COVER CROPS AND COVER CROP RESIDUES REGULATE RATES OF SOIL MICROBIAL DECOMPOSITION AND PLANT AVAILABLE NITROGEN

By

Nzube Prisca Egboluche

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Crop and Soil Sciences – Master of Science

2020

ABSTRACT

COVER CROPS AND COVER CROP RESIDUES REGULATE RATES OF SOIL MICROBIAL DECOMPOSITION AND PLANT AVAILABLE NITROGEN

By

Nzube Prisca Egboluche

Changes in soil microbial community and activities can influence the nutrient cycling and its availability to plants. I conducted a one-year soil incubation study to: evaluate the effects of short- and long-term N additions on the soil microbial activities and how cover crop residue mixtures influence ecosystem functions. Two legume residues, *Vicia villosa* - hairy vetch (V) and *Pisum sativum* - field pea (P) with C:N ratio 12.8 and 10.5 respectively and two non-legume residues, *Pennisetum glaucum* - pearl millet (M) and *Lolium multiflorum* - rye grass (R) with C:N ratio 37.8 and 14.3 respectively were used in this study. Soils amended with V and R had greater inorganic N compared to M and P residues while in soils with residue mixtures I observed non-additive synergistic (NAS) effect on inorganic N at the initial and final stage of the incubation period. Long-term N addition increased inorganic N but was apparent with short-term N fertilizer. Addition of both short- and long-term N increased β-1,4,-glucosidase (BG), β-D-1,4-cellobiohydrolase (CBH), phenol oxidase (PO), peroxidase (PER) and acid phosphatase (PHOS) with the exception of Leucine-aminopeptidase (LAP) and urease enzyme activities. There was non-additive antagonistic (NAA) effects on cellulase, total oxidase, β -1,4,-N-acetyl glucosaminidase (NAG) and NAS effects on LAP, urease and PHOS enzyme activities. The addition of residues likely stimulated microbial growth, but the NAA effect of residue mixtures on C acquisition enzyme (cellulase and total oxidase) activities suggests development of C limitation.

This thesis is dedicated to my loving family and friends. To all my mentors and colleagues who continued to inspire and challenge me. Thank you all for always believing in me.

ACKNOWLEDGEMENTS

This research was made possible by funding from the Michigan State University AgBioResearch Project GREEEN and a New Innovator Award from the Foundation for Food and Agriculture Research.

I cannot express enough thanks to my major advisor Dr. Lisa Tiemann for her immense guidance, continued support and encouragement during the course of my study here at Michigan State University. Her positive attitude and support to her graduate students especially to me is admirable and certainly appreciated. In addition, the guidance committee has been pivotal throughout the duration of my research - to Drs. Sieglinde Snapp, Daniel Brainard and Kimberly Cassida, I offer my sincere appreciation for the learning opportunity you all have provided to me.

The completion of my research could not have been accomplished successfully without the support of my lab team, Alexia, Darian, Amanda, Daniel, Yang, and the undergraduates; Laney, Megan, Bana, Evan and Katheryne who helped in one way or the other. You all deserve a trip to Disney! My deepest gratitude goes out to all my friends for their encouragement mostly; Uzo, Roselyn, and Elizabeth.

I give special thanks to the MasterCard Scholarship Program for their financial support, which allowed me to focus more on my academics. My dream of advancing my education in the U.S.A wouldn't have been easy without the MasterCard program. Big thanks! Finally to my family especially my parents, words cannot express how much I am grateful to you all for being there for me; your prayers, unfailing support and encouragement throughout my years of studies. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

C: carbon

N: nitrogen

CC: Cover crops

SOC: soil organic carbon

TN: total nitrogen

C:N; carbon-to-nitrogen ratio

$NO3^-$- N: nitrate

$NH4^+$ -N: ammonium

BG: β-1,4,-glucosidase

CBH: β -D-1,4-cellobiohydrolase,

NAG: β -1,4,-N-acetyl glucosaminidase

PHOS: acid phosphatase

LAP: Leucine-aminopeptidase

PER: peroxidase

PO: Phenol oxidase

TOX: Total oxidase

EEA: extracellular enzyme activities

NAA: Non-additive antagonistic

NAS: Non-additive synergistic

MEI: mixture effect index

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1. Introduction

1.1 Cropping system diversification with cover crops

Minimizing environmental risks and biodiversity loss, while increasing agricultural productivity are major challenges for row cop agriculture. Maintaining high productivity requires agricultural systems that conserve natural soil richness, enhance plant productivity and specifically promote agricultural sustainability. Crop diversity is an important strategy that can improve environmental protection and lead to more sustainable agriculture (Vitousek and Hooper, 1994; Liebman and Davi, 2000; Hobbs et al., 2008; Tiemann et al., 2015). Increases in crop diversity can have large impacts on the functions of agroecosystems through disease and pest reduction, increase in organic matter decomposition and nutrient cycling, and increases in belowground biodiversity (Vitousek and Hooper, 1994; Hooper et al., 2005; Smith et al., 2008; McDaniel et al., 2014). High biodiversity has been shown to improve the stability of ecosystems, plant productivity and resource use efficiency (Hooper et al., 2005). In particular, cover crops are a form of diversification that has been recognized as a major driver of the overall positive diversity effects in diversified cropping systems (McDaniel et al., 2014b; Tiemann et al., 2015). Although it is recognized that diversification in row-crop agriculture through the addition of cover crops can be beneficial to soil health and subsequent cash crops, very little is known about mechanisms underlying these benefits.

The use of cover crops (legumes and non-legumes) offer many benefits whether incorporated as residue or integrated into the cropping system. Some of these benefits include; improved soil quality and productivity (Sainju et al., 2008), increased soil water

retention (DuPont et al., 2009), and availability of nitrogen (N) (Zhou et al., 2012). Due to these benefits there is increased use of cover crop by farmers. These benefits can be obtained by careful selection of appropriate crop species. For example, cereal cover crops have been shown to produce large biomass; therefore these should be used if the aim is to build up SOM and reduce weed growth. While non-leguminous cover crops do not fix N, but they can positively affect soil N availability by scavenging residual fertilizer or legume N. Leguminous cover crops make N available through atmospheric N fixation, increase soil N availability, plant N uptake and the yield of subsequent crops (Kuo and Sainju, 1998; Ćupina et al., 2011). Several studies have shown increased benefits when more than one cover crop is used, either in a rotation or incorporated as residues compared to individual cover crops. For example, a mixture of annual rye and hairy vetch has been shown to decrease nitrate leaching and increase soil N for the subsequent cash crops (Ranells and Wagger, 1997; Kuo and Sainju, 1998; Kuo and Jellum, 2002; Teasdale et al., 2008; Nair and Ngouajio, 2012). Currently, agricultural producers and crop consultants believe diverse mixtures of cover crops can increase the functionality of an agroecosystem through interspecific interactions. Cover crop mixtures can also have negative effects on the subsequent crop through the process of N immobilization, allelopathy and physical interference (Kuo and Jellum, 2002). However, whether or not these benefits can be realized in row crop system and the mechanisms leading to increased soil system benefits through cover crop diversity still remain unclear.

1.2 Cover crop diversity and decomposition processes

Soil microorganisms are intimately associated with plant communities (Gartner

and Cardon, 2004; Bohme et al., 2005) and it is expected that cover crop diversification should have impacts on soil microbes and soil processes they mediate. Indeed, the quantity and quality of cover crops can influence crop residue decomposition rates and microbial processes (Bending et al., 2002; Zhou et al., 2012; Nair and Ngouajio, 2012; McDaniel et al., 2014). While some aspects of cover crop impacts on microbial communities remain understudied, we know that residue quality influence the nature of soil microbial communities. For example, fungi are the key decomposers of low quality (high C:N and N poor) residues while bacteria are the major degrader of higher quality (low C:N ratio and N rich) residues (Bending et al., 2002; Liu et al., 2007; McDaniel et al., 2014; Stewart and Moturi, 2015).

Plant residue's C:N ratio reflects differences in residue tissue chemistry resulting in faster or slower decomposition rates. For example, Grandy et al., (2013) reported a faster decomposition rate (23% mass remaining) of corn residues with C:N ratio 60.9 ± 4.9 than wheat residues (37% mass remaining) of C:N ratio 111 ± 5.0 after one year of decomposition. Other studies reported faster decomposition of residues with low C:N ratio e.g legume residues (Bending et al., 2002; Gartner and Cardon, 2004; Liu et al., 2007). However, with this variation in residue characteristics, it is still unclear how the interactions of two or more cover crop residues regulate the soil biodiversity.

