). Enzyme assays measure specific substrates that may be cleaved only by a particular enzyme. The extracellular enzyme activities (EEAs) measured are considered potential activities, because they assess enzyme activities in the laboratory under controlled temperature conditions, with high water availability and mixing with the substrate, via the creation of a well-mixed soil slurry (Wallenstein et al., 2010; Henry, 2012). Biotic factors, such as plant and microbial community composition and microbial biomass, can directly determine extracellular enzyme production. In addition to secreting their own enzymes from roots, plants produce root exudates that can prime microbes to degrade organic matter, which affects both microbial enzyme production and microbial biomass in soil close to the root surface (Arnosti et al., 2014). Abiotic factors affecting the soil microclimate, such as temperature, soil moisture, pH, and soil texture, can also influence the availability and activity of enzyme substrates and organic matter quality, resulting in spatial and temporal differences in potential EEAs (Allison and Vitousek, 2005; Arnosti et al., 2014).

1.5 Climate Warming Impacts on Soil Carbon

Nutrient cycling and decomposition are two dominant processes that define ecosystem functioning in soils, and these processes largely depend on plant-soil interactions. Small changes in biotic factors, such as microbial biomass and activity, as well as abiotic factors, including temperature and soil pH, can alter plant-soil interactions and therefore directly and indirectly impact nutrient cycling and decomposition. For example, temperature can directly impact decomposition by upregulating microbial activity, but also indirectly impact decomposition by decreasing soil moisture due to the warming-drying effect (Allison and Treseder, 2008).

In general, experimental soil warming has led to increased plant productivity, soil respiration, decomposition, and nutrient mineralization (Rustad et al., 2001). The effects of warming on soil carbon are, however, complicated by the integrated effects of warming on soil temperature and moisture, along with substrate and nutrient availability (Melillo et al., 2017). Short-term studies have, in some cases, demonstrated that warming can increase enzyme production by promoting increased microbial biomass and activity; however, warmer temperatures also can increase microbial enzyme efficiency, which

means microbes can produce fewer enzymes to meet their needs (Koch et al., 2007). In other short-term experiments, warming has been found to stimulate carbon turnover, leading to changes in carbon pools (Xu et al., 2012). Although the effects of warming on soil functioning have been researched extensively in field experiments, there has been a lack of consensus, possibly because direct warming treatment effects can be confounded by indirect effects of warming on plant productivity (Jonasson et al., 1999; Classen et al., 2015). For example, it is unknown if warming effects on decomposition are due to indirect effects on plant community composition and litter production, or direct effects on microbial activity.

Long-term field experiments (>10 years) have provided evidence that short-term warming effects may not translate to the long-term. For example, soil carbon losses in a 26-year field experiment were not uniform over time and alternated between periods of significant carbon loss and periods of undetectable carbon loss (Melillo et al., 2017). Another long-term experiment similarly found that warming initially upregulated microbial activities, increasing soil carbon losses, but this loss was temporary due to a progressive depletion of substrate availability over time (Walker et al., 2018). Several factors affect the timing and magnitude of soil carbon loss, including changes in microbial community composition and biomass, as well as microbial carbon use efficiency, and evidence suggests warming induced changes in the short-term can dampen over longer time periods (Melillo et al., 2017). Warming can also alter nitrogen cycling by stimulating nitrogen turnover and microbial growth, which can contribute to both increased soil nitrogen availability and increased soil nitrogen losses, often with important consequences for soil carbon cycling (Wu et al., 2012).

As described above, in addition to increasing soil surface temperatures, warming has further indirect implications for litter and SOM decomposition by altering litter and soil moisture content (Cheng and Huang, 2016), which can be confounded with the direct effects of warming (Allison and Treseder, 2008). Experimental drought treatments are shown to decrease microbial biomass, while increasing extracellular enzyme activities (Alster et al., 2013). Under drought conditions, soil microbes must increase enzyme production to compensate for the limited diffusion of enzymes (Allison and Vitousek,

2005). However, when water availability in soil is sufficient, warmer temperatures increase decomposition rates due to the upregulation of microbial activity, with the caveat that these responses can decline over the longer term (Wu et al., 2012). The effects of warming treatments also vary seasonally. In the summer, the effects of a warming treatment at the soil surface can be small relative to the fluctuating, hot summer air temperatures, and a thick plant canopy combined with the litter layer also can insulate the soil from overhead warming (Sharratt, 2002). In turn, over winter, the snowpack provides insulation, resulting in relatively stable soil temperatures and protection from extreme drops in air temperature (Henry, 2008). However, when warming decreases or eliminates the snowpack, soil freezing intensity and soil temperature variability can increase, which can stress soil microbes and plant roots, with implications for both carbon and nitrogen cycling (Bell et al., 2010). Future climate projections suggest that with increases in winter temperature, the frequency and intensity of soil freeze-thaw cycles may increase in northern temperate regions (Henry, 2008; Schuerings et al., 2014).

1.6 Effects of Increased Atmospheric Nitrogen Deposition on Soil Carbon

Changes in nitrogen inputs to a system has a high capacity for altering soil function and carbon storage, and soil functional responses to chronic nitrogen inputs often change over time. Chronic nitrogen addition can increase foliar nitrogen (the nitrogen found in plant leaves or needles), which subsequently alters the C:N ratio of the plant litter, which can have implications for litter decomposition rates (Aber et al., 1998; Yue et al., 2017). Nitrogen mineralization, and net primary productivity (NPP), which describes the rate at which energy is stored as biomass in plants, also can change over time with nitrogen addition (Aber et al., 1998). While nitrogen addition typically increases NPP in the short-term, chronic nitrogen addition can promote soil acidification; the latter can decrease soil fertility over time as a result of important plant nutrients (e.g. calcium and magnesium) being released from soil particles making them susceptible to leaching, due to exchange with the hydrogen ions released from excess plant uptake of NH_4^+ (Chapin et al., 2002). In addition, when the nitrogen becomes saturated (i.e. when its availability in the soil no

longer limits plant or microbial growth), soil inorganic nitrogen can accumulate, and soil nitrogen losses tend to increase (Zhong et al., 2015).

As addressed above, soil nitrogen cycling interacts strongly with carbon cycling, especially when nitrogen availability limits plant growth and decomposition (Fernández-Martínez et al., 2014). While bulk soil respiration as an indicator of microbial activity and decomposition is not always affected by treatments such as nitrogen fertilization, the latter can have a strong effect on microbial biomass (Wallenstein et al., 2006; Khalili et al., 2016), along with the production of extracellular enzymes that target carbon and phosphorus acquisition (Allison et al., 2008). Previous long-term studies have found that nitrogen fertilization suppresses lignin-degrading enzyme production, reducing the decomposition of lignin-dense, recalcitrant plant matter (Carreiro et al., 2000; Saiya-Cork et al., 2002). However, chronic nitrogen addition also can increase the production of cellulose-degrading enzymes (Saiya-Cork et al., 2002; Cenini et al., 2015; Chen et al., 2018), and can increase carbon storage in soils (Khalili et al., 2016; Chen et al., 2018). For example, in a grassland system after 19 years of nitrogen addition, increases in soil carbon sequestration in the heavy fraction of soil were found, but there was no change to the light fraction (Cenini et al., 2015), suggesting that microbes may produce more metabolites under nitrogen fertilization that encourage organic matter and mineral-associations. Similar to the effects of warming, nitrogen application can also cause changes in plant community composition and production, indirectly impacting soil functional responses (Rinnan et al., 2007). However, given the slow rates of turnover of individuals in plant communities relative to those of soil microbes, there can be multi-year time lags in such responses (Parmesan, 2006; Komatsu et al., 2019).

1.7 The WINNTER (WINter, warming and Nitrogen addition in Temperate Ecosystems Research) Field Experiment

In 2006, the WINNTER (WINter, warming and Nitrogen addition in Temperate Ecosystems Research) experiment was established at a temperate old field site in London, Ontario, Canada, to examine the individual versus combined effects of warming and nitrogen addition on plants and soil. The term ‘old field’ describes plant communities that establish following the abandonment of agricultural land (Gibson and Newman, 2019).

An old field was selected for WINNTER primarily because the relatively small stature of the plants allowed for warming and nitrogen treatments to be administered to multiple plants within 1 m^2 field plots, allowing community-level (i.e. changes in the relative abundances or composition of species) and ecosystem-level (i.e. productivity, decomposition, and nutrient cycling) responses to be assessed. Moreover, old fields and other grass-dominated ecosystems are widespread globally and important in the context of global carbon cycling (Cramer and Hobbs, 2007).

The factorial experiment consisted of control plots, nitrogen addition plots, warming plots, and plots with the combination of nitrogen addition and warming. Within two years, aboveground plant productivity began to respond strongly to nitrogen addition (Hutchinson and Henry, 2010), and this response remained strong through the following five years of the experiment, with significant effects of warming on plant productivity in some years (Henry et al., 2015). However, despite these strong increases in aboveground plant biomass, warming and nitrogen addition treatments had few significant effects on belowground responses, such as net nitrogen mineralization (Turner and Henry, 2010), extracellular enzyme activities, and microbial biomass (Bell et al., 2010; Bell and Henry, 2011). Although the decoupling of above and belowground responses to the treatments was unexpected, it was hypothesized that cumulative effects would likely result in the emergence of belowground effects over the longer term. However, it also was noted that the specific aboveground and belowground responses observed in the first few years of the experiment could have been caused by interactions of the treatments with annual variation in weather. Therefore, in 2019, new control, warming and nitrogen addition treatment plots were established to compare short-term (1-2 year) versus long-term (14-15 year) responses under identical weather conditions. No new combined nitrogen addition and warming plots were established in 2019 due to logistical constraints, but few significant interactions between nitrogen addition and warming had been observed over the first seven years of the experiment (Henry et al., 2015).

1.8 Thesis Objectives

The overall objective of my thesis was to compare the short-term (1-2 year) versus the long-term (14-15 year) effects of warming and nitrogen addition treatments on soil

carbon and microbial activity in a grass-dominated, temperate old field. I hypothesized that warming and nitrogen addition treatments would affect previously established plots more than newly established plots.

My specific objectives were to:

Objective 1: *Evaluate the treatment effects of warming and nitrogen on the quantity and quality of carbon in soil organic matter fractions.* – I used density fractionation to divide bulk soil into distinct organic matter fractions, then ran FTIR analyses to determine the composition and quantity of the different carbon compounds (e.g. polysaccharides and lignin) found in each fraction. I predicted the amount of labile carbon (FLF and OLF) recovered in the warming and nitrogen addition plots would be less than the amount recovered in the control plots due to the stimulation of microbial activity in the former, and that these effects would be more pronounced in the old plots than in the new plots. I also predicted that the warming and nitrogen treatments would have a higher proportion of recalcitrant carbon (i.e. lignin) than the control plots as a consequence of increased decomposition of more labile compounds, and that these effects also would be more pronounced in the old plots than the new plots.

Objective 2: *Evaluate the treatment effects of warming and nitrogen on potential soil extracellular enzyme activities (EEAs).* – I examined the potential EEAs of five microbial enzymes (N-acetyl-glucosaminidase [NAG], phosphatase, β-glucosidase, phenol oxidase, and peroxidase). Enzymes were a combination of hydrolase and oxidase enzymes, and included a variety of C-, N- and P-acquiring enzymes. I predicted that in response to nitrogen addition, microbial functioning would change. Specifically, oxidative enzyme production would decrease, and C- and P-acquiring hydrolase enzyme production would increase. I also predicted that enzyme efficiencies would increase with warming and therefore result in decreased enzyme production, leading to reduced potential activities. In both cases, I predicted these effects would be more pronounced in the old plots than in the new plots.

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Chapter 2

2 Short-term vs. long-term warming and nitrogen treatment effects on soil carbon and microbial activity in a temperate old field

2.1 Introduction

Elements of global change, such as climate warming and atmospheric nitrogen deposition, have the potential to exert very strong influences on ecosystem functioning in the coming decades (Millennium Ecosystem Assessment, 2005). Both factors vary with latitude; climate warming is projected to intensify with increasing latitude (IPCC, 2021), whereas atmospheric nitrogen deposition is highest in regions that feature a high density of industrial or agricultural activities (Galloway et al., 2004). Northern temperate regions are therefore expected to experience both factors to a relatively high degree over the next century. Grass-dominated communities can be particularly informative and tractable for studying the effects of climate warming and atmospheric nitrogen deposition on ecosystems. Specifically, the productivity of these systems is typically nitrogen limited, and the relatively small stature of the herbaceous species makes them convenient for the administration of treatments at the plot level in field experiments (Gibson and Newman, 2019). Grasslands, and other grass-dominated systems such as old fields, also are widespread globally, and they comprise approximately 37% of the Earth's terrestrial surface (O'Mara, 2012) and store approximately 30% of all terrestrial carbon (Scurlock and Hall, 1998).