Decomposition is a complex and important process in nutrient cycling. SOM decomposition is a microbial mediated process during which organic materials are converted into inorganic constituents. Wickings et al. (2012) demonstrated that the interaction between residue quality and microbial community structure influenced the chemical composition of decomposing residue. Individual residues vary in chemical

composition including often-measured C, N and lignin content (Bending et al., 2002; Meier et al., 2010), but also in other chemical constituents (Wickings et al., 2012), hence, there will be more diverse chemical complexity with mixture of residues. This diversity in chemical composition may greatly affect SOM turnover and stabilization, nutrient cycling and soil microbial community structure (Wickings et al., 2012), and also may control microbial N mineralization or immobilization processes (Bending et al., 2002; Meier et al., 2010). These differences in chemical composition have also resulted in rapid loss of decomposable compounds (such as starch and protein) and build-up of more recalcitrant compounds such as lignin and phenols (Wagger et al., 1998; Bending et al., 2002, Liu et al., 2007; Sylvain et al., 2011; Wickings et al., 2012).

Diversity of residue mixtures may either result in additive or non-additive effects on the decomposition process. Additive effects result when the observed response of microbes to residue mixtures is similar to the mean response of microbes to the individual residues. Non-additive effects result when the observed values due to a residue mixtures differs from the expected mean value of the individual species (Gartner and Cardon, 2004; Liu et al., 2007; Meier et al., 2010; Butenschoen et al., 2014). Non-additive effects of residue mixtures on microbial decomposition can either produce a synergistic (positive interaction or faster mass loss) or antagonistic effect (negative interaction or slower mass loss) (Gartner and Cardon, 2004; Bonanomi et al., 2014; Liu et al., 2007; Sylvain et al., 2007; McDaniel et al., 2014), with a synergistic effect being the most frequently observed mixture effect (>65%)(Gartner and Cardon, 2004). Non-additive synergistic interactions are expected to occur when high quality residues are mixed with low quality residues as a result of a net N transfer between the two materials

(Bonanomi et al., 2014). However, Hooren et al., (2003) also reported possible occurrence of antagonistic effects on decomposition of residue mixtures if one of the residues contained a high proportion of difficult to decompose compounds such as lignin. These contrasting responses may be due to factors such as the type and diversity of residue mixture, different climatic conditions (e.g temperature and moisture), microclimatic conditions (Gartner and Cardon, 2004, Meier et al., 2010; Butenschoen et al., 2014) and edaphic conditions (Eskelinen et al., 2009). Therefore, it is poorly understood how residue mixtures affect the rate of decomposition processes, N mineralization and immobilization.

1.3 Nitrogen availability and decomposition processes

Microbial activity has been shown as a major factor controlling soil nutrient pools. Change in the community structure and activity of soil microbes can influence the cycling and transformation of N and its availability to plants. Increased N addition has been found to affect nutrient cycling and rates of decomposition by influencing microbial functions, such as production of the extracellular enzymes that drive residue decomposition (Tiemann & Billings, 2011; Zhou et al., 2012). Most microorganisms are saprotrophs and as such are primarily limited by sources of C for energy and growth. However, during the growing season, in soils with low N, plants and microorganisms compete for N, which may result in a shift towards microbial N limitation (Zhang et al., 2008; Tiemann and Billings, 2011). When N is added to the soil microorganisms overcome N limitation and when applied long-term, this can lead to increased microbial biomass as C available to microbes is also increased through primary productivity,

which is also enhanced by N addition (Tiemann and Billings, 2011; Geisseler and Scow, 2014).

Overall, it is expected that N addition could narrow the overall organic matter C:N ratio thus shifting microbial biomass to be more bacteria dominated (Keeler et al., 2009). The results of long-term N addition studies are variable with some reporting negative effects of N addition on microbial biomass (Carriero et al., 2000; Hobbie, 2008; Keeler et al., 2009; Zhang et al. 2016), some reporting neutral effects (Zak et al., 2006; Tiemann and Billings, 2011) and some positive effects (Alon and Steinberger, 1999; Hobbie 2000, Saiya-Cork 2002; Geisseler and Scow, 2014). The sources of variation in the result of these studies could partly be a result of litter quality and different rates of N addition (Hobbie, 2005; Knorr et al., 2005). For example, in a meta-analysis, Knorr et al., (2005) reported increased decomposition of high quality litter with N addition, but reduced decomposition rates of low quality litter. Another potential explanation for these inconsistencies is that N additions can affect residue decomposition rates both directly and indirectly. Direct effects of N addition are a result of alteration in microbial functions such as extracellular enzyme production (Hobbie, 2008; Yevdokimov et al., 2008; Zhou et al., 2012; Geisseler et al., 2016), while indirect effects of N addition are usually related to alteration of residue and/or substrate quality (Hobbie, 2005; Tiemann and Billings, 2011; Ramirez et al., 2012).

The general objective of this study is to examine how cover crop diversity and N addition influence soil microbial activities, decomposition rates and plant available N. I conducted a soil laboratory incubation study of cover crop residues of different qualities including: *Lolium multiflorum* (ryegrass); *Pennisetum glaucum* (Canadian pearl millet)

Pisum sativum (Field pea)*;* and *Vicia villosa* (hairy vetch). Soils with a history of different levels of N application were mixed with residues individually and in mixtures to determine a) the effects of both long- and short-term N application on microbial activities, residue decomposition rates and N availability and; b) effects of cover crop mixtures vs. single cover crops on soil microbial activities. I hypothesized that there will be positive, non-additive synergistic (NAS) mixture effects with long-term N addition on residue decomposition rates and N mineralization in most diverse residue mixtures irrespective of the N addition dose. Also, I predicted a positive interaction between crop residue mixtures and N availability such that the addition of N will increase decomposition rates at the initial stages of residue breakdown and decrease decomposition rates at the later stages.

LITERATURE CITED

LITERATURE CITED

Alon, A., & Steinberger, Y. (1999). Effect of nitrogen amendments on microbial biomass, aboveground biomass and nematode population in the Negev Desert soil. Journal of Arid Environments 41: 429–441.

Bending, G.D., Mary, T. K., & Julie, J. E. (2002). Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Soil Biology & Biochemistry 34:1073-1082

Bohme, L., Langer, U., & Bohme, F. (2005). Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. Agriculture, Ecosystems and Environment, 109(1–2), 141–152.

Bonanomi, G., Manuela, C., Guido, I., & Stefano, M. (2014). Nitrogen transfer in litter mixture enhances decomposition rate, temperature sensitivity, and C quality changes. Plant Soil 381:307–321

Butenschoen, O., Valentyna, K., Mark, M., Franca, M., Dorothee, S., & Stefan, S. (2014). Litter mixture effects on decomposition in tropical montane rainforests vary strongly with time and turn negative at later stages of decay. Soil Biology & Biochemistry 77:121e128

Carreiro, M. M., Sinsabaugh, R. L., Repert, D. A., & Parkhurst, D. F. (2000). Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81:2359–65

Ćupina, B., Manojlovic, M., Krstic, D.j., Ĉabilovski, R., Mikic, A., Ignjatović-Ćupina, A., & Eric, P. (2011). Effect of winter cover crops on the dynamics of soil mineral nitrogen and yield and quality of Sudan grass [Sorghum bicolor (L.) Moench]. AJCS 5(7): 839-845 ISSN:1835-2707.

DuPont S.T., Ferris, H., & Van, H. M. (2009). Effects of cover crop quality and quantity on nematode-based soil food web and nutrient cycling. Appl Soil Ecol 41:157–167.

Eskelinen, A., Stark, S., & Männistö, M. (2009). Links between plant community composition, soil organic matter quality and microbial communities in contrasting tundra habitats. Oecologia,161(1):113-23.

Gartner, T.B., & Cardon, Z.G. (2004). Decomposition dynamics in mixed-species leaf litter. Oikos104:230–246

Geisseler, D., & Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms - A review. Soil Biology and Biochemistry, 75, 54–63.

Geisseler, D., Lazicki, P. A., & Scow, K. M. (2016). Mineral nitrogen input decreases microbial biomass in soils under grasslands but not annual crops. Applied Soil Ecology, 106, 1–10.

Grandy, A. S., Salam, D. S., Wickings, K., McDaniel, M. D., Culman, S. W., & Snapp, S. S. (2013). Soil respiration and litter decomposition responses to nitrogen fertilization rate in no-till corn systems. Agriculture, Ecosystems and Environment, 179, 35–40.

Hobbie, S. E. (2008). Nitrogen effects on decomposition: A five-year experiment in eight temperate sites. Ecology, 89(9), 2633–2644.

Hobbie, S. E., & Vitousek, P. M. (2000). Nutrient limitation of decomposition in Hawaiian orests. Ecology 81:1867– 1877.

Hobbs, P.R., Sayre, K., & Gupta, R. (2008). The role of conservation agriculture in sustainable agriculture. Philos. Trans. R. Soc. Lond. B 363, 543–555

Hooper, D. U., Chapin , F. S., Ewel , J. J., Hector, A., Inchausti, P., Lavorel, S., & Lawton, J. H. (2005). Effects of biodiversity on eco- system functioning: A consensus of current knowledge. Ecological Monographs 75 : 3 – 35 .

Hoorens, B., Aerts, R., & Stroetenga , M. (2003). Does initial litter chemistry explain litter mixture effects on decomposition? Oecologia 137: 578–586.

Keeler, B. L., Hobbie, S. E., & Kellogg, L. E. (2009). Effects of long-term nitrogen addition on microbial enzyme activity in eight forested and grassland sites: implications for litter and soil organic matter decomposition. Ecosystems, 12(1), 1–15.

Knorr, M., Frey, S. D., Curtis, P. S. (2005). Nitrogen additions and Litter Decomposition: A Meta-Analysis. Ecology, 86(12), 3252–3257.

Kuo, S., & Jellum, E. J. (2002). Influence of winter cover crop and residue management on soil nitrogen availability and corn. Agronomy Journal, 94(3), 501–508.

Kuo, S., & Sainju, U.M. (1998). Nitrogen mineralization and availability of mixed leguminous and non-leguminous cover crop residues in soil. Biol Fertil Soils 26:346–353.

Liebman, M., & Davis, A.S. (2000). Integration of soil, crop and weed management in low-external-input farming systems. Weed Res 40:27–47.

Liu, P., Sun, O. J., Huang, J., & Han, L. L. X. (2007). Nonadditive effects of litter mixtures on decomposition and correlation with initial litter N and P

concentrations in grassland plant species of northern China. Biol Fertil Soils 44:211–216.

McDaniel, M.D., Grandy, A.S., Tiemann, L.K., & Weintraub, M.N. (2014). Crop rotation complexity regulates the decomposition of high and low quality residues. Soil Biology & Biochemistry 78: 243e254

McDaniel, M.D., Tiemann, L.K., & Grandy, A.S. (2014). Does agricultural crop diversity enhance soil microbial biomass and organic matter dynamics? a meta-analysis. Ecological Applications 24, 560e570.

Meier, C. L., & Bowman, W. D. (2010). Chemical composition and diversity influence non-additive effects of litter mixtures on soil carbon and nitrogen cycling: Implications for plant species loss. Soil Biology and Biochemistry, 42(9), 1447–1454.

Nair, A., & Ngouajio, M. (2012). Soil microbial biomass, functional microbial diversity, and nematode community structure as affected by cover crops and compost in an organic vegetable production system. Applied Soil Ecology, 58, 45–55.

Ramirez, K. S., Craine, J. M., & Fierer, N. (2012). Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. Global Change Biology, 18(6), 1918–1927.

Ranells, N. N., & Wagger, M. G. (1997). Winter annual grass-legume bicultures for efficient nitrogen management in no-till corn. Agriculture, Ecosystems and Environment, 65(1), 23–32.

Sainju, U.M., Senwo, Z.N., Nyakatawa, E.Z., Tazisong, I.A., & Reddy, K.C. (2008). Soil carbon and nitrogen sequestration as affected by long-term tillage, cropping systems, and nitrogen fertilizer sources. Agric Ecosyst Environ 127:234–240

Saiya-Cork, K., Sinsabaugh, R., & Zak, D. (2002). The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biology and Biochemistry, 34(9), 1309–1315

Smith, R., K. Gross, & Robertson, G. (2008). Effects of crop diversity on agroecosystem function: crop yield response. Ecosystems 11:355–366.

Stewart, C. E., Moturi, P., Follett, R. F., & Halvorson, A. D. (2015). Lignin biochemistry and soil N determine crop residue decomposition and soil priming. Biogeochemistry, 124(1), 335–351.

Sylvain, C., Jean, W., Olaf, B., Damien, B., & Stephan, H. (2011). Litter composition rather than plant presence affects decomposition of tropical litter mixtures. Plant Soil 343:273–286.

Tiemann, L. K., & Billings, S. A. (2011). Changes in variability of soil moisture alter microbial community C and N resource use. Soil Biology and Biochemistry, 43(9), 1837–1847.

Tiemann, L. K., Grandy, A. S., Atkinson, E. E., Marin-Spiotta, E., & McDaniel, M. D. (2015). Crop rotational diversity enhances belowground communities and functions in an agroecosystem. Ecology Letters, 18(8), 761–771.

Vitousek, P., & Hooper, D. (1994). Biological diversity and terrestrial ecosystem biogeochemistry, in anonymous biodiversity and ecosystem function. In: Schulze, E., Mooney, H.A. (Eds.), Biodiversity and Ecosystem Function. Springer Verlag, Berlin, Heidelberg, pp. 3–14.

Wagger, M. G., Cabrera, M. L., & Ranells, N. N. (1998). Nitrogen and carbon cycling in relation to cover crop residue quality. Journal of Soil and Water Conservation, 53(3), 214-218.

Waldrop, M. P., Zak, D. R., & Sinsabaugh, R. L. (2004). Microbial community response to nitrogen deposition in northern forest ecosystems. Soil Biology and Biochemistry, 36(9), 1443–1451.

Wickings, K., Stuart G.A., Sasha, C. R., & Cory, C. C. (2012). The origin of litter chemical complexity during decomposition. Ecology Letters,15: 1180–1188.

Yevdokimov, I., Gattinger, A., Buegger, F., Munch, J. C., & Schloter, M. (2008). Changes in microbial community structure in soil as a result of different amounts of nitrogen fertilization. Biology and Fertility of Soils, 44(8), 1103–1106.

Zak, D.R., William, E. H., Matthew, J. T., Kurt, S. P., & Andrew, J. B. (2006). Microbial cycling of c and n in northern hardwood forests receiving chronic atmospheric NO_3 deposition. Ecosystems 9: 242–253.

Zhang, N., Shiqiang, W., Linghao, L., Jie, B., Mingming, Z., & Keping, M. (2008). Impacts of urea N addition on soil microbial community in a semi-arid temperate steppe in northern China. Plant Soil 311:19–28

Zhang, W., Chao, L., Yang, Q., Wang, Q., Fang, Y., & Wang, S. (2016). Litter quality mediated nitrogen effect on plant litter decomposition regardless of soil fauna presence. Ecology, 97(10), 2834–2843.

Zhou, X., Chen, C., Wu, H., & Xu, Z. (2012). Dynamics of soil extractable carbon and nitrogen under different cover crop residues. J Soils Sediments 12:844–853

CHAPTER TWO: LONG- AND SHORT-TERM NITROGEN ADDITIONS INCREASE DECOMPOSITION OF COVER CROP RESIDUES IN AGRICULTURAL SOILS

ABSTRACT

Understanding the pattern of soil microbial response to increasing nitrogen (N) availability is important for better understanding of agroecosystem function. In a year-long laboratory incubation, I examined how long- and short-term N addition influence soil microbial enzyme activities: β-1,4-glucosidase, β-D-1, 4-cellobiohydrolase (CBH), phenol oxidase, peroxidase (PER), β-1,4,-N acetylglucosaminidase (NAG), Leucine aminopeptidase (LAP), urease and acid phosphatase (PHOS), related to residue decomposition and plant available N. Five types of residues (none; *Pisum sativum (*field pea); *Vicia villosa* (hairy vetch); *Pennisetum glaucum* (pearl millet) & *Lolium multiflorum* (ryegrass) ranging in C:N ratios from 10.5 to 37.8, were incubated for 362 days with soil sampled from a 12-year field experiment involving a rotational sequence of no-till corn-soy-wheat. I collected soil samples during the corn phase of the rotation from three out of the nine plots; F1, F5 and F9. Every third year, inorganic N fertilizer was applied in these plots F1=0, F5=134, and F9=291 kg N ha^{-1} y^{-1}. In the lab, cover crop residues were added to these soils and fertilized with urea at either 0 or 0.013 g N per 100 g of dry soil to start the incubation. I observed increase in total soil C and N (2.2 and 0.15 mg/g soil respectively) with residue addition relative to control (no residue addition), which disappeared by the end of the incubation with rapid residue decomposition. Long-term N addition increased; inorganic N at the beginning (37 day) and end of the incubation (362 day), most enzyme activities except LAP and urease and was more evident with short-term N addition. The V and R increased the inorganic N pool at day 37 compared to P and M residues. My results revealed that increased microbial activities by N additions could be attributed to alleviation of C limitation of soil microbes.

2.1 Introduction

Nitrogen (N) has been recognized as the major nutrient element limiting plant growth and is the most applied nutrient to increase crop yields (Saiya-Cork et al., 2002; Keeler et al., 2009; Geisseler et al., 2014; Chen et al., 2014). Each year, about 68 million tons of N is applied to agricultural land worldwide with a total cost of approximately $44 billion (Chen et al., 2014). However, increased N additions pose risks to the environment through increased nitrate leaching (Keeler et al., 2009; Chen et al., 2014; Venter et al., 2016), nitrous oxide emissions (Bouwman et al., 2002a), decreased soil pH and alteration of a wide range of biological processes such as decomposition (Saiya-Cork et al., 2002). It is important to understand the mechanisms through which N additions influence soil processes such as decomposition and nutrient cycling if we want to improve N use efficiency and the negative effects of N loss on the environment.