The soils of grass-dominated systems are large reservoirs for carbon, and global change has the potential to alter the carbon balances of these soils substantially over the next century (Cerli et al., 2012). However, the effects of global change drivers on soil carbon and nitrogen cycling often have been studied over relatively short time spans (e.g. 1-5 years). These short-term experiments can be unrepresentative of longer-term trends if they coincide with anomalous weather conditions, such as extremely wet or dry periods (Henry et al., 2015). Grass-dominated systems, in particular, can alternate among years in functioning as net carbon sources or sinks as a result of interannual weather variability

(Novick, 2004). It also is not clear whether initial effects in field experiments may be transient, and to what extent cumulative effects on soil carbon may emerge over longer time scales (Bell et al., 2010; Bradford et al., 2016). For example, when water availability is sufficient, warm temperatures can increase decomposition rates due to upregulation of microbial activity, but these responses can decline over time (Wu et al., 2012). Long-term warming experiments (>10 years) have provided evidence that reductions in microbial activity and biomass may result from labile carbon pools becoming depleted over time, which can limit further carbon losses (Melillo et al., 2017). In addition, plant responses may be an important indirect driver of soil carbon responses to global change (Rinnan et al., 2007), and long-term studies (>10 years) of plant productivity and community composition have revealed lag periods in response to global change drivers, with long-term experiments showing more pronounced differences between treatment and control conditions over time (Parmesan, 2006; Komatsu et al., 2019).

Typically, the high levels of carbon in soil make it difficult to detect significant changes in total soil carbon in global change experiments (Song et al., 2012). However, fractionation techniques can be used to isolate distinct soil organic matter (SOM) carbon pools to provide more refined information. The heavy fraction of soil is large and is composed of mineral-associated organic matter (MaOM) that remains relatively stable in response to environmental change (Golchin et al., 1994; Song et al., 2014). In contrast, the light fraction of soil, the more labile fraction, appears as two distinct fractions, the free light fraction (FLF) and the occluded light fraction (OLF); carbon composing the OLF is highly labile, similar to the FLF, but is protected in soil aggregates, which must be dispersed prior to chemical analysis, and this fraction typically has proportionally less lignin than the FLF (Henry et al., 2005; Cerli et al., 2012). Following fractionation, analytical techniques, such as Fourier-transform infrared (FTIR) spectroscopy, can be used to assess the chemical composition of the different carbon fractions (Matějková and Šimon, 2012), which can provide further insight into how global change treatments can affect carbon quality. While experiments examining the effects of warming and nitrogen addition treatments on distinct soil carbon fractions have been uncommon, there is evidence that warming can decrease the FLF and OLF, while having no detectable effect

on the heavy fraction of organic matter (Song et al., 2012). However, in some cases warming has had no effect on the free light fraction (Henry et al., 2005). Similarly, chronic nitrogen addition may decrease FLF carbon over time (Song et al., 2014), or have no effect on FLF carbon (Cenini et al., 2015). Variation in the time scales of experiments may be an important factor in explaining this variation in results among studies.

The mechanisms underlying changes in soil carbon in global change experiments can be further explored by assessing functional components of microbial activity. For example, while microbial functions, such as bulk soil respiration, do not always respond significantly to nitrogen fertilization, there may be a strong effect on the production of extracellular enzymes that target carbon and phosphorus acquisition (Allison et al., 2008). Since the nitrogen cycle and carbon cycle are directly linked, when nitrogen availability limits plant growth and decomposition, implications for the carbon cycle may exist (Fernández-Martínez et al., 2014). When nitrogen is no longer limiting, in the case of nitrogen addition treatments, the production of C- and P-acquiring hydrolase enzymes is increased (Carreiro et al., 2000). In addition, while nitrogen-rich litter tends to decompose rapidly, long-term studies have found that nitrogen fertilization can suppress lignin-degrading enzyme activities, which reduces the decomposition of lignin-dense, recalcitrant plant matter (Saiya-Cork et al., 2002). In response to warming, microbes can initially increase enzyme production, accelerating decomposition (Rustad et al., 2001), but this response can diminish in the long-term if substrates are depleted (Walker et al., 2018). The drying effect caused by warming also can reduce enzyme production in some cases (Allison and Treseder, 2008), but in many other cases warming treatments have had little to no effect on microbial enzyme activities in field experiments (e.g. Bell and Henry, 2011; Henry, 2012). However, many of these results have been obtained from short-term experiments.

The goal of my study was to measure soil carbon and microbial activities to compare short-term (1-2 year) versus long-term (14-15 year) warming and nitrogen addition treatment differences using a field experiment conducted in a northern temperate old field. I predicted there would be a divergence between the short-term and long-term plots,

due to cumulative effects in the long-term plots that would alter soil carbon and microbial activities over time. Specifically, I examined the quantity of soil carbon fractions (FLF and OLF) and the carbon quality of the fractions using FTIR over the short- and long-term with warming and nitrogen addition treatments. In addition, I assessed the potential activities of five extracellular enzymes (N-acetyl-glucosaminidase [NAG], phosphatase, β-glucosidase, phenol oxidase, and peroxidase). I predicted the amount of labile carbon (FLF and OLF) recovered in the warming and nitrogen addition plots would be less than the amount recovered in the control plots, and I also predicted that the warming and nitrogen treatments would have a higher proportion of recalcitrant carbon (i.e. lignin) than the control plots. I predicted in both cases that the proxies measured would differ between old and new plots, with greater divergence in the old plots. For the extracellular enzymes, I predicted that decreased enzyme production would occur with warming due to warming increasing enzyme efficiencies, leading to reduced potential activity in the laboratory for old plots. I also predicted there would be greater activity of C- and P-acquiring enzymes with nitrogen addition, and reduced activity of oxidative enzymes. As with the soil carbon proxies, I predicted that extracellular enzyme activities would differ more from the control in the old plots.

2.2 Materials and Methods

2.2.1 Site Description

The study was performed at a temperate old field site located in London, Ontario, Canada, on the property of the Agriculture and Agri-Food Canada Southern Crop Protection and Food Research Centre (43°01′46″N, 81°12′52″W). The old field was taken out of agricultural production over 30 years ago and seeded with grasses. The vegetation at the site has remained dominated by Kentucky bluegrass (*Poa pratensis* L.) and smooth brome (*Bromus inermis* Leyss.), with the forbs Canada thistle (*Cirsium arvense* L.), common milkweed (*Asclepias syriaca* L.), white heath aster (*Aster ericoides* L.), tall goldenrod (*Solidago altissima* L.), and the legume bird's-foot trefoil (*Lotus corniculatus* L.) present at lower densities. The soil is a silt loam glacial till (Hagerty and Kingston, 1992) composed of approximately 50% sand, 41% silt and 9% clay, with an average pH of 7.6 (Bell et al., 2010).

2.2.2 Experimental Design

A randomized factorial block design experiment was established at the site in late 2006, with 1 m^2 circular plots assigned to either warming or ambient temperature, and either with nitrogen addition or without nitrogen addition (n=10 per treatment combination, 40 old plots total; Turner and Henry 2009). An additional 10 new warmed plots were established in October 2019, and 10 new nitrogen addition plots were established in early spring 2020. In addition, 10 new control plots were designated to test for potential long-term disturbance effects caused by the frequent plant and soil sampling in the original control plots. The expanded experiment therefore contained a total of 70 plots. No new warming and nitrogen fertilization combination treatment plots were established due to logistical constraints. However, previous studies conducted in the site found few significant warming by nitrogen addition effects (Bell et al., 2010; Henry et al., 2015). Warming was administered year-round using 150 W ceramic infrared heaters (Zoo-Med Laboratories, San Luis Obispo, CA, USA) at a height of 50 cm, which increased surface soil temperatures by approximately 2 °C without producing photosynthetically active radiation (Harte et al., 1995). Nitrogen was added annually during early spring as aqueous NH_4NO_3 at a rate of 2 g m^{-2} followed by the addition of 4 g m^{-2} of slow-release NH_4NO_3 pellets in early summer. The total addition rate of 6 g m^{-2} (60 kg/ha) is consistent with the high estimate of the predicted increase in nitrogen addition expected by 2050 in the study region (Galloway et al., 2004). Plot-level microclimate conditions were also monitored hourly using soil temperature probes (5 cm depth) and soil moisture probes measuring volumetric water content (5 cm depth).

2.2.3 Soil Carbon Analysis

2.2.3.1 Density Fractionation

For density fractionation, I used a method adapted from Henry et al. (2005), based on the methods of Six et al. (2001). Soil samples were collected 5 August, 2020, using a 15 cm deep, 1.5 cm diameter corer, and the soil was oven-dried at 60 °C for five days. Once dry,

the soil was passed through a 2 mm sieve to remove large root fragments, small rocks, and other debris. The soil cores were homogenized, and 14 g of dry soil was weighed into 50 ml centrifuge tubes. Next, 25 ml of sodium polytungstate (NaPT) with a density of 1.85 g cm^{-3}, was added to each tube. Samples were then shaken at 60 Hz for two hours and centrifuged for 10 min at 2100 × g (RCF) (2300 rpm). The supernatant containing the FLF of organic matter was vacuum filtered through a 1.2 µm nylon filter membrane that was weighed prior to use, and then rinsed with deionized water. The filters with the FLF were then left to oven dry at 60 °C. The pellet was redispersed in NaPT, and the previous step was repeated to collect the remainder of the FLF. To fraction out the OLF following the second FLF filtration, the pellet was redispersed in 0.5% sodium hexametaphosphate and shaken again for two hours to break up soil aggregates. Following centrifugation and decanting of the sodium hexametaphosphate supernatant, NaPT was returned to the pellet and centrifugation in NaPT was repeated to recover the OLF. Filter membranes were dried at 60 °C for three days and then weighed to estimate fraction recovery. The FLF and OLF were then recovered for analysis by gentle scraping of the membrane. The FLF was further fractioned by size by sieving (282 µm) to separate the fine organic particles from the more obvious root fragments. To prepare for FTIR analysis, a ball mill with stainless steel balls was used to grind each sample.

Statistical Analyses

The amounts recovered from fractionation for each fraction were analyzed to determine treatment effects using three two-way factorial block ANOVAs. The first ANOVA tested warming and plot age effects for the unfertilized plots only, the second tested nitrogen and plot age effects for the ambient temperature plots only, and the third examined the interactions between warming and nitrogen for the old plots only. Two-way ANOVAs were chosen instead of a three-way ANOVA, because no new nitrogen and warming combination treatment plots were established, rendering the experiment only partially factorial, not fully factorial. Prior to statistical analysis, the data were square root-transformed. Data transformations and ANOVAs were run using RStudio (Version 1.4.1106), and the outliers indicated in the boxplots were included in the analysis (see Figures 2.3, 2.4, and 2.5).

2.2.3.2 FTIR Analysis

Soil organic matter fractions were analyzed for carbon quality using ATR (attenuated total reflection)-FTIR. FTIR absorbance spectra were collected using a Nicolet 380 FTIR spectrometer equipped with a Smart MIRacle^{TM} Single Reflection ATR accessory and a ZnSe crystal plate (PIKE Technologies, Inc., WI, USA) following the method developed by James (2020). For each experimental field plot, FTIR data were collected for both the particulate and root debris fractions of the FLF (referred to hereafter as the FLF < 282 µm and FLF > 282 µm), and the OLF. The average of 32 scans at a 4 cm^{-1} resolution over a wavenumber range of 400-4000 cm^{-1} was collected for each organic matter fraction recovered (FLF < 282 µm, FLF > 282 µm, and OLF) and three replicates, which were then averaged, were collected for each fraction to create a representative spectrum for each sample (Figure 2.1). Corrections were applied for all spectra for the baseline, ATR, and atmospheric CO_2 and H_2O, using OMNIC^{TM} Series Software (Thermo Fisher Scientific Inc., WI, USA). Absorption peaks used as indicator peaks for organic matter quality are listed in Table 2.1 and were identified according to Niemeyer et al. (1992), Zaccheo et al. (2002), and Boeriu et al. (2004).

Table 2.1: List of wavenumbers for indicator peaks used for Fourier-transform infrared spectroscopy analysis.

Wavenumber (cm^{-1})	Characterization	Reference
1030	Polysaccharides	Zaccheo et al. (2002)
1265	Lignin backbone	Niemeyer et al. (1992)
1371	Aliphatic compounds	Boeriu et al. (2004)
1426	Humic acids	Boeriu et al. (2004)
1515	Lignin/phenolic backbone	Zaccheo et al. (2002)
1650	Aromatic structures	Zaccheo et al. (2002)
1720	Carboxylic acids	Niemeyer et al. (1992)
2920	Fats, waxes, and lipids	Niemeyer et al. (1992)

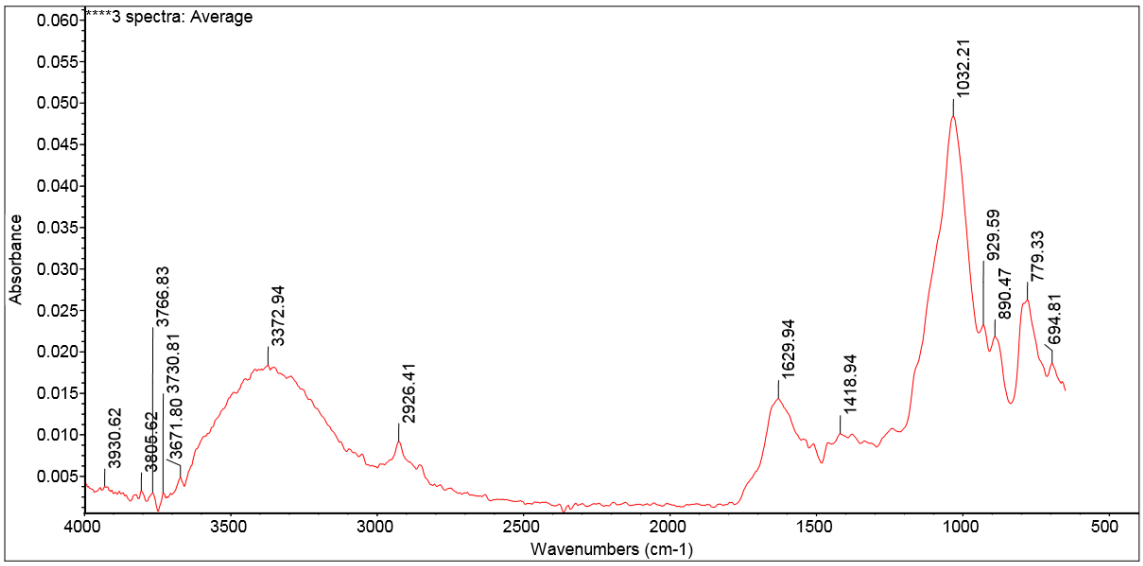

Figure 1.1: Example of Fourier-transform infrared spectroscopy spectrum collected using Nicolet 380 FTIR spectrometer equipped with a Smart MIRacle^{TM} Single Reflection ATR accessory and a ZnSe crystal plate produced by OMNIC^{TM} Series Software. Values on the spectrum indicate the wavenumber, and the peak heights represent the corresponding absorbance intensities.