Inorganic N fertilization has a profound impact not only on aboveground communities but also on belowground communities especially soil microorganisms (Zhang et al., 2008; Treseder 2008; Zhou et al., 2012; Geisseler et al., 2014; Geisseler et al., 2016; Zhang et al., 2016). Long-term effects of inorganic N inputs on aboveground communities have been adequately characterized, but the effects of N addition belowground are still poorly understood. Soil microorganisms provide numerous ecosystem functions including decomposition of organic materials, nutrient cycling (Nair et al., 2012; Zhou et al., 2012;) and biotransformation of organic pollutants (Bohme et al., 2005; Geisseler et al., 2016). Increased N addition can alter and interrupt these important soil processes by affecting microbial community structure and functions

(Alon and Steinberger 1999; Waldrop et al., 2004; Treseder 2008; Zhou et al., 2012). Alterations in microbial community function with N addition could be a result of a shift in microbial limitation from N to C, which results in a shift in the physiology of microorganisms and rates of decomposition (Zhang et al., 2008; Treseder 2008). Although effects of N addition on microbial communities appears to be highly variable, it appears that residue quality (e.g. residue C:N ratio) plays a central role. For example Tiemann and Billings, (2011) reported an increase in enzyme activities that degrade labile C compounds with N fertilization, which was associated with increases in plant residue quality. Additionally, Geisseler and Scow, (2014) reported that increases in plant productivity due to N additions have a positive impact on microbial biomass in agricultural soils.

Studies have also shown that soil microbial functions are affected differently by long-term (several years to decades) and short-term (days to a few years) N additions (Alon and Steinberger 1999; Zhang et al., 2008; Zhong et al., 2010; Zhou et al., 2012; Geisseler and Scow 2014; Geisseler et al., 2016). In general, long-term N additions can indirectly increase microbial biomass and activity as the microorganisms respond positively to the increases in plant productivity and/or reduced plant C:N ratio that accompanies N additions (Ramirez et al., 2010; Tiemann and Billings, 2011; Fierer et al., 2012; Geisseler & Scow, 2014). In contrast, short-term N addition can lead to reductions in microbial biomass and respiration rates (Hallin et al., 2009; Lupwayi et al., 2011). However, these generalities do not always hold true with studies showing microbial responses to long-term N additions to be positive (Alon and Steinberger, 1999; Zhong et al., 2010; Zhou et al., 2012; Grandy et al., 2013), negative (Treseder

2008; Zhang et al., 2008; Ramirez et al., 2012) and neutral (Zak et al., 2006; McCrankin et al., 2008). For example, Treseder 2008 observed a 15% decline in microbial biomass under the highest levels of N fertilization in longer duration studies. Contrastingly, Zhong et al. (2010) reported increased microbial biomass and activities with N fertilizer addition in a 21-year experiment. In short-term N addition studies, we also see variable results depending on the soil type, duration of incubation, and N dosage. For example, Yevdokimov et al., (2008) found no effect of N additions on soil microbial biomass in a 30 day laboratory incubation of soil collected under annual crops while Stark et al., (2007) found increased microbial biomass and functional diversity after 10 days with addition of 100 kg N ha^{-1} as urea, but these changes were not sustained over the 91 day incubation period. Because microbial communities play a key role in controlling soil processes, it is crucial to understand their response to N additions and how those responses change the critical soil functions they mediate.

The addition of N could affect nutrient cycling and rates of residue decomposition by influencing microbial processes such as N mineralization and extracellular enzyme production (Hobbie 2008; Yevdokimov et al., 2008; Tiemann and Billings 2011; Zhou et al., 2012). Residue decomposition rates generally depend on the plant residue chemistry, type and rate of N addition (Saiya-Cork et al., 2002; Knorr et al., 2005; Zhou et al., 2012), and climatic conditions (Wang et al., 2015). Residue decomposition is an important process controlling nutrient availability for both plants and soil microbes. However, studies have demonstrated increased (Saiya-Cork et al., 2002; Zak et al., 2006; Wang et al., 2015), decreased (Hobbie 2008; Chen et al., 2013; Zhang et al., 2016) and no change (Prescott 1995; Knorr et al., 2005; Grandy et al., 2013) in

decomposition rates with N addition. The effects of inorganic N additions on litter/residue decomposition are highly variable across systems and have been attributed to several mechanisms including differences in plant residue chemistry (Knorr et al., 2005; Coq et al., 2011; Wickings et al., 2012; Wang et al., 2015; Zhang et al., 2016) microbial C or N use efficiencies (Hobbie, 2005; Grandy and Neff 2008) and indirect effects of changes in plant communities and/or productivity (Tiemann and Billings, 2011; Butenschoen et al., 2014; Geisseler & Scow, 2014). In addition, previous studies have reported initial increases in decomposition rates, while at the later stage, decomposition rates decreased with N addition (Saiya-Cork et al., 2002; Knorr et al., 2005).

The majority of studies assessing inorganic N addition effects on litter decomposition have been conducted in natural systems, where N application rates mimicking N deposition are low relative to agricultural systems. This, along with a paucity of studies, creates uncertainty about the effects of agricultural N fertilization on litter/residue decomposition (McDaniel et al., 2016). Furthermore, N addition has been shown to have a positive influence on soil microbial mineralization and is thus likely to increase soil N availability (Grandy et al., 2013). Therefore, it remains unknown if historical or long-term N additions change microbial communities such that they may respond to more immediate or short-term N additions in an unexpected manner.

The inconsistency of responses to N inputs on residue decomposition and nutrient cycling, calls for more investigation. Part of the difficulty is the lack of understanding with regards to differences in short-term versus long-term N additions because there are very few studies that examine effects of both simultaneously.

Therefore I addressed this knowledge gap using soils from a long-term N addition gradient field study to which I added cover crop (CC) residues of varying quality. This soil incubation study also included short-term N additions, in order to determine the effects of both long- and short-term N additions on soil microbial activities related to CC residue decomposition and N mineralization. I hypothesized that; 1) microbial communities from long-term N addition soils would exhibit increased decomposition activity with greater net N mineralization across all residue types; 2) short-term N addition would increase microbial decomposition activities only with addition of high quality (low C:N) CC residues; 3) the interaction between short- and long-term N additions would further increase soil microbial activities related to CC residue decomposition and N mineralization especially with low C:N residues.

2.2 Materials and Methods

2.2.1 Study Site

Soils for this research were collected from the Resource Gradient Experiment established in May 2005 at the Michigan State University W.K Kellogg Biological Station (KBS). The mean annual rainfall and temperature are 890mm and 9.7^{o}C respectively (KBS 2015). The soils are classified as Kalamazoo (fine-loamy) and Oshtemo (coarse-loamy) mixed, mesic, Typic Hapludalfs (Alfisols) that developed on glacial outwash. The experiment is set-up in a corn-soy-wheat rotation that the corn and wheat phases receives nine different N fertilization rate ranging from 0-291 kg N ha^{-1} y^{-1} (corn) and 0-180 kg N ha^{-1} y^{-1} (wheat). The experimental plots, which are 5 m x 30 m, are

randomized across two sets of four blocks, with one set of blocks irrigated and the other not.

2.2.2 Laboratory Soil Incubation Experimental Design

Soils for the laboratory incubation were collected in December 2015, after the soy phase of the rotation so that no N fertilizer was applied to the site in 2015. Soils were collected from three of the nine corn N addition rates F1 = 0, F5 =134, and F9 = 291 kg N ha^{-1} y^{-1} within the four irrigated blocks only. At the same plots during the wheat phase the fertilization rates were F1= 0, F5= 90, F9= 180 kg N ha^{-1} y^{-1}. I collected soils from irrigated plots in order to reduce the effects of moisture variability, which is prevalent without irrigation. Five 2.5 cm (diameter) soil cores were randomly collected (0-15 cm depth) from the three N rate plots within each of four blocks (n=12). The soils were arranged in coolers and transported to the laboratory at Michigan State University. Fresh soils were passed through an 8 mm mesh sieve, and a sub-sample was dried at 60 ^{o}C to determine gravimetric soil moisture. I determined water-holding capacity (WHC) by saturating 5 g dry soil placed in Whatman #4 filter paper fitted into funnels. These were covered to prevent evaporative loss and allowed to drain overnight (Fierer and Schimel, 2002).

The amount of water added to maintain the soil at 50 % WHC was estimated from the gravimetric moisture content of the soil. Cover crop residues were collected from farmer's field in late October, oven dried and then chopped to 2 mm size pieces. Four different CC residues were used including two grasses, *Lolium multiflorum* - ryegrass (R) and *Pennisetum glaucum* - pearl millet (M), and two legumes *Vicia villosa* -

hairy vetch (V) and *Pisum sativum* - field pea (P). A total of 0.5 g of each individual CC residue was completely homogenized with the equivalent of 100 g dry soil (5 mg residue g^{-1} soil). Duplicate sets of soils mixed with residues were placed in 10cm long by 6.35 cm wide PVC cores, sealed at the bottom with course filter paper. These were then placed on top of a layer of marbles in 950 ml Mason jars. A thin layer of water was added to the bottom of the jars to help maintain soil moisture. Soils were incubated at 22 ^{o}C with loosely placed lids on the jars that were frequently removed to allow for gas exchange.

To one of the duplicate sets of the soils in jars, I added 0.013 g N (urea) per 100 g of dry soil to observe the impact of short-term N additions. Approximately 10g of soils were sub-sampled from the jars using a spatula on days 37, 110, 204, and 362 of the incubation. Cumulative value of the variable extracellular enzyme activity was calculated by converting the enzyme activity that was measured as hourly rates to daily rates and then multiplying by days elapsed between sampling dates:

$$EEA_{cum} = (EEA_{hourly}) * 24 * (T_{days})$$

2.2.3 Soil organic C and Total Nitrogen

Soil organic C (SOC) and total nitrogen (TN) were measured on day 1 after the addition of residue treatments and at the end of the incubation period (day 362). Subsamples were oven dried, finely grounded, sieved through a 2 mm mesh, and finely ground prior to analysis on an elemental analyzer (Costech ECS 4010; Costech Analytical Technologies Inc., Valencia, CA, USA).

2.2.4 Extracellular enzyme activity and inorganic nitrogen

Soil extracellular enzyme activities (EEA) and soil inorganic N were measured at each point of subsampling throughout the incubation period. Soil extracts for inorganic N analysis were obtained by shaking 8 g of fresh soil with 40 ml of 0.5 M K_2SO_4 on orbital shaker for 1 hr. The resulting slurries were filtered with Whatman #1 filter paper and stored at 4 °C prior to analysis. The soils were assayed for eight enzymes involved in decomposition and nutrient cycling as described by Saiya-Cork et al., (2002) with slight adjustment. I measured the C acquisition enzymes β-1,4-glucosidase (BG), β-D-1, 4-cellobiohydrolase (CBH), phenol oxidase (PO) and peroxidase (PER), the C and N acquisition enzyme β-1,4,-N-acetylglucosaminidase (NAG), N acquisition enzymes Leucine aminopeptidase (LAP) and urease (UR) and acid phosphatase (PHOS). Soil slurries were made with 1 g of soil previously stored frozen, at -80 °C and 125 ml of a 50 mM acetate buffer adjusted to an average soil pH of 6.5. The mixture was homogenized for 30s with an immersion blender. The soil slurries were continuously stirred with a magnetic stir bar while 200 µl aliquots were dispensed into 96-well microplates using a multi-channel pipette. All assays, except PO, PER, and urease, used fluorescently labeled substrates, with the fluorescent molecule, 4-methylumbelliferone or 7-amino-4-methylcoumarin, fluorescing when cleaved from the target substrate by the enzyme of interest. The oxidative enzymes, PO and PER, received 50 µl of 25 mM L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate, with PO assays additionally receiving 10 µl of 0.3 % H_2O_2. Urease plates received 10 µl of 20 mM urea as substrate. I incubated soil slurries at 22 ^{0}C for 18 hrs. After the incubation, hydrolytic enzyme plates received 10 µl of 0.5 M NaOH to increase pH to a level optimal

for MUB and MC fluorescence before plates were read on a Synergy HT1 plate reader (BioTek Instruments, Inc., Winooski, VT USA) at 355 nm excitation and 450 nm emission wavelengths. Color change in the L-DOPA substrate for PO and PER plates was assessed spectrophotometrically at 460 nm.

Determination of ammonium concentrations after addition of urea was used to assess urease activity and soil extractable ammonium was done using the same colorimetric method (Sinsabaugh et al., 2000). Each well in a 96-well plate containing either soil slurry (for urease) or soil extract received 40 µl of both ammonia salicylate and ammonia cyanurate solutions prepared per manufacturer's instructions (Hach, Loveland, CO, USA). Color change related to ammonium concentration was determined at 610 nm on the Synergy HT1 plate reader. To determine concentration of nitrate in soil extracts, I used a 96-well plate colorimetric assay based on Doane and Horwath (2003) with one adjustment. Instead of using a chemical reduction agent to convert nitrate to nitrite, I used nitrate reductase (AtNaR2; Nitrate reductase from Arabidopsis thaliana, EC 1.7.1.1; NECi, Lake Linden, MI, USA). Color change related to final nitrite concentrations was determined spectrophotometrically on the Synergy HT1 plate reader at an absorbance wavelength of 540 nm.

2.2.5 Statistical analysis

Statistical analyses were accomplished using SAS (SAS Institute, Cary, SC). Using PROC GLIMMIX three-way ANOVA was used to analyze the main effects and interactions of long-term (field) and short-term (laboratory) N applications and residue addition treatments, on soil microbial functions, residue decomposition rates and plant N

availability. Normality of residuals was checked using normal probability and boxplot. Any variables that were non- normal or showed low heterogeneity were appropriately transformed (square root transformation) to meet variance analysis assumptions. Homogeneity of variances was checked using Levene's test. Where interactions were not significant, pairwise comparisons were done within significant main effects using LS means tables. When interactions were significant; we used the slice function within SAS to compare difference among treatments and to look at individual comparisons for each level of interaction. The results are reported statistically significant at α= 0.05.

2.3 Results

There was no effect of long-term N treatment on soil organic C and total N before the addition of the residue treatments (Table 2.2). On day 1 of the incubation after residue addition, control soils had lower soil organic C and total N compared to soils treated with residue, but this difference disappeared by day 362, regardless of residue type and long- or short-term N additions (Fig. 2.1a, b, c & d). On day 1, there were no treatment interaction effects but a significant main effect of long-term N addition and residue treatments ($p<.0001$), while on day 362, there was no treatment effect on total organic carbon and nitrogen ($p>0.05$) (Table 2.3).

2.3.1 Inorganic nitrogen

Net N mineralization and nitrification were measured at the initial stage (day 37) of the incubation. There was a significant interaction of long- and short-term N addition treatment on net N mineralization and nitrification (Table 2.4). Short-term N addition

resulted in greater N mineralization and nitrification in soils with 291N long-term N addition (Fig. 2.2a & b). Among the residues, hairy vetch had higher net N mineralization and nitrification rates than the millet and control treatment but not different with field pea and rye (Table 2.4, Fig. 2.2a & b).

The control treatment had low soil NH_4^+ compared to hairy vetch, pearl millet and ryegrass (p=0.009) on day 37 (Table 2.4, Fig. 2.3a). There was no treatment effect on NH_4^+ on day 110 and 204. On day 362, soil NH_4^+ was greater in 134N long-term N treatment than 0N and 291N kg ha^{-1} y^{-1} (p=0.002) (Fig. 2.3d).

There was a treatment interaction effect of long- and short-term N addition on soil inorganic NO_3^- on day 37 and day 362. On day 37, short-term N additions resulted in greater NO_3^- in soils with the 291 compared to 0 long-term N addition rate (p<.0001) (Fig. 2.4a). On day 362, soil without short-term N addition had greater nitrate in 291N compared to 0N and 134N long-term N levels (p=0.013), while soil with short-term N addition showed the opposite trend with greater nitrate found in soils from 0N compared to 134N and 291N long-term N treatments (p=0.019) (Fig 2.4d). Residue treatment affected soil NO_3^- regardless of short or long-term N addition rate at all timings other than day 362 (Table 2.4). On day 37, soils with hairy vetch residues had higher NO_3^- concentration than the other CC residue and control treatments (Fig. 2.4a), while on day 110 and 204 annual ryegrass and hairy vetch were higher in NO_3^- concentration than other residue treatments and control (Fig. 2.4b & c). At the final stage of the incubation day 362, there was a significant three-way interaction of long-term N, short-term N and residue treatments on soil NO_3^- (Table 2.4). Soil from 291N amended with V residue without short-term N addition had greater NO_3^- concentration (p=0.0002) while with

short-term N addition control (no residue) treatment in 0N long-term N treatment had lower soil NO_3^- (p=0.0005) (Fig.2.4 d).