Statistical Analyses

Peak absorption intensities were collected and classified for the peaks of interest noted in Table 2.1. Peak absorption intensities were analyzed for each peak within each treatment using three two-way factorial block ANOVAs, similar to those used for the density fractionation experiment. The ANOVAs were performed using RStudio (Version 1.4.1106), and the outliers indicated in the boxplots (see Figure 2.6, and Appendices A-F) were included in the statistical analysis. The first ANOVA tested warming and plot age effects, the second tested nitrogen and plot age effects, and the third examined the interactions between warming and nitrogen for the old plots only.

2.2.4 Microbial Activity Analysis

2.2.4.1 Enzyme Assays

Enzyme assays were conducted following a protocol by Sinsabaugh et al. (2003) and adapted by Henry et al. (2005) and Saiya-Cork et al. (2002). The activities of three hydrolase enzymes (N-acetyl-glucosaminidase [NAG], phosphatase, and β-glucosidase), were analyzed using methylumbelliferone-tagged substrates (4-MUB- N-acetyl-β-D-glucosaminide, 4-MUB- phosphate, 4-MUB-β-D-glucopyranoside, respectively). The activities of two oxidase enzymes (phenol oxidase and peroxidase) were analyzed using L-3,4-dihydroxyphenyl-alanine (L-DOPA) as a substrate. Soil cores were taken from each sample plot over two two-week periods, one in mid-spring (10-26 May) and the other in early summer (12-23 July) and were processed within 24 hours of collection. Soil cores were homogenized, and subsamples were taken for moisture content analysis. A sample of soil was measured out to 1 g and blended with 125 ml of 50 mM, Tris buffer, with a pH of 7.5, to match the site soil pH. Using a multichannel pipette, eight 200 µl aliquots were taken for analysis while the soil suspension was stirred and dispensed into black 96-well microplates for hydrolase enzymes, or clear flat-bottomed microplates for the oxidase enzymes.

For hydrolase enzymes, 50 µl of 200 µM substrate solutions were added to the soil suspension in eight sample wells. Blank wells received 200 µl of the soil suspension and

50 µl of Tris buffer. Negative controls received 50 µl of 200 µM substrate solution and 200 µl of Tris buffer. Hydrolase enzymes also required both a quench standard and reference standard. The quench standard received 200 µl of soil suspension and 50 µl of 10 µM methylumbelliferone (MUB) standard. The reference standard received 200 µl of Tris buffer and 50 µl of 10 µM MUB standard. Eight replicate wells were used for each blank, negative control, quench, and reference standard. Plates were incubated in the dark at room temperature for the appropriate length of time (45 mins for NAG and phosphatase, and 3 hours for β-glucosidase) then 10 µl of 1M NaOH was added to each well to stop the reaction. Fluorescence was measured using a multi-detection microplate reader (SpectraMax® M2e with SoftMax® Pro software) with 365 nm excitation and 450 nm emissions filters. To measure oxidase enzymes for each sample, 50 µl of 25 mM L-DOPA substrate solutions were added to the 200 µl of soil suspension in sample wells. For peroxidase only, 10 µl of 0.3% hydrogen peroxide (H_2O_2) was also added to each well in the assay. Similarly, blank wells received 200 µl of the soil suspension and 50 µl of Tris buffer, and negative controls received 50 µl of 200 µM substrate solution and 200 µl of Tris buffer. Eight replicate wells were used for each sample, blank, and negative control. Phenol oxidase and peroxidase assays were incubated in the dark at room temperature for 1 hour, and then absorbance was measured at 450 nm using the same multi-detection microplate reader as previously mentioned.

Statistical Analyses

Extracellular enzyme activities were calculated based on absorbance or fluorescence values measured for each enzyme and converted to nmol h^{-1} per gram of soil dry weight (dw). Using RStudio (Version 1.4.1106), three two-way factorial block ANOVAs were used to test for treatment effects on EEAs. The first tested warming and plot age effects, the second two-way factorial block ANOVA tested nitrogen and plot age effects, and the third examined the interactions between warming and nitrogen for the old plots only. All outliers indicated in the boxplots (See Figure 2.7, 2.8 and 2.9) were included in the analysis.

2.3 Results

2.3.1 Soil Temperature and Soil Moisture Data

Soil temperature and soil moisture varied between the two years of observation (2020 and 2021). The soil temperature and moisture probe data in Figure 2.2 (Craig, 2021, p.27) showed that soils were warmer in the early part of the growing season (1 March to 31 July – when the dominant grasses are most active) in 2021 compared to 2020. Mean air temperature for this period was 12.7 °C (± 3.9 SE) in 2020, and 13.0 °C (± 3.3 SE) in 2021, and mean precipitation was 69.8 mm (± 11.8 SE) in 2021, and 63.4 mm (± 8.3 SE) in 2020 for this period (Environment Canada, National Climate Data and Information Archive, Historical Data). Soil temperature was on average 13.7 °C (± 0.1 SE) for the warmed plots and 12.5 °C (± 0.1 SE) for the ambient temperature plots for 2020. In 2021, average soil temperatures were on average 15.6 °C (± 0.11 SE) in the warmed plots and 13.9 °C (± 0.11 SE) in the ambient temperature plots. Volumetric water content for the warmed plots in 2020 was on average 0.31 (± 0.002 SE), and 0.33 (± 0.002 SE) for the ambient temperature plots compared to 2021, which was 0.31 (± 0.001 SE) on average for warmed plots and 0.32 (± 0.001 SE) for ambient temperature plots.

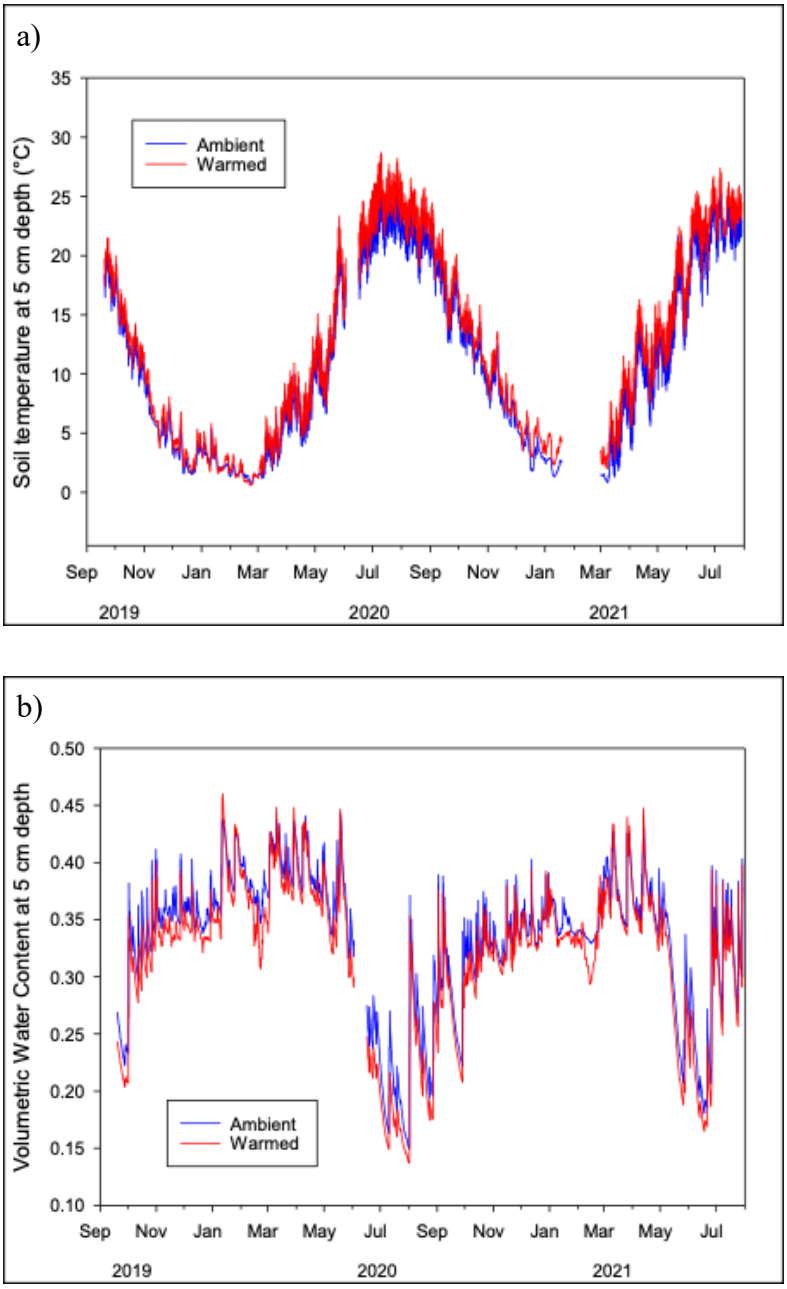

Figure 2.2: (a) Soil temperature and (b) volumetric water content for ambient temperature and warmed plots at 5 cm depth from September 2019 to July 2021.

Note. Adapted from "Long-term vs. Short-term Plant Responses to Warming and Nitrogen Addition in a Temperate Old Field," by B, Craig, 2021, *Electronic Thesis and Dissertation Repository*. 8194. (https://ir.lib.uwo.ca/etd/8194).

2.3.2 Soil Organic Matter Fraction Recovery

In the old plots, nitrogen addition alone increased FLF recovery (P_N=0.018; Table 2.2; Figure 2.3), but FLF recovery was not significantly affected by warming, nor was there a significant interaction between warming and nitrogen addition. In contrast to the increased FLF recovery in the old plots with nitrogen addition, FLF recovery declined in the new plots with nitrogen addition, as evidenced by the significant interaction between nitrogen and plot age ($P_{N\times age}$=0.015; Table 2.2; Figure 2.4). There were no significant treatment effects on the ratio of FLF > 282 µm to FLF < 282 µm recovery; the mean FLF > 282 µm recovered was 0.077 g (± 0.004 SE) and the mean FLF < 282 µm recovered was 0.073 g (± 0.004 SE) across all treatments. OLF recovery declined with independent nitrogen (P_N=0.016) and warming (P_w=0.036) treatments in the old plots (Table 2.2; Figure 2.3). However, OLF recovery increased significantly with nitrogen addition in the old plots, in combination with warming ($P_{W\times N}$=0.002; Table 2.2; Figure 2.3).

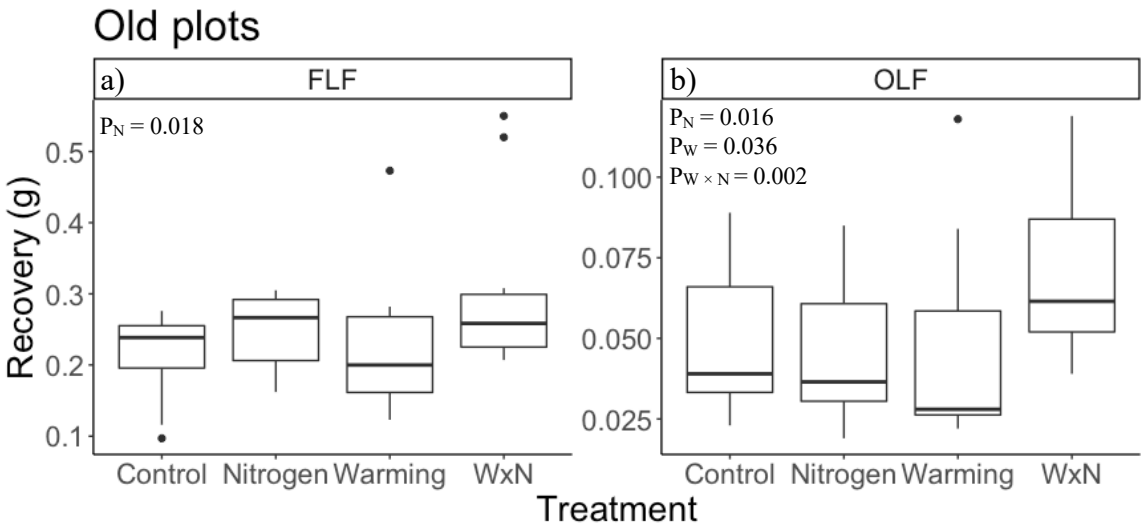

Figure 2.3: Changes in a) free and b) occluded light soil fractions in response to nitrogen addition, warming, and nitrogen by warming treatments (W×N) (n=10). Boxes indicate the upper quartile (75th percentile) and the lower quartile (25th percentile) which make up the interquartile range (IQR) of the dataset, and the median is represented by the line within the box. Whiskers are drawn up to largest data point that is 1.5 times the IQR, and black circles represent outliers that lie more than 1.5 times outside the IQR. P-values from three two-way ANOVAs are displayed and shown in Table 2.2. The data were square-root transformed prior to statistical analysis.