2.3.2 Extracellular enzyme activity (EEA)

There were variations in response among the eight EEA measured. Effects of short-term N and long-term N on EEA were similar across sampling dates so I will report results as cumulative EEA only. There was no significant correlation between soil inorganic N and EEA (data not shown). Cumulative BG enzyme activity was determined by an interaction between short- and long-term N treatments (Table 2.5). In particular, short-term N addition resulted in greater increases in BG activity as long-term N addition rate increased and was greatest in 291N treatment (p<.0001) (Fig.2.5a). In contrast, without short-term N addition, BG activity was lower in 134N treatment compared to the 0N and 291N long-term N treatments. There was also a significant interaction of long- and short-term N addition treatments on CBH enzyme activity (Table 2.5). With short-term N addition, I observed declines in CBH activity as long-term N level increased and was lowest in 291N treatment (p=0.0003). While without short-term N addition there was no difference in CBH by long-term N treatment (P>0.05) (Fig.2.5b). There was a significant interaction between long- and short-term N addition and also between long-term N and residue treatments on oxidase activity: peroxidase (PER) and phenol oxidase (PO) (Table 2.5). In soils without short-term N addition the PO activity was lower in 291N treatment compared to 0N and 134N treatments (p<.0001) (Fig. 2.5c). There was also a significant interaction of long- and short-term N addition and between long-term N addition and residue treatment on PER activity (Table 2.5).

There was no N treatment interaction effect on NAG enzyme activity (Table 2.5). The NAG activity increased with increase in long-term N addition levels. The NAG activity was higher in soil with short-term N treatment (Fig. 2.6a). There was no treatment effect on LAP and urease activities. Only the long-term N addition treatment had a significant effect on PHOS activity (Table 2.5), with the greatest activity found in 134N compared to 0N and 291N treatments ($p=0.0019$) (Fig. 2.6d).

Cover crop residue effects on soil enzyme were only detected for PO, PER and NAG enzymes (Table 2.5). For NAG, these effects were independent of N treatment, with vetch residue having greater NAG than other residue treatments ($p=0.0067$) (Fig. 2.6a). For PO and PER, residue effects varied with long-term N addition but not short-term N addition treatment (Table 2.5). In particular, PO activity was lower in control (no residue) treatment in soils that received 0N long-term N addition rate compared to soils mixed with residues ($p=0.03$) (Fig. 2.5c). Similar to PO activity, the residue treatment effects showed similar effects on PER activity (Fig. 2.5d).

2.4 Discussion

Cover crop residues can increase the N availability in the soil (Lawson et al., 2011; Zhou et al., 2016), but the interactive effects of N additions and N availability in CC residues (i.e C:N ratios ranging from 12 to 38) on residue decomposition and N mineralization has rarely been studied. I conducted a laboratory incubation of soil with different CC residues that allowed me to assess effects of both short- (immediate/lab N) and long- (field N) term N additions on decomposition and N availability without the complicating factor of environmental variability. This study provides insight on how N

additions influence soil microbial activities that directly affect soil processes, such as residue decomposition and other agroecosystem functions, such as N mineralization. I also use this one-year laboratory incubation to address if residues interact with N additions to change decomposition dynamics.

2.4.1 N addition effects on N mineralization and CC residue decomposition

I observed that at the start of the incubation (day 1), the total soil C and N contents were significantly lower in control soil (no residue) as expected, but surprisingly, on day 362 the total C and N of residue treated soils were not significantly different from control soils, regardless of residue type, historical field N addition levels or short-term N additions in the laboratory. Although we expected to see decreased soil C and N over the course of a year long incubation (Sainju et al., 2000), finding no evidence of CC residue additions after a year was somewhat surprising, although this has been observed previously in agricultural soils under similar incubation conditions (McDaniel et al., 2016). In other year-long soil incubations undergoing residue additions researchers have found total soil organic C and N to be significantly greater compared to control soils (no residue additions) at the end of the incubation (Chen et al., 2014). Also Li et al. (2013) reported higher C and N mineralization in soil amended with CC residues compared to control soil after 56-day incubation. These results are likely connected to starting total soil organic C and N content as the Chen et al. (2014) was conducted in soils with much higher soil organic C and N stocks than soil in this study or those used in McDaniel et al., (2016).

In addition to residue types, both long- and short-term N addition had significant effects on decomposition and N mineralization. After 37 days of incubation, short-term N addition increased net N mineralization and nitrification rates with high long-term N addition rates (134N and 291N) and was higher in hairy vetch than in millet and control (no residue) treatments. Similar to this result Zhang et al. (2008) found higher net mineralization and nitrification rates in soils treated with mineral fertilizer compared to control. Also O'Connell et al. (2015) reported increased potential N mineralization with CC treatment relative to bare-soil and was greater in legume-dominated residues than grass. Changes in NH_4^+ and NO_3^- between day 1 and day 37 indicate the influence of N addition on soil N availability. N additions and CC residues increased NO_3^-- N more than NH_4^+-N. This could have been due to high potential for nitrification at my study site. In addition, different soil microbes use different forms of N depending on the energy required for the breakdown, which could result in preferential assimilation of NH_4^+-N rather than NO_3^-- N (Hobbie 2005).

Soil NO_3^--N increased with the interaction of short-term N and long-term N additions. Soil NO_3^--N was greater in soil from 291N field N level with short-term N addition at day 37 and without short-term N addition at day 362. This result is consistent with previous research at this study site. Grandy et al. (2013) reported greater increases in soil inorganic N following short-term N fertilizer in treatments with greater long-term N addition rate (291N kg $ha^{-1}y^{-1}$). My result was also consistent with other studies for example Stark et al. (2007) reported increase in mineral N in soils treated with urea N after 91 days of incubation.

The present study showed that the initial increase in C content due to addition of cover crop C could stimulate microbial growth especially the copiotrophs (Hu et al., 1997; Fontaine et al., 2003). Copiotrophs are fast growing microbes with high resource demands and are generally limited more by C than N. In my study, when CC residues of C:N ratio ranging from 11 to 38 were added, decomposition was rapid and complete. The highly disturbed nature of row crop production systems in conjunction with relatively high plant productivity due to field N fertilization has likely increased belowground C inputs and altered microbial communities with a shift towards dominance by copiotrophs (Ramirez et al., 2010; Ramirez et al., 2012). In addition, my results showed evidence of increased soil inorganic N pool with N additions and residue treatments especially hairy vetch and ryegrass.

2.4.2 Effect of N addition on EEA related to CC decomposition and N mineralization

Extracellular enzymes are N-rich thus; their production is tightly regulated by N availability. Here, I observed that short-term and long-term N additions influenced activities of different enzymes. In the presence of short-term N, long-term N addition had a large effect on BG, CBH and oxidase activities. Presumably, this was because the long-term supply of N was critical for production of the enzymes responsible for residue decomposition prior to the stat of the incubation (Hobbie, 2008; Bradley et al., 2006). This supports the idea that communities shifted from N to C limitation with high rates of enzyme production and residue decomposition and may explain the complete decomposition of added residues over the course of 362-day incubation period we

observed in our study, indicating that microbial enzyme activities are good indicators of resource demand (Sinsabaugh et al., 2002).

In the present study, the BG and CBH activities involved in metabolism of relatively labile polysaccharides did not show similar patterns of response with N addition. In particular, the interaction of long- and short-term N additions increased BG but decreased CBH activity. Consistent with these results are several other studies that reported increased activity of one or more enzymes involved in the degradation of labile C with N addition (Ajwa et al., 1999; Carreiro et al., 2000; Saiya-Cork et al., 2002; Tiemann and Billing, 2011; Grandy et al., 2013). For example, at the same study site, Grandy et al. (2013) reported increased hydrolase enzyme activities with N addition and no change in oxidative enzyme. In addition, studies have shown that N addition increased activities of hydrolase enzymes and the decomposition rate of low lignin content residues, while decreasing decomposition rate of high lignin content residues (Frey et al., 2004; Zak et al., 2006; Hobbie, 2008; Saiya-Cork et al., 2002, Tiemann and Billings, 2011). Additionally, in a meta-analysis, Whittinghill et al. (2012) found a 9 % increase in cellulose decomposition and a 30% decrease in lignin decomposition with N addition. However, in contrast with our result, Ramirez et al. (2012) observed strong decline in BG activity across 28 N-fertilized soils, which was linked to a reduction in microbial biomass. Overall, these results highlight the variability of N addition effects due to complex interaction with residue chemistry and soil extant soil C and N stocks.

Oxidative enzyme activities decreased with increase in long-term N addition rates and were lower in soil treated with short-term N addition. Contrastingly, Grandy et al. (2013) reported no change in oxidase with N addition in the same study site. Several

other studies have demonstrated negative or no change for oxidative enzyme activities with N addition, although these studies primarily focused on long-term N addition (Saiya-Cork et al., 2002; Hobbie, 2008; Keeler et al., 2009; Tiemann and Billings, 2011; Zhou et al., 2012). N addition could hinder the synthesis of oxidative enzymes (Zak et al., 2006; Hobbie, 2008; Knorr et al., 2005) or form recalcitrant compounds with the breakdown products of lignin (Hobbie, 2008), thereby causing a decrease in oxidative enzyme activity. Reduction of lignin-degrading enzyme activity with N addition could be one of the mechanisms that may explain the negative feedback of N addition on decomposition rates that studies often observe (Knorr et al., 2005; Hobbie, 2008). However, in this study I added residues that have relatively low lignin concentrations. Therefore, any negative responses of PER and PO to N addition would have very little effect on the decomposition rate of the residues.