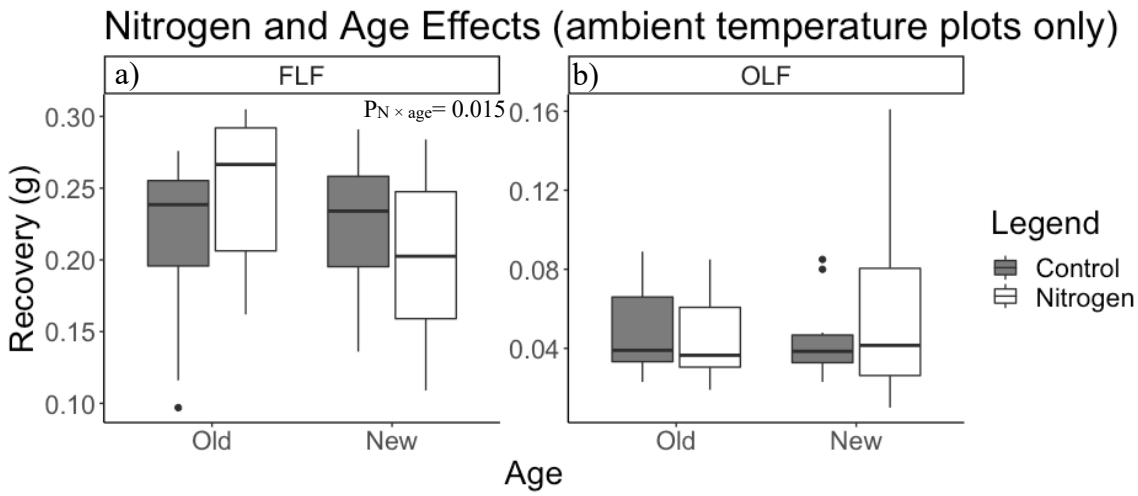

Figure 2.4: Changes in a) free and b) occluded light soil fractions recovered from nitrogen and age effects plots (ambient temperature plots only) in response to nitrogen treatments and plot age (n=10). Boxplot and p-value details as described in Figure 2.3. The data were square-root transformed prior to analysis.

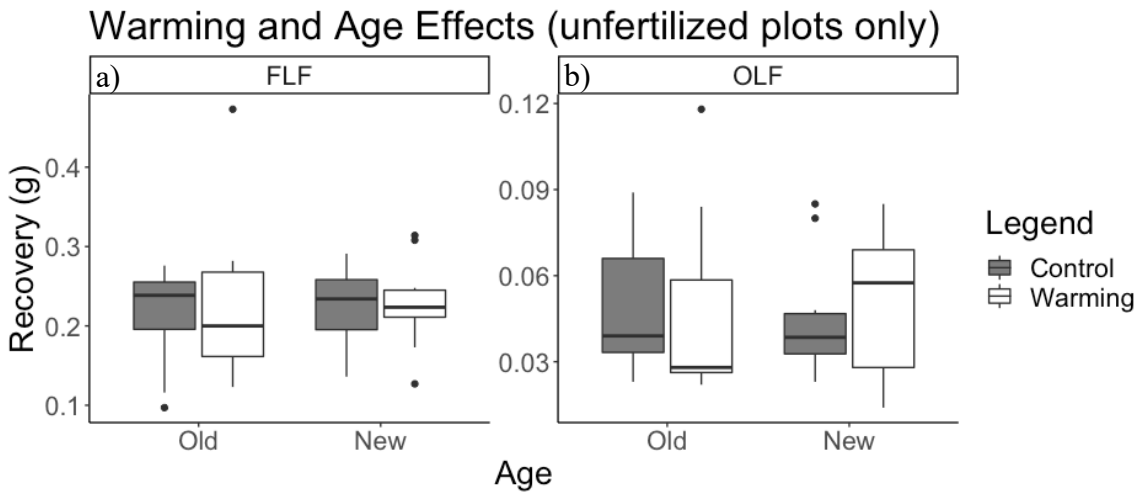

Figure 2.5: Changes in a) free and b) occluded light soil fractions recovered from the warming and age effects plots (unfertilized plots only) in response to warming treatments and plot age (n=10). Boxplot and p-value details as described in Figure 2.3. The data were square-root transformed prior to analysis.

Table 2.2: Summary of P-values from three two-way ANOVAs measuring effects of warming and nitrogen treatments and plot age on soil organic matter fraction recovery.

		Fraction	
Plot Type	Treatment	FLF	OLF
Old Plots	N (1)	**0.018***	**0.016***
	W (1)	0.228	**0.036***
	W × N (1)	0.486	**0.002****
	Df error (27)		
Nitrogen and Age Effects (ambient temperature plots only)	N (1)	0.473	0.840
	Age (1)	0.116	0.777
	N × age (1)	**0.015***	0.409
	Df Error (27)		
Warming and Age Effects (unfertilized plots only)	W (1)	0.670	0.914
	Age (1)	0.673	0.839
	W × age (1)	0.865	0.488
	Df Error (27)		

W - Warming; N - Nitrogen; Age - Plot age; FLF - Free light fraction; OLF - Occluded light fraction. Asterisks denote significant results (*** < 0.001, ** 0.001 - 0.01, * 0.01 - 0.05). Degrees of freedom for treatments and error are in parentheses.

2.3.3 FTIR

There were few significant treatment effects on the FTIR peak intensities. The polysaccharide peak intensity of the FLF < 282 μm decreased significantly with warming in the old plots (P_W=0.013; Table 2.3, Figure 2.6). See Appendix A for nitrogen by plot age treatment effects, and Appendix D for warming by plot age treatment effects. For the FLF > 282 μm, the warming treatment reduced the lignin peak intensity in combination with nitrogen addition, but lignin peak intensity was greater without nitrogen addition, although not statistically significant ($P_{W\times N}$=0.055; Table 2.4, Figure 2.6). See Appendix B for nitrogen by plot age treatment effects, and Appendix E for warming by plot age treatment effects. There were no significant treatment effects on any of the intensities of the indicator peaks for the OLF (Table 2.5). See Appendix C for nitrogen by plot age treatment effects, and Appendix F for warming by plot age treatment effects.

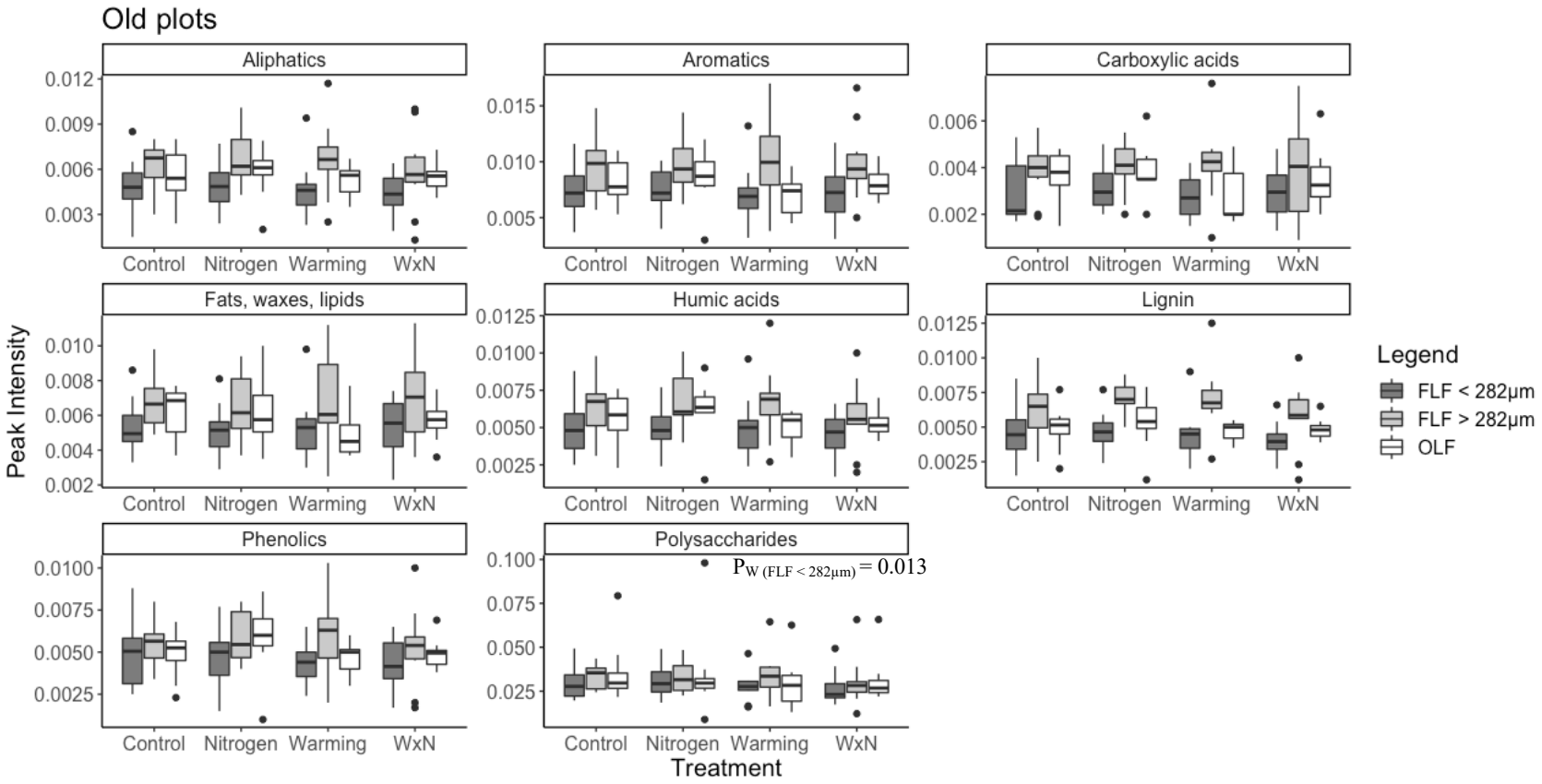

Figure 2.6: Absorbance peak intensities from Fourier-transform infrared spectroscopy (FTIR) analysis for the free light fraction (FLF) < 282 µm, FLF > 282 µm, and occluded light fraction (OLF) from the old plots in response to warming and nitrogen addition treatments for the various indicator peaks as mentioned in Table 2.1 (n=10). Details of boxplots explained in Figure 2.3. P-values from three two-way ANOVAs are displayed and shown in Table 2.3, Table 2.4, and Table 2.5.

Table 2.3: Summary of P-values from three two-way ANOVAs measuring the treatment effects of warming and nitrogen treatments and plot age on the peak intensities of the free light fraction (FLF) < 282 µm of soil organic matter using Fourier-transform infrared spectroscopy.

		Indicator Peaks							
		Polysaccharides	*Lignin*	*Aliphatics*	*Phenolics*	*Humic Acids*	*Aromatics*	*Carboxylic acids*	*Fats, waxes, lipids*
Plot Type	Treatment								
Old Plots	N	0.844	0.740	0.626	0.609	0.448	0.924	0.243	0.811
	W	**0.013***	0.229	0.333	0.149	0.465	0.563	0.385	0.905
	W × N	0.467	0.408	0.645	0.837	0.592	0.924	0.793	0.811
Nitrogen and Age Effects (ambient temperature plots only)	N	0.869	0.811	0.602	0.345	0.745	0.869	0.933	0.681
	Age	0.626	0.212	0.480	0.315	0.485	0.347	0.454	0.953
	N × age	0.542	0.707	0.628	0.501	0.959	0.869	0.340	0.860
Warming and Age Effects (unfertilized plots only)	W	0.372	0.895	0.931	0.736	0.852	0.958	0.591	0.902
	Age	0.239	0.895	0.813	0.591	0.894	0.975	0.107	0.882
	W × age	0.303	0.662	0.700	0.367	0.709	0.531	0.939	0.805

W - Warming; N - Nitrogen; Age - Plot age. Asterisks denote significant results (*** < 0.001, **0.001-0.01, *0.01 – 0.05). Degrees of freedom are as shown in Table 2.2.

Table 2.4: Summary of P-values from three two-way ANOVAs measuring the treatment effects of warming and nitrogen treatments and plot age on the peak intensities of the free light fraction (FLF) > 282 µm of soil organic matter using Fourier-transform infrared spectroscopy.

		Indicator Peaks							
		Polysaccharides	*Lignin*	*Aliphatics*	*Phenolics*	*Humic Acids*	*Aromatics*	*Carboxylic acids*	*Fats, waxes, lipids*
Plot Type	Treatment								
Old Plots	N	0.402	0.643	0.717	0.647	0.602	0.881	0.938	0.979
	W	0.819	0.695	0.732	0.810	0.551	0.759	0.718	0.617
	W × N	0.552	0.055	0.236	0.290	0.186	0.964	0.643	0.617
Nitrogen and Age Effects (ambient temperature plots only)	N	0.875	0.383	0.495	0.809	0.575	0.751	0.375	0.373
	Age	0.986	0.589	0.630	0.665	0.801	1.000	0.422	0.908
	N × age	0.856	0.304	0.666	0.511	0.624	0.958	0.799	0.783
Warming and Age Effects (unfertilized plots only)	W	0.570	0.432	0.378	0.664	0.626	0.672	0.524	0.729
	Age	0.988	0.271	0.306	0.739	0.763	0.988	0.963	0.992
	W × age	0.897	0.320	0.321	0.591	0.732	0.977	0.721	0.730

W - Warming; N - Nitrogen; Age - Plot age. Asterisks denote significant results (*** < 0.001, **0.001-0.01, *0.01 – 0.05). Degrees of freedom are as shown in Table 2.2.

Table 2.5: Summary of P-values from three two-way ANOVAs measuring the treatment effects of warming and nitrogen treatments and plot age on the peak intensities of the occluded light fraction (OLF) of soil organic matter using Fourier-transform infrared spectroscopy.

		Indicator Peaks							
		Polysaccharides	*Lignin*	*Aliphatics*	*Phenolics*	*Humic Acids*	*Aromatics*	*Carboxylic acids*	*Fats, waxes, lipids*
Plot Type	Treatment								
Old Plots	N	0.946	0.626	0.630	0.276	0.461	0.321	0.350	0.592
	W	0.488	0.533	0.619	0.311	0.240	0.228	0.235	0.169
	W × N	0.897	0.845	0.953	0.592	0.689	0.633	0.677	0.490
Nitrogen and Age Effects (ambient temperature plots only)	N	0.915	0.745	0.778	0.313	0.516	0.771	0.639	0.585
	Age	0.805	0.957	0.900	0.653	0.578	0.541	0.397	0.671
	N × age	0.949	0.812	0.860	0.634	0.727	0.837	0.927	0.629
Warming and Age Effects (unfertilized plots only)	W	0.354	0.534	0.695	0.585	0.426	0.232	0.797	0.167
	Age	0.798	0.960	0.927	0.967	0.820	0.875	0.581	0.315
	W × age	0.948	0.843	0.977	0.991	0.004	0.485	0.169	0.920

W - Warming; N - Nitrogen; Age - Plot age. Degrees of freedom are as shown in Table 2.2.

2.3.4 Extracellular Enzyme Assays

Potential extracellular enzyme activities differed between sampling periods, and the activities of individual enzymes differed with treatments, with hydrolase enzymes typically having greater treatment responses than oxidase enzymes (Table 2.6). For the enzyme assays conducted in May, NAG activity was greater with warming (P_W=0.047; Figure 2.7) in the old plots. In July, the latter effect was no longer significant, and the old plots had significantly less NAG activity (P_{age}=0.013; Figure 2.8 and Figure 2.8) than the new plots. Also in July, there was a significant interaction between warming and nitrogen for NAG activity ($P_{W\times N}$=0.047; Figure 2.7), with the latter increasing in response to nitrogen addition, but only in the warmed plots. In May, there was a significant interaction between warming and plot age for phosphatase activity, with less phosphatase activity in the new plots with warming, but greater phosphatase activity in the old plots ($P_{W\times age}$=0.024; Figure 2.9). A similar interaction between nitrogen and age occurred with phosphatase having greater activity in the old plots with nitrogen addition, and less activity in the new plots with nitrogen, but this interaction was not statistically significant ($P_{N\times age}$=0.087; Figure 2.8). In May, there was a significant interaction between warming and plot age for β-glucosidase activity ($P_{W\times age}$=0.041). β-glucosidase activity was greater with warming in the old plots but had reduced activity in new plots (Figure 2.9). Warming alone also significantly increased β-glucosidase activity (P_W=0.039) in old plots (Figure 2.7). In July, β-glucosidase activity increased significantly with nitrogen addition (P_N=0.028) in the old plots (Figure 2.7).

Phenol oxidase activity was unchanged regardless of treatment at either time point, but in May peroxidase activity was greater in the old plots with nitrogen addition (P_N=0.021; Figure 2.7). In July, the warming treatment effects on peroxidase (P_W=0.076) were not statistically significant (Figure 2.9), but there was a strongly significant interaction between warming and nitrogen ($P_{W\times N}$ =0.001) in the old plots (Figure 2.7).

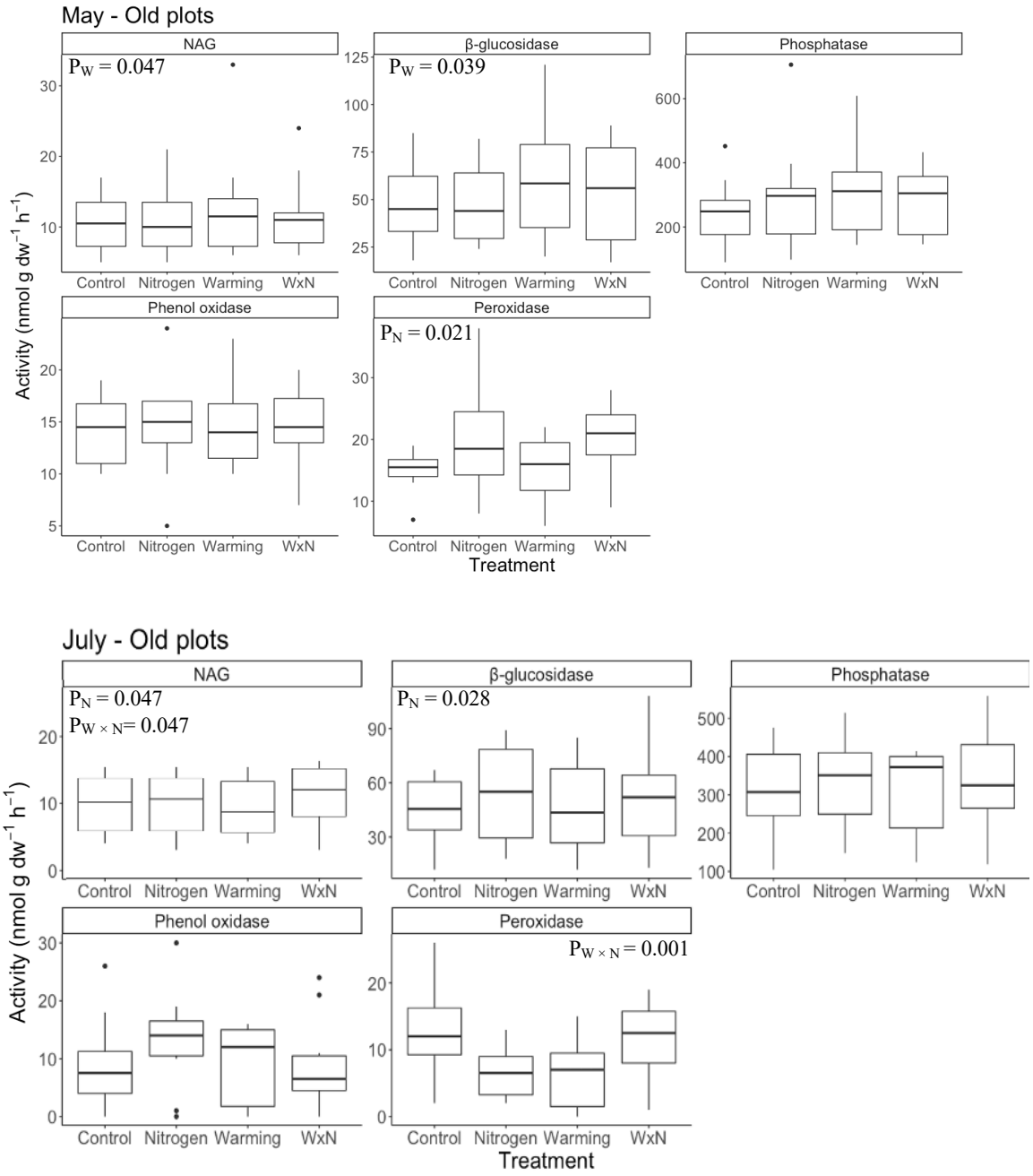

Figure 2.7: Potential extracellular enzyme activities in the old plots from enzyme assays in May and July in response to nitrogen addition, warming and their combination (n=10). Details of boxplots explained in Figure 2.3. P-values from three two-way ANOVAs are displayed and shown in Table 2.6.

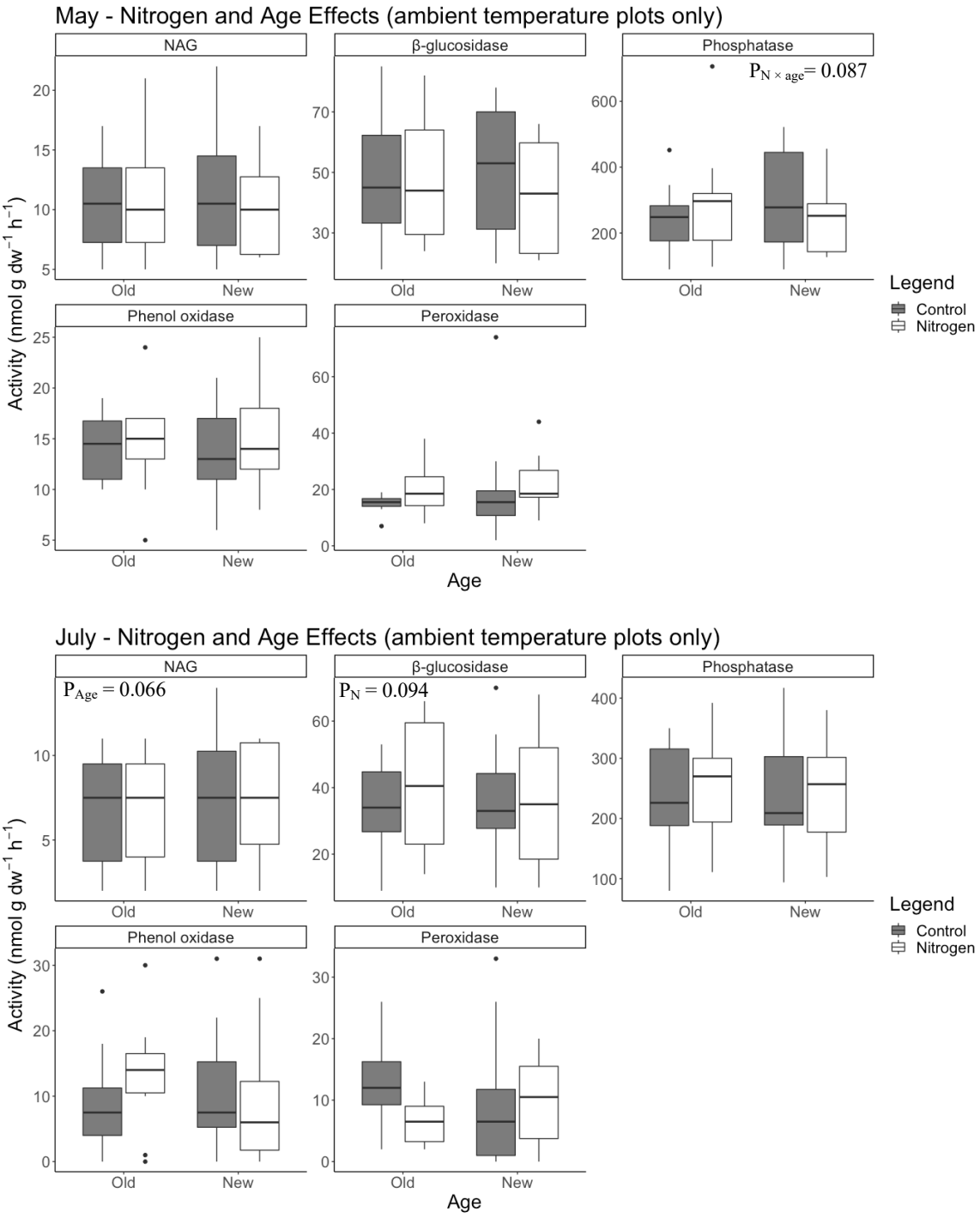

Figure 2.8: Potential extracellular enzyme activities in the nitrogen and age effects plots (ambient temperature plots only) from enzyme assays in May and July in response to nitrogen addition and plot age (n=10). Boxplot details as described in Figure 2.3. P-values from three two-way ANOVAs are displayed and shown in Table 2.6.