The N-acquiring enzymes, urease, LAP and NAG showed varied responses with N addition. Urease and LAP enzyme activities displayed no change in response to N addition in both residue treatments and control. Similar to my result, Tiemann and Billings (2011) reported that LAP showed no significant difference between N fertilized treatments but differed in sampling dates. I observed increase in NAG enzyme activity with N additions. The NAG enzyme activity increased with increase in long-term N addition rate and was greater in soil with short-term N treatment. Also NAG enzyme activity was higher in residue treatment compared to control. Being consistent with our result in the same site, Grandy et al. (2013) reported a periodical increase in NAG enzyme activity in response to N input. Grandy et al. (2013) argued that increased chitin-degrading enzyme (NAG) provided additional evidence for increased enzyme

activities involved in the decomposition of relatively labile substrates. Further, other studies have shown similar response (Ajaw et al., 1999; Saiya-Cork et al., 2002; Keeler et al., 2009) while some reported negative or no change in NAG activity with N addition (Zhou et al., 2012).

Phosphatase enzyme activity is associated with mineralization of organic phosphorous (P) to inorganic P (Zhou et al., 2012) and also an essential element for plant and microbial growth. In soils with high N input, P becomes a limiting element. Soil microbes are known to be N limited (Zhou et al., 2012; Geisseler et al., 2014) therefore, microbial activity could be majorly controlled by N availability. N addition might shift EEA away from N acquisition and toward P acquisition (Sinsabaugh et al., 2002). In my study, phosphatase enzyme activity slightly decreased with an increasing long-term N addition rate. Slight declines in PHOS activity in response to N addition could be a result of a reduction in the PHOS isoenzyme (Enrique et al., 2008) or could be attributed to declines in microbial populations responsible for mineralizing P (Keeler et al., 2009). Previous studies have demonstrated increased phosphatase activity with N addition. For example, Keeler et al. (2009) reported increased alkaline phosphatase (AP) activity after 7 years of N fertilization, suggesting that N addition stimulated microbial activity and the demand for P.

2.5 Conclusions

Residue decomposition and N release following short- and long-term N addition were measured in a one-year laboratory incubation experiment. Short-term N addition increased N mineralization and inorganic N content while long-term N addition showed

large increase in the presence of short-term N addition. Legumes and ryegrass residues resulted to higher soil inorganic N than pearl millet. The interaction of short- and long-term N additions also increased soil inorganic N availability suggesting a potential increase in soil N pool and soil fertility level. Decreased total soil C and TN content at the end of the incubation shows that the majority of the CC residue C had been mineralized. This short-term persistence of cover crop C suggests the inclusion of C either through rotation or residue addition is required for C accumulation. In addition, residue types had little to no impact on soil microbial activities related to decomposition except on NAG and oxidative EEA. The addition of N largely influenced the decomposition rate and led to rapid and complete decomposition of CC residue. Similar to the previous studies that reported greater net primary production with repeated N addition (LeBauer and Treseder, 2008), the 12 years of N addition in my study site might have shifted microbial communities with a shift towards microbial group that are more C than N limited. Therefore, increased soil microbial activities related to decomposition of residues by N addition could be attributed to alleviation of C limitation on soil microbes. This indicates that soil microbes play an important role in mediating residue decomposition.

APPENDIX

Table 2.1. Chemical characteristics of cover crop residues.

Cover crops	C (%)	N (%)	C:N ratio	Lignin (%)	NDF (%)	ADF (%)	Cellulose (%)
Hairy vetch (V)	43.5	3.4	12.8[c]	8.4	39.2-48.5	30.3-39.7	28.8
Field pea (P)	44.1	4.3	10.5[d]	2.4	41.0	27.6	23.6
Annual rye (R)	41.8	2.9	14.3[b]	4.6	52.0	32.0	22.6
Pearl millet (M)	42.1	1.1	37.8[a]	4.7	46.5	28.4	28.4

Significant treatment effects at $p \leq 0.05$; different lower case letters are significantly different. NDF=neutral detergent fiber, ADF= acid detergent fiber, and cellulose contents were adapted from literature as follows: V from Hu et al. 1997; Lawson et al. 2011; P from Murungu et al. 2011;Mustafa & Seguin, 2003; R from Aganga et al. 2004; Bending et al. 1999; M from Raphael et al. 2016.

Table 2.2. Properties of soil from the W. K. Kellogg Biological Station Nutrient Gradient Experiment prior to the start (day 0) of a lab incubation study by field N addition level. Values are means followed by the standard errors in parentheses.

Field N Treatment (kg N ha^{-1})	Total soil C (mg g^{-1} soil)	Total soil N (mg N g^{-1} soil)	pH	C:N
0N	16.661(0.04)[ns]	1.425(0.01)[ns]	6.85[ns]	9.495 (0.02)[ns]
134N	16.127(0.15)[ns]	1.311(0.02)[ns]	6.76[ns]	9.952 (0.07)[ns]
291N	17.387(0.13)[ns]	1.404(0.01)[ns]	6.86[ns]	10.147(0.07)[ns]

Significant treatment effect at $p \leq 0.05$; ns = non-significant.

Table 2.3. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N and residue and their interaction on total soil carbon and total nitrogen of soil from the W. K. Kellogg Biological Station Nutrient Gradient Experiment on the initial stage (day 1) and final stage (day 362) of soil incubation study.

	Treatment effects	Total carbon	Total nitrogen
Day 1	Field N (FN)	<.0001	<.0001
	Lab N (LN)	-	-
	Residue (R)	<.0001	<.0001
	FN*LN	-	-
	FN*R	-	-
	LN*R	-	-
	FN*LN*R	-	-
Day 362	Field N (FN)	-	-
	Lab N (LN)	-	-
	Residue (R)	-	-
	FN*LN	-	-
	FN*R	-	-
	LN*R	-	-
	FN*LN*R	-	-

† Presented are p-values of treatments effect across each sampling time. Significant treatment effect at $p \leq 0.05$, (-)=not significant.

Table 2.4. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N and residue and their interaction on net N mineralization, nitrification at day 37 and soil inorganic N at different sampling points over the one-year of soil incubation.

	Treatment effects	Day 37	Day 110	Day 204	Day 362
Net N mineralization	Field N (FN)	-	na	na	na
	Lab N (LN)	<.0001	na	na	na
	Residue (R)	0.0187	na	na	na
	FN*LN	0.0107	na	na	na
	FN*R	-	na	na	na
	LN*R	-	na	na	na
	FN*LN*R	-	na	na	na
Nitrification	Field N (FN)	-	na	na	na
	Lab N (LN)	<.0001	na	na	na
	Residue (R)	0.0287	na	na	na
	FN*LN	0.0147	na	na	na
	FN*R	-	na	na	na
	LN*R	-	na	na	na
	FN*LN*R	-	na	na	na
NH_4^+-N	Field N (FN)	-	-	-	0.0008
	Lab N (LN)	-	-	-	-
	Residue (R)	0.0086	-	-	-
	FN*LN	-	-	-	-
	FN*R	-	-	-	-
	LN*R	-	-	-	-
	FN*LN*R	-	-	-	-
NO_3^--N	Field N (FN)	-	-	-	-
	Lab N (LN)	<.0001	-	-	-
	Residue (R)	0.0299	0.0007	0.0012	0.0002
	FN*LN	0.0149	-	-	0.0008
	FN*R	-	-	-	-
	LN*R	-	-	-	-
	FN*LN*R	-	-	-	0.007

† Presented are p-values of treatment effects across each sampling time; day 37, day 110, day 204, and 362 day. Significant treatment effect at $p \leq 0.05$, (-) = not significant and (na)= not applicable.

Table 2.5. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N and residue and their interaction on cumulative extracellular enzyme activities (EEA) during a one-year soil incubation.

Treatment effects	BG	CBH	PO	PER	NAG	LAP	UREASE	PHOS
Field N (FN)	0.0474	-	-	-	0.0247	-	-	0.0327
Lab N (LN)	<.0001	0.0007	<.0001	0.0062	<.0001	-	-	-
Residue (R)	-	-	<.0001	0.0011	0.0067	-	-	-
FN*LN	<.0001	0.0254	<.0001	<.0001	-	-	-	-
FN*R	-	-	<.0001	<.0001	-	-	-	-
LN*R	-	-	-	-	-	-	-	-
FN*LN*R	-	-	-	-	-	-	-	-

Presented are significant p-values ($\alpha \leq 0.05$) (-)=not significant. Extracellular enzyme abbreviations are BG= β-1,4,-glucosidase, CBH= β -D-1,4-cellobiohydrolase, PO= phenol oxidase, PER= peroxidase, NAG= β -1,4,-N-acetyl glucosaminidase, LAP= Leucine-aminopeptidase and PHOS= acid phosphatase.