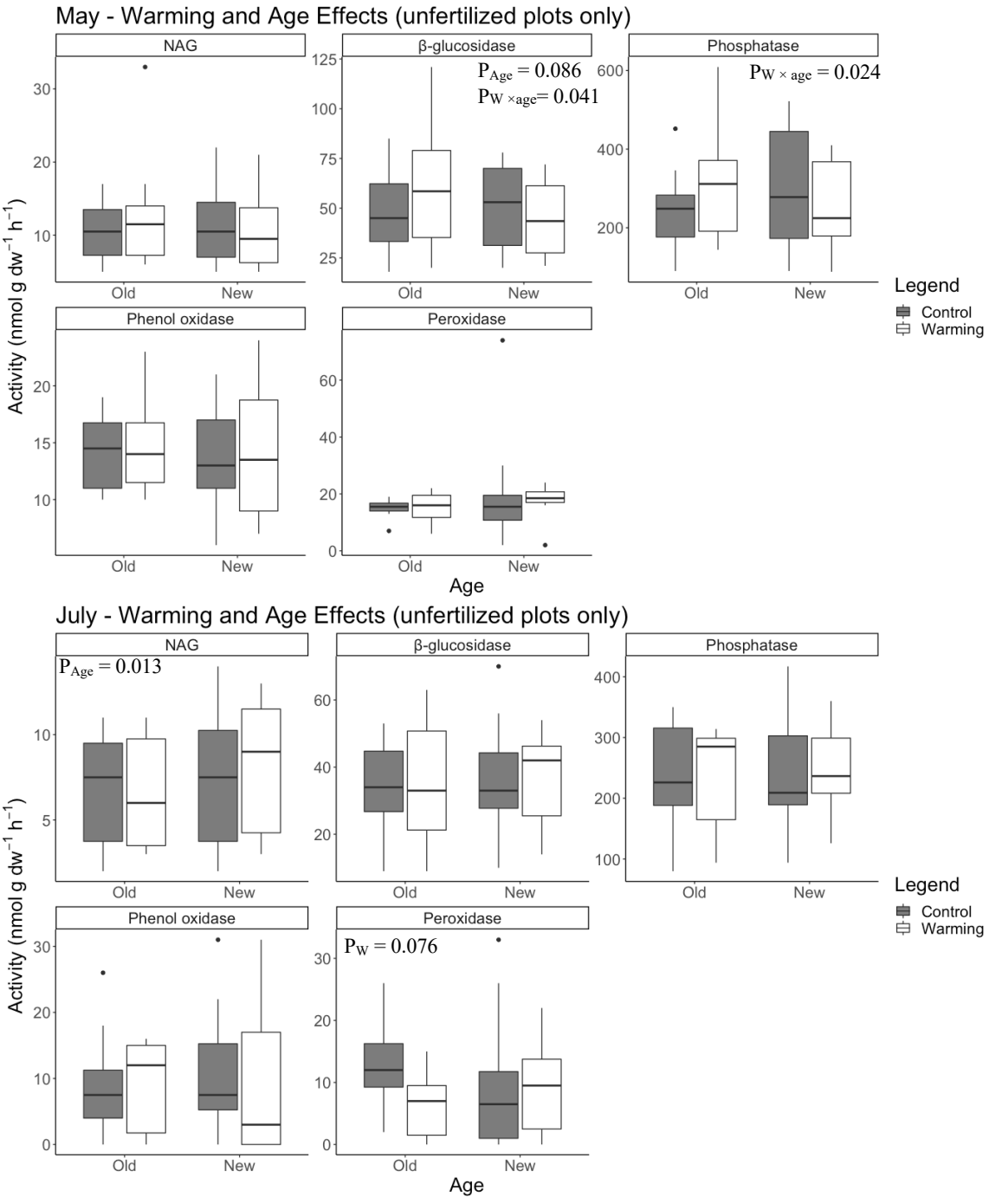

Figure 2.9: Potential extracellular enzyme activities in the warming and age effects plots (unfertilized plots only) from enzyme assays in May and July in response to warming treatments and plot age (n=10). Boxplot details as described in Figure 2.3. P-values from three two-way ANOVAs are displayed and shown in Table 2.6.

Table 2.6: Summary of P-values from three two-way ANOVAs measuring effects of warming and nitrogen treatments and plot age on enzyme production.

		May					July				
Plot Type	Treatment	*NAG*	*Bglu*	*Phos*	*Phenol Ox*	*Perox*	*NAG*	*Bglu*	*Phos*	*Phenol Ox*	*Perox*
Old Plots	N	0.642	0.458	0.702	0.512	**0.021***	**0.047***	**0.028***	0.108	0.237	0.622
	W	**0.047***	**0.039***	0.452	0.378	0.776	0.257	0.975	0.976	0.156	0.622
	W × N	0.323	0.600	0.315	0.668	1.000	**0.047***	0.642	0.932	0.214	**0.001*****
Nitrogen and Age Effects (ambient temperature plots only)	N	0.575	0.176	0.827	0.776	0.372	0.662	0.094	0.222	0.448	0.153
	Age	0.911	0.541	0.998	0.494	0.308	0.066	0.661	0.929	0.554	0.908
	N × age	0.152	0.287	0.087	0.361	0.668	0.662	0.132	0.535	0.142	0.141
Warming and Age Effects (unfertilized plots only)	W	0.312	0.448	0.762	0.245	0.754	0.793	0.789	0.978	0.520	0.076
	Age	0.368	0.086	0.964	0.329	0.272	**0.013***	0.405	0.644	0.639	0.938
	W × age	0.121	**0.041***	**0.024***	0.388	0.630	0.196	0.817	0.978	0.639	0.150

W - Warming; N - Nitrogen; Age - Plot age; NAG - N-acetyl-glucosaminidase; Phos - phosphatase; Bglu - β-glucosidase; Phenol Ox - Phenol oxidase; Perox - Peroxidase. Asterisks denote significant results (*** < 0.001, **0.001-0.01, *0.01 – 0.05). Degrees of freedom are as shown in Table 2.2.

2.4 Discussion

2.4.1 Soil Organic Matter Fraction Recovery

I predicted that nitrogen addition would stimulate labile carbon breakdown, which would lead to a decrease in FLF and OLF recovery. While this prediction was evident in the new plots, the opposite response was observed for the old plots. This nitrogen addition by plot age interaction may be explained by the fact that the quantity and quality of labile SOM in soil is affected by both microbial carbon consumption and by carbon inputs from plant litter and roots. In the case of the new plots, the direct effect of nitrogen addition could have enhanced microbial activities, whereas there were no significant above and belowground plant growth responses in the first year of treatment (Craig 2021), which would have precluded an increase in plant soil carbon inputs. In contrast, the old plots exhibited a strong plant response to nitrogen addition, including a significant increase in root biomass (Craig 2021), which appeared to be an important contributor to the FLF. Therefore, my prediction appeared to overlook the important role of variation in plant responses in driving the overall FLF quantity with nitrogen addition in the old plots. In other systems, effects of nitrogen treatments likewise have been inconsistent between short and long-term experiments. Specifically, in a short-term grassland nitrogen addition experiment, nitrogen increased the FLF of soil carbon (Khalili et al., 2016), whereas in another experiment it had no effect (Song et al., 2014). In a long-term nitrogen fertilization experiment, there was no effect of nitrogen addition on the light density fraction recovered, but nitrogen addition did increase soil carbon in the heavy fraction (Cenini et al., 2015), and there is evidence that nitrogen increases total soil carbon stocks (Tong et al., 2014). It has been suggested that inconsistent results from nitrogen addition experiments on soil carbon pools have been due to site-specific responses (Kazanski et al., 2019), or abiotic factors such as soil pH (Song et al., 2014), but my results emphasize that treatment effects on plant biomass production and litter inputs also may play an import role in explaining this variation. Importantly, lags in plant responses to nitrogen addition can modulate changes in soil carbon over time.

While previous studies have reported that warming treatments can decrease the soil carbon light fraction (Song et al., 2012) and total carbon stocks (Peplau et al., 2021), short-term warming experiments nevertheless have failed to detect effects of warming on soil organic carbon content in surface soils (Guan et al., 2018) and subsoils (Jia et al., 2019). I consequently predicted the amount of labile carbon recovered in the old warming plots would be less than the amount recovered in the control plots and new warming plots. Contrary to my prediction, there were no significant warming effects on FLF recovery in either the old or new plots, likely due to the relatively weak warming treatment. Mean soil temperature was only increased by 1.2°C and 1.7°C in 2020 and 2021, respectively, with the warming treatment. However, given there has not been a consistent effect on warming on plant productivity in the experiment (Henry et al., 2015), carbon inputs to the soil may not have differed substantially over time among treatments.

As for the OLF, although it is comprised of labile carbon, the latter is protected from microbial decomposition within soil aggregates. While this results in a slower carbon turnover rate than the FLF, the quality of the aggregated carbon often is preferred by microbes for decomposition (Golchin et al. 1994; Kölbl et al., 2004; Wander et al., 2004; Riggs et al., 2015). Therefore, treatment effects on soil aggregate formation and breakdown are key in driving the quantity of OLF in response to changes in edaphic conditions. Nitrogen addition treatments can increase SOM aggregate formation by stimulating plant root biomass, and microbial aggregation of organic matter, which is consistent with nitrogen treatments increasing carbon accumulation in soils (Song et al., 2014; Riggs et al., 2015), however, the results of my study contradict this. In contrast, it was observed that the OLF decreased with warming, which is consistent with some previous research (Guan et al., 2018), but it is also sometimes unaffected despite enhanced microbial decomposition (Schnecker et al., 2016). My observation that OLF recovery increased with the combination of warming and nitrogen addition in the old plots is difficult to explain, given these contrasting effects. Nevertheless, the latter remains an intriguing result, given that interactive effects of the warming and nitrogen addition treatments were largely absent through the first seven years of the experiment (Henry et al., 2015).

2.4.2 Carbon Quality

Warming and nitrogen addition treatments can alter SOM quality, even in short-term experiments (Sun et al., 2019). I therefore predicted that treatment effects on labile carbon decomposition would vary the carbon quality of the FLF and OLF, and in particular, the remaining proportion of recalcitrant compounds. However, the FTIR spectral data demonstrated that carbon quality of the different soil organic matter fractions did not differ with experimental treatments. Density fractionation separates soil carbon coarsely into fractions associated with relatively labile carbon (FLF and OLF) versus relatively recalcitrant carbon (the heavy fraction), and I further separated the FLF by particle size into the FLF < 282 μm, which was very fine, indistinguishable material, and the FLF > 282 μm, which was composed of larger, more recognizable root debris fragments. Peak intensities for the FLF > 282 μm demonstrated that this fraction contained consistently greater lignin, humic acids, aliphatic, and aromatic carbon compounds than the FLF < 282 μm, but both the FLF > 282 μm and FLF < 282 had similar polysaccharide content. The composition of the OLF was more variable but was similar to that of the FLF < 282 μm. Although the treatments affected the amounts of these fractions recovered (as discussed in the previous section), my results indicate that the density and size fractionation steps were effective in separating the resulting fractions into pools of uniform carbon quality. One significant result I observed was that warming altered the carbon composition of the polysaccharides in the FLF < 282 μm. This soil carbon fraction is composed of soluble saccharides that feature a rapid turnover rate (Wander, 2004; Sequeira et al., 2011), and as described above, warming can reduce labile carbon pools, especially in the short-term, by stimulating microbial decomposition (Song et al., 2012; Melillo et al., 2017; Li et al., 2018).

Although not statistically significant, in the old plots, the lignin peak for the FLF > 282 μm declined with warming when nitrogen was applied, but peak intensity was higher with warming alone. This contrasts with previous research, which found that nitrogen addition impedes the breakdown of recalcitrant organic matter by reducing microbial biomass and decreasing oxidase enzyme activities (Carreiro et al., 2000; Song et al., 2014). Because the OLF is protected in aggregates, it is possible that this material was

not accessible for microbes to degrade, regardless of treatment (Wander, 2004), and I did not observe a change in the carbon composition of the OLF that corresponded with the combined warming by nitrogen addition effect on OLF recovery described above for the old plots.

2.4.3 Extracellular Enzyme Activities

In support of my prediction, there were greater enzyme activities in the old plots than in the new plots. It was expected that microbial activities, and therefore function, would have shifted over time as a result of cumulative treatment effects. Although there was variability in EEAs between sampling periods for each enzyme, it appeared that microbes responded to treatments by altering production of hydrolase enzymes more so than the oxidase enzymes. Typically, nitrogen addition stimulates enzyme activities, specifically increasing hydrolase enzyme production (Allison et al., 2008; Song et al., 2014; Khalili et al., 2016; Zhang et al., 2019). Warming can also stimulate C-acquiring hydrolase enzyme activities in the short-term, but this response decreases over time as substrate availability decreases (Li et al., 2018; Walker et al., 2018). As I predicted, the activities of two hydrolase enzymes (NAG and β-glucosidase) were greater with nitrogen addition, but the lack of a change in phosphatase production with nitrogen addition in the old plots was unexpected. When nitrogen limitation is alleviated, it is expected that other important nutrients, such as phosphorus, become limiting, resulting in increasing P-acquiring enzyme production by microbes, and plants (Calleiro et al., 2000); however, this was not the case in my experiment.

Given that nitrogen addition typically suppresses the production of oxidase enzymes by microbes (Calleiro et al., 2000; Saiya-Cork et al., 2002; Henry et al., 2005; Keeler et al., 2009), it was unexpected for the peroxidase activities measured in the old plots with the combination treatment of warming and nitrogen addition, to be greater than the control. Warming appeared to negate the negative effect of nitrogen on peroxidase activity. As previously mentioned, warming can stimulate C-acquiring hydrolase enzymes (Li et al., 2018; Walker et al., 2018), and the increased substrate availability from plant litter and roots in old plots (Craig, 2021) may also explain why, contrary to my prediction, the

enzyme activities in the old plots were greater with the warming treatment. The observation that warming effects were stronger in the spring compared to the summer was consistent with the warming treatment having less of an effect on soil temperature in summer due to the insulating effect of the plant canopy (Hutchison and Henry, 2010).

2.4.4 Conclusions

Overall, the warming and nitrogen addition effects on the light fraction of SOM and microbial activities appeared to be strongly influenced by indirect treatment effects on plant growth. This mechanism may similarly explain why the effects of warming and nitrogen treatments on EEAs were negligible in the early years of the experiment in the old plots (Bell and Henry, 2010). Because the OLF is formed when old pools of carbon become aggregated during decades of root growth and is often lost due to cultivation, conversion of cultivated land to old fields may therefore negate the losses of this fraction if quantities of OLF carbon increase in old fields, revealing additional ecological benefits of old field systems (Golchin et al., 1994). Questions regarding long-term carbon storage in the presence of warming temperatures and atmospheric nitrogen deposition may potentially be addressed by analyzing changes in carbon of the soil heavy fraction, despite the slow response of this pool to environmental variation. Organic matter in the heavy fraction has extremely long residence times, and is less susceptible to microbial degradation, and therefore has a greater capacity for carbon sequestration than the light fraction.

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Chapter 3

3 Conclusions and Future Directions

3.1 Research Findings

My study revealed warming and nitrogen treatment effects on both soil organic matter fraction recovery and extracellular enzyme activities. Overall, the old plots exhibited greater differences from controls than the new plots, which reinforces the concept that results of short-term experiments measuring soil carbon and microbial responses may not reflect long-term responses, likely due in part to lags in plant responses to the treatments. Other key observations were that once the density and size fractionation steps were complete, there was little variation among treatments in carbon quality, and that microbial activities of hydrolase enzymes were more affected by treatments than those of the oxidase enzymes.

3.2 Methodological Considerations and Potential Limitations

The soil fractionation methods I used were physical fractionation techniques. These methods separate soil fractions by density or particle size and are based on the physical associations and bioavailability of the organic matter for decomposition (von Lützow et al., 2007). Chemical methods can also be used for soil fractionation. Chemical fractionation can remove the mineral component of soil, isolating organic matter for analysis; however, chemical fractionation methods do not allow the differentiation between the free and occluded soil organic matter found in aggregates if both components are soluble in the extracting agent (von Lützow et al., 2007). Another advantage of using density for fractionation as opposed to chemical fractionation is that separating by density maintains the integrity of the organic matter fractions (Cerli et al., 2012). The light fraction of SOM is generally a good representation of the active organic matter in soil, but the differentiation between the light fraction and heavy fraction using density fractionation is approximate (von Lützow et al., 2007). The heavy fraction of organic matter was not analyzed in my study because it is composed of strongly bound mineral-associated organic matter that cannot be completely separated and therefore it is

not typically available for microbial degradation, and it is relatively insensitive to environmental change (Golchin et al., 1994; Cerli et al., 2012; Song et al., 2012). Nevertheless, by analyzing changes only in the light fraction, the results from my experiment were limited to just the active pool, and responses of the passive pool (which are relevant to longer-term soil carbon sequestration) remained unknown.

The extracellular enzyme assays I used to assess microbial activity also have potential limitations. In the field, many factors determine enzyme activities in soil, and these include soil structure, temperature, soil moisture, and pH, as well as substrate availability, and as biotic factors including plant community composition (Sinsabaugh, 2010). Many of these factors can only be replicated *in vitro* to a certain extent, for example by maintaining a similar pH to the soil pH by using buffer. However, this same buffer can alter the structural composition of soil samples, which can impact measured enzyme activities. In addition, the enzymes measured are temperature sensitive, and the measurement of enzyme activities in the laboratory is only indicative of total potential activity in soil (Allison and Vitousek, 2005; Koch et al., 2007, Allison et al., 2010), and not actual activity. Moreover, laboratory assays measure the entire pool of enzymes, even if there are enzymes associated with organic matter or mineral particles that would not encounter their substrates *in situ*, and the use of a soil slurry in the laboratory provides ample water, whereas enzyme diffusion (and thus contact with substrate) can be limited in the field when soil moisture is low (Steinweg et al., 2012). Therefore, while the use of enzyme assays can provide a quick estimation of microbial functional activity, actual EEAs in the field likely differ.

Lastly, a further limitation of my thesis was the lack of microbial biomass estimates to complement the enzyme assay data. While my project was originally intended to include microbial biomass estimates obtained via a differential cell staining technique to determine active fungal and bacterial biomass, timing constraints forced us to omit these analyses. Estimates of fungal and bacterial biomass could have been used to infer to what extent changes in EEAs were driven by changes in microbial biomass versus changes in enzyme production per unit microbial biomass.

3.3 Directions for Future Research

Because the global carbon and nitrogen cycles are highly intertwined, and ecosystem functioning relies strongly on these cycles, understanding changes in soil nitrogen as a result of climate change and nitrogen addition treatments would further enhance the understanding of carbon cycling in the experiment. Northern temperate grasslands are often nitrogen-limited, and there is therefore a high potential for changes in soil nitrogen dynamics in response to long-term warming and nitrogen addition. Previous short-term results from the experiment (i.e. the initial years for the old plots) indicated no treatment effects on net nitrogen mineralization rates (Turner and Henry, 2010), but found that soil ^{15}N retention varied with warming and nitrogen addition (Turner and Henry, 2009).

Long-term global change experiments typically see greater deviation in treatment responses from the controls over time (Komatsu et al., 2019). Another long-term experiment by Melillo et al. (2017) examined the effect of warming on soil carbon losses from soil and found that responses over time occurred in phases. Initially, soil carbon losses increased, but they leveled out after ten years. However, after another seven years of continuous warming, soil carbon losses began to increase again, suggesting that long-term responses can continue to change. Therefore, a further assessment of soil carbon in my experiment in another 10 years could yield further insights. In addition, although my study examined the effects of two global change drivers on soil carbon and microbial activities, community responses are more frequently observed with greater than three global change drivers (Komatsu et al., 2019). A long-term multifactorial experiment observing the effects of water addition/drought treatments in addition to warming and nitrogen would therefore be further informative. For example, the negative effects of water addition on labile carbon and nitrogen pools can be mitigated by nitrogen addition (Khalili et al., 2016) and the soil drying effect caused by warming may be relieved by water addition (Allison and Treseder, 2008).

3.4 Conclusions

Experiments examining warming and nitrogen addition treatment effects on soil carbon quantity and composition and microbial activities are common, but many experiments

have been conducted over relatively short time periods, and in addition to not addressing potential long-term cumulative effects, the results of short-term studies can be affected by interannual weather variation (Henry et al., 2015). My add-on to a pre-existing experiment was established to control for the effects of weather variability in short-term experiments and understand the extent to which cumulative effects on soil carbon and microbial activities may occur over time. The results from my study suggest that short-term soil carbon and microbial responses are not representative of the cumulative effects that can occur over longer periods. Warming and nitrogen treatment effects were more pronounced in old plots compared to new plots, and these long-term changes can have implications for future ecosystem functioning. Results from long-term studies examining soil responses to elements of global change in a variety of ecosystems can be applied to global change models, allowing the models to make more accurate predictions of how ecosystems might respond to future environmental conditions (Burns et al., 2013). Whether a soil system acts as a net carbon source or sink depends on many ecological factors, and as global temperatures continue to increase and atmospheric nitrogen deposition persists, results from this study will contribute to the understanding of how temperate soils in herbaceous systems may respond to global change.

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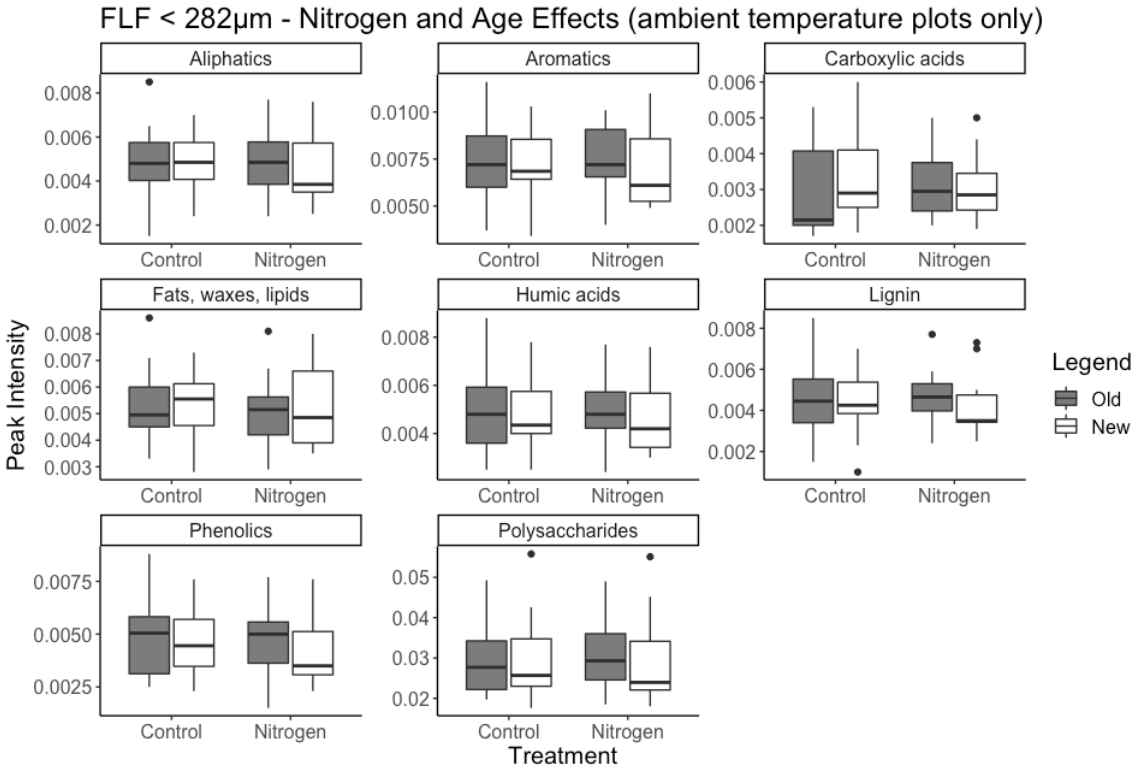
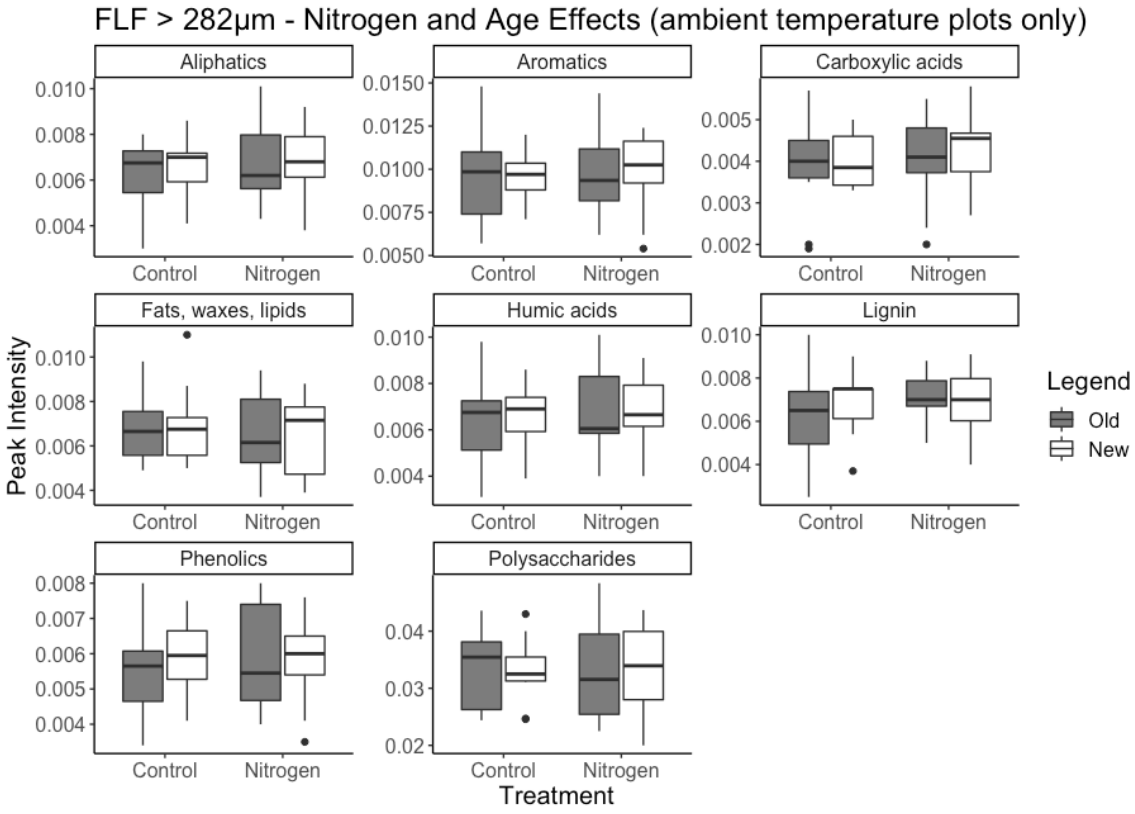
Appendices

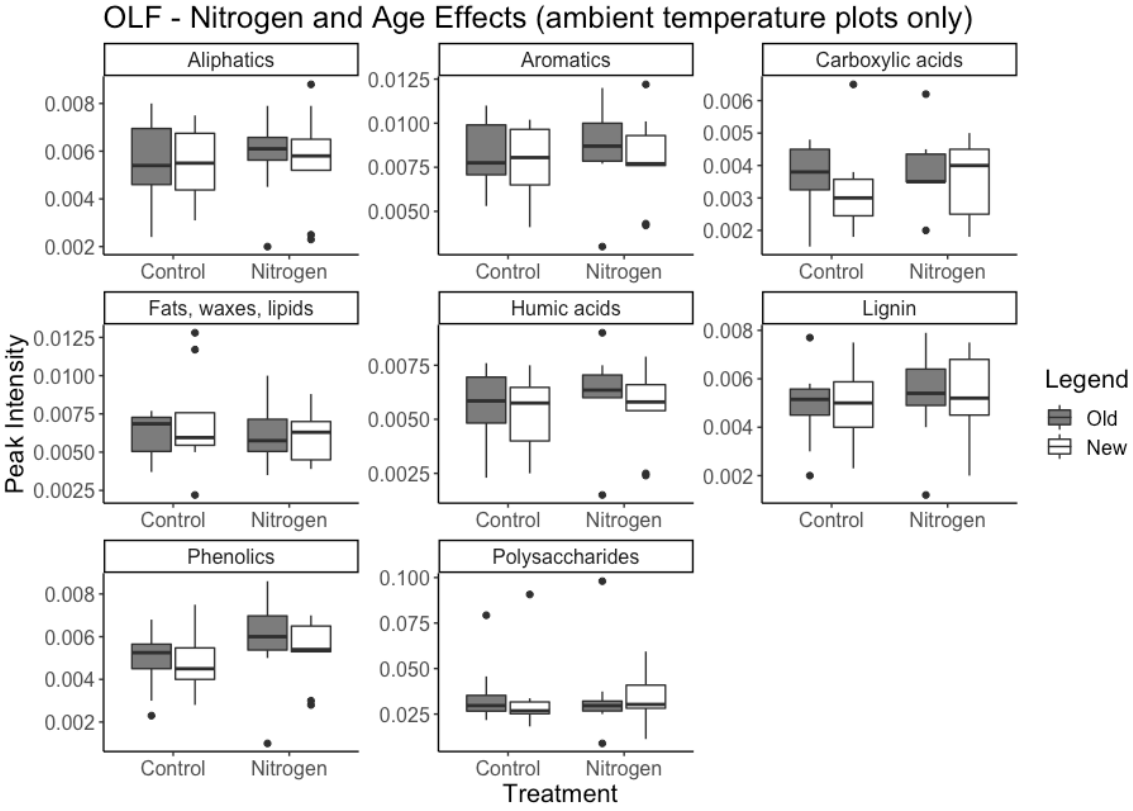
Appendix A: Absorbance peak intensities measured with Fourier-transform infrared spectroscopy (FTIR) for the free light fraction (FLF) < 282 µm from the nitrogen and age effects plots (ambient temperature plots only) for various indicator peaks (n=10). Boxplot details as described in Figure 2.3.



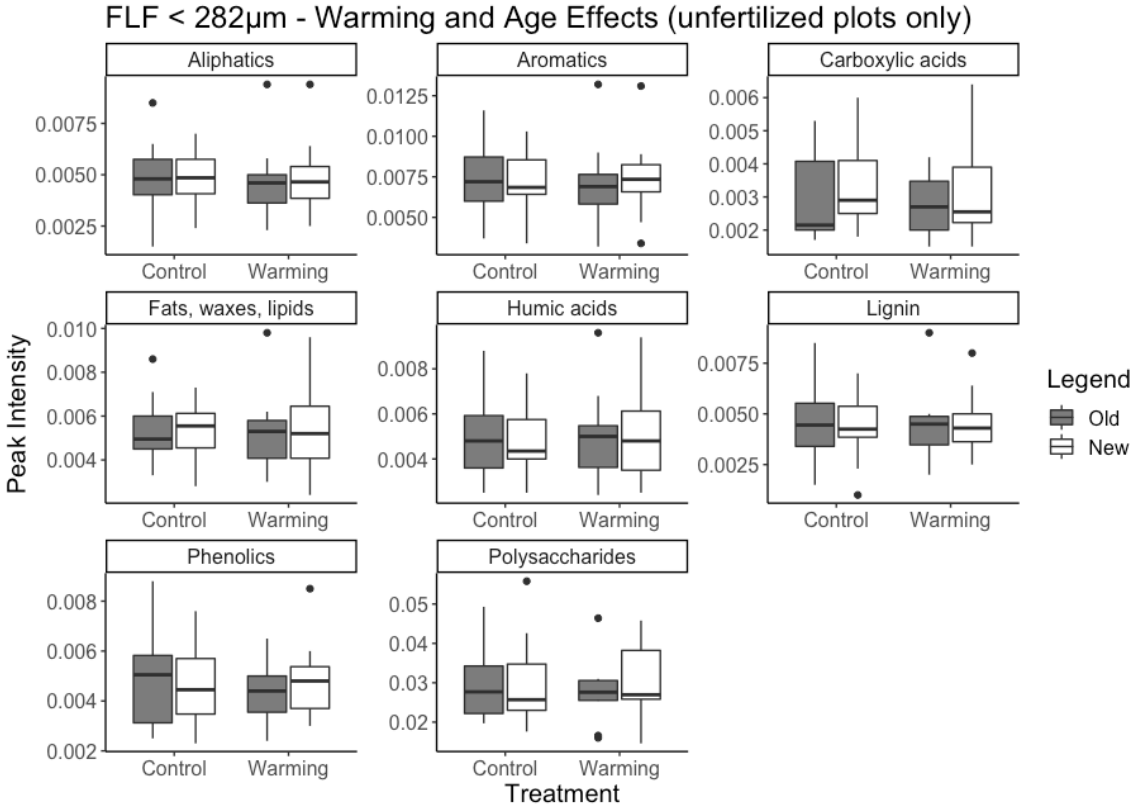
Appendix B: Absorbance peak intensities measured using Fourier-transform infrared spectroscopy (FTIR) for the free light fraction (FLF) > 282 µm from nitrogen and age effects plots (ambient temperature plots only) for various indicator peaks (n=10). Boxplot details as described in Figure 2.3.



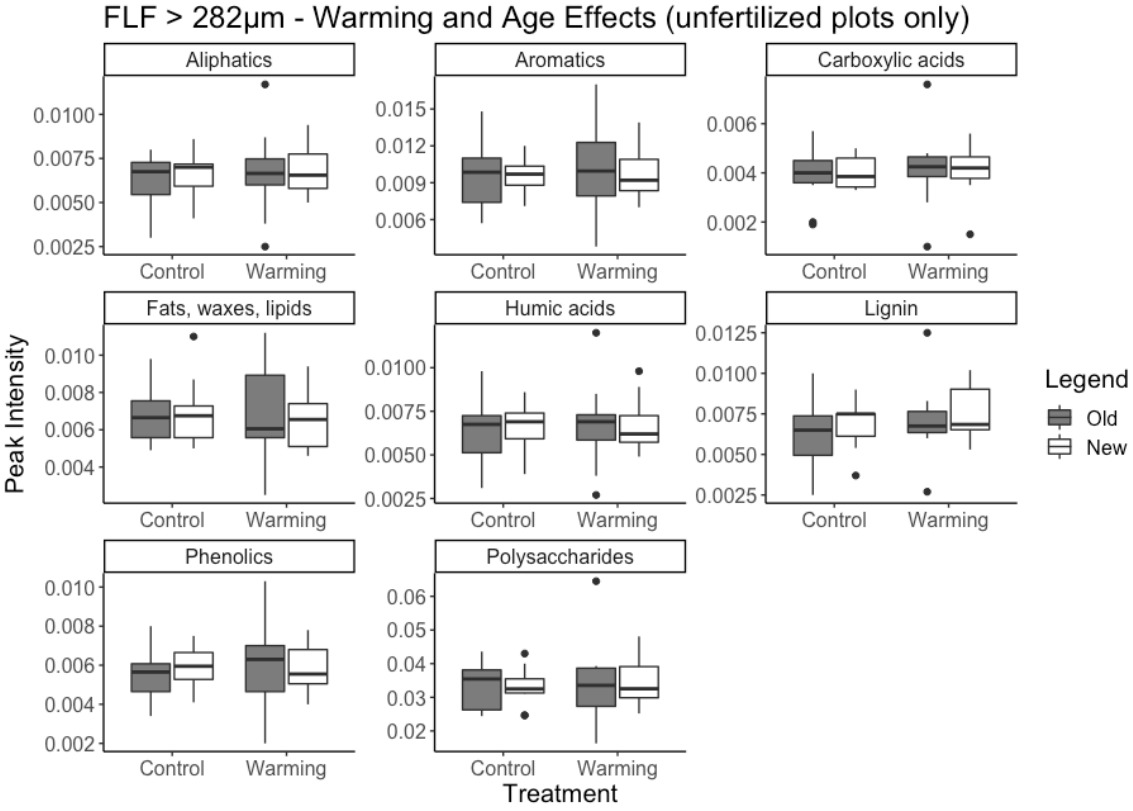
Appendix C: Absorbance peak intensities measured using Fourier-transform infrared spectroscopy (FTIR) for the occluded light fraction (OLF) from nitrogen and age effects plots (ambient temperature plots only) for various indicator peaks (n=10). Boxplot details as described in Figure 2.3**.**



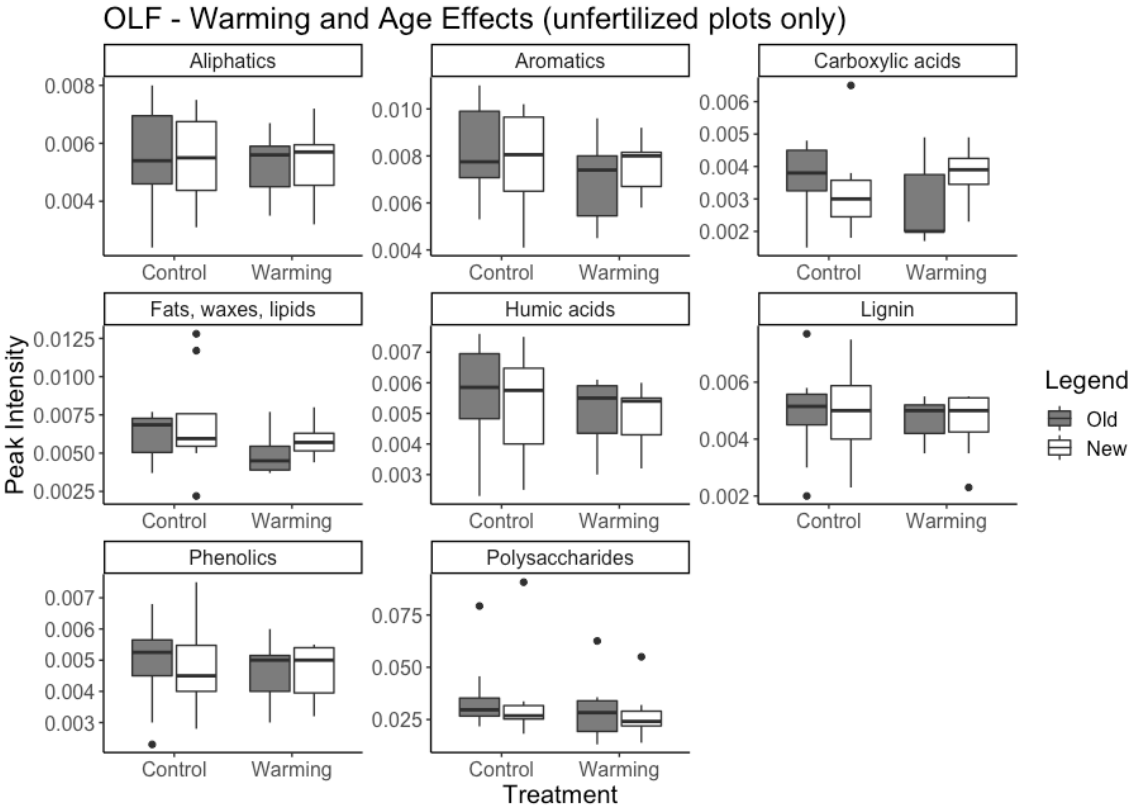
Appendix D: Absorbance peak intensities measured using Fourier-transform infrared spectroscopy (FTIR) for the free light fraction (FLF) < 282 µm from warming and age effects plots (unfertilized plots only) for various indicator peaks (n=10). Boxplot details as described in Figure 2.3.



Appendix E: Absorbance peak intensities measured using Fourier-transform infrared spectroscopy (FTIR) for the free light fraction (FLF) > 282 µm from warming and age effects plots (unfertilized plots only) in response to warming treatments and plot age for various indicator peaks (n=10). Boxplot details as described in Figure 2.3.



Appendix F: Absorbance peak intensities measured using Fourier-transform infrared spectroscopy (FTIR) for the occluded light fraction (OLF) from warming and age effects plots (unfertilized plots only) for various indicator peaks (n=10). Boxplot details as described in Figure 2.3.



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