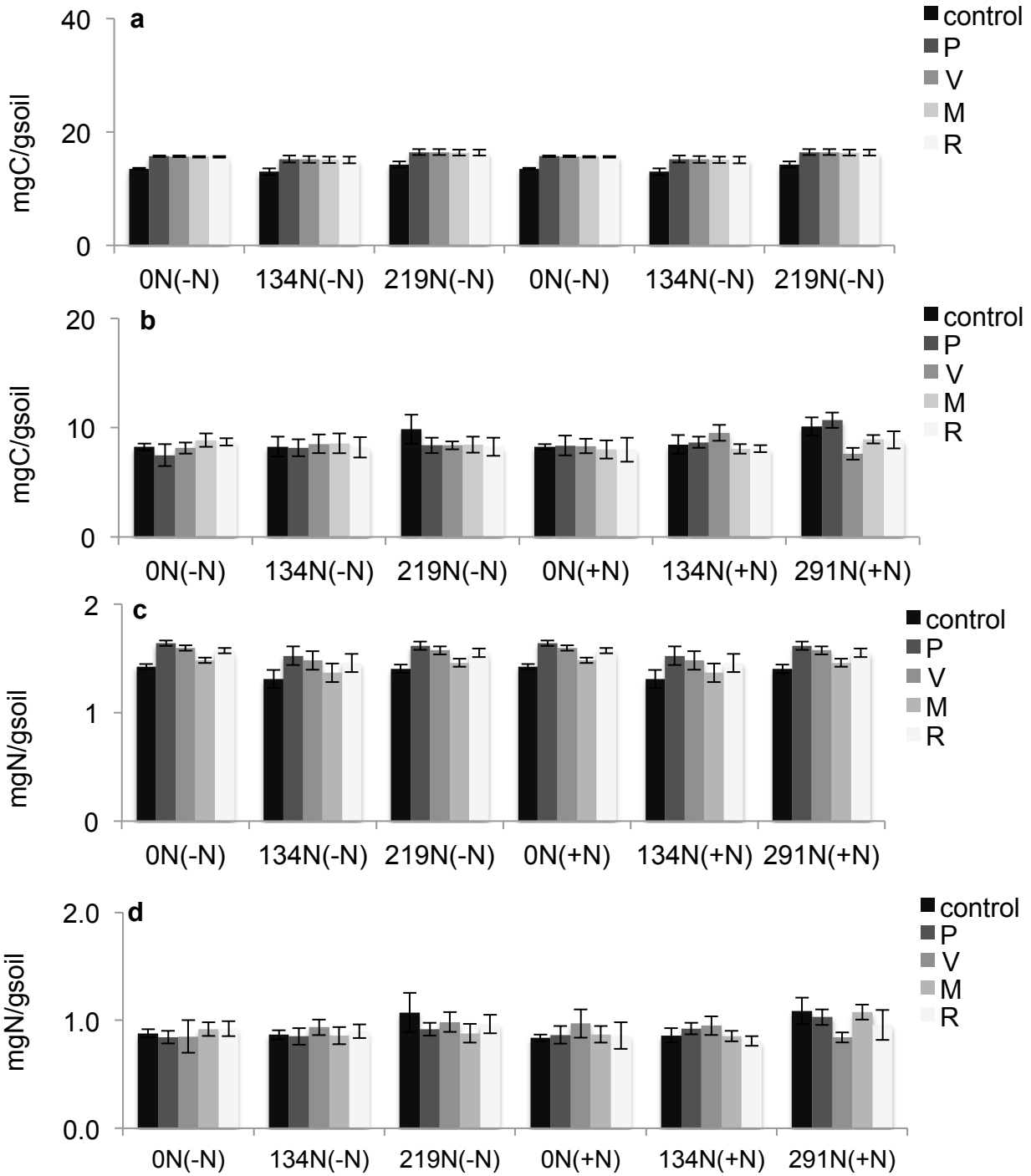

Figure 2.1. Total soil organic C (extant soil C plus added residue C) at day 1 (a), and day 362 (b); and total soil N (extant soil N plus added residue N) at day 1 (c) and day 362 (d) of soil mixed with cover crop residues (P=field pea, V=hairy vetch, M=Austrian pearl millet, R=annual ryegrass) that received long-tern N additions of 0, 134 and 291 N kg ha^{-1} y^{-1} for 12 years. Samples with short-term N addition are indicated with +N. Different letters indicate significant differences between the residue treatments. Bars represent means ± one standard error of the mean (n=4).

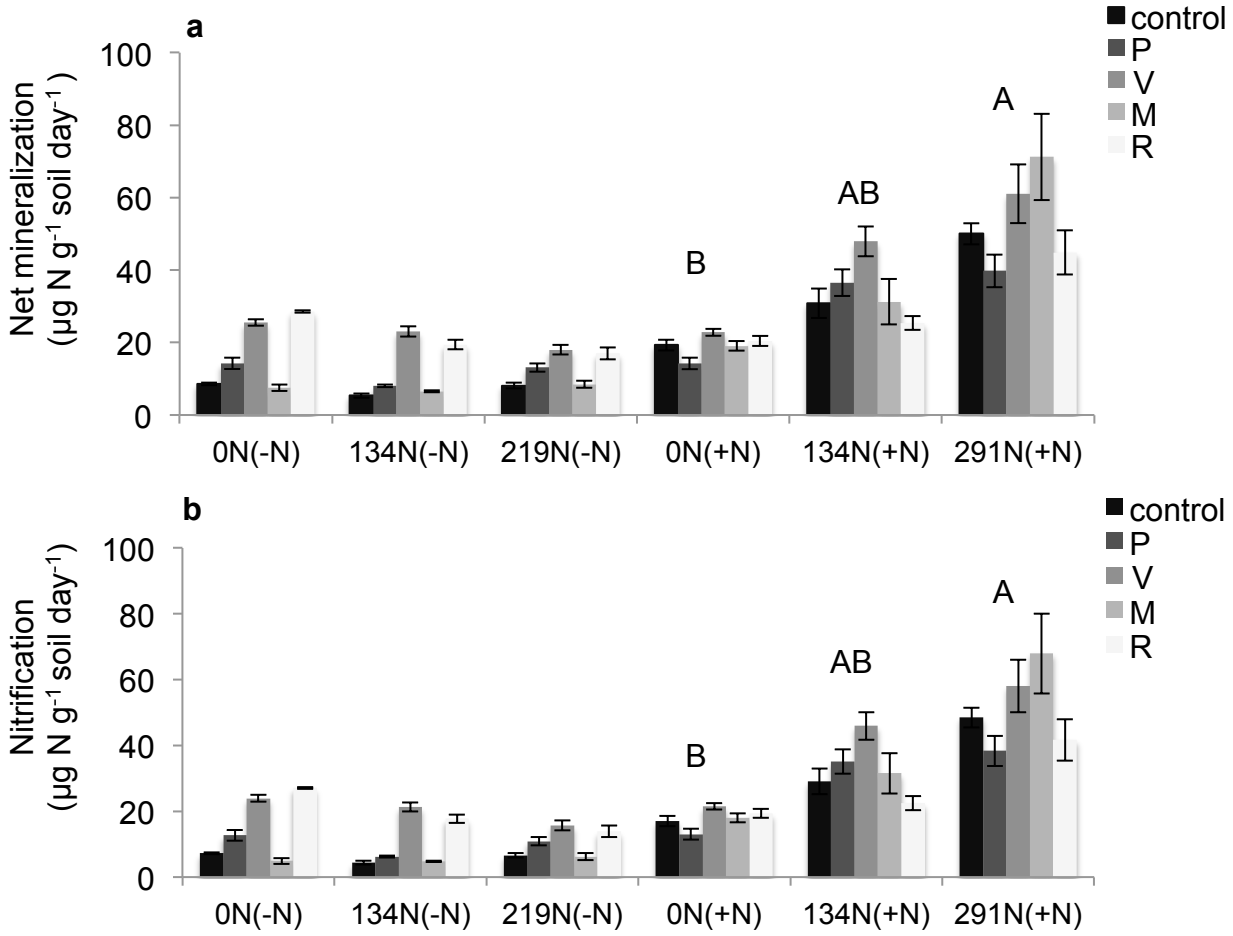

Figure 2.2. The net N mineralization (a) and nitrification (b), rates of soil mixed with individual cover crop residues (P=field pea, V=hairy vetch, M=Austrian pearl millet, R=annual ryegrass) that received long-tern N additions of 0, 134 and 291 N kg ha^{-1} y^{-1} for 12 years. Short-term N additions are indicated with +N. Different letters indicate significant differences between long-term (field) N treatment with or without short-term N addition. Bars represent means ± one standard error of the mean (n=4).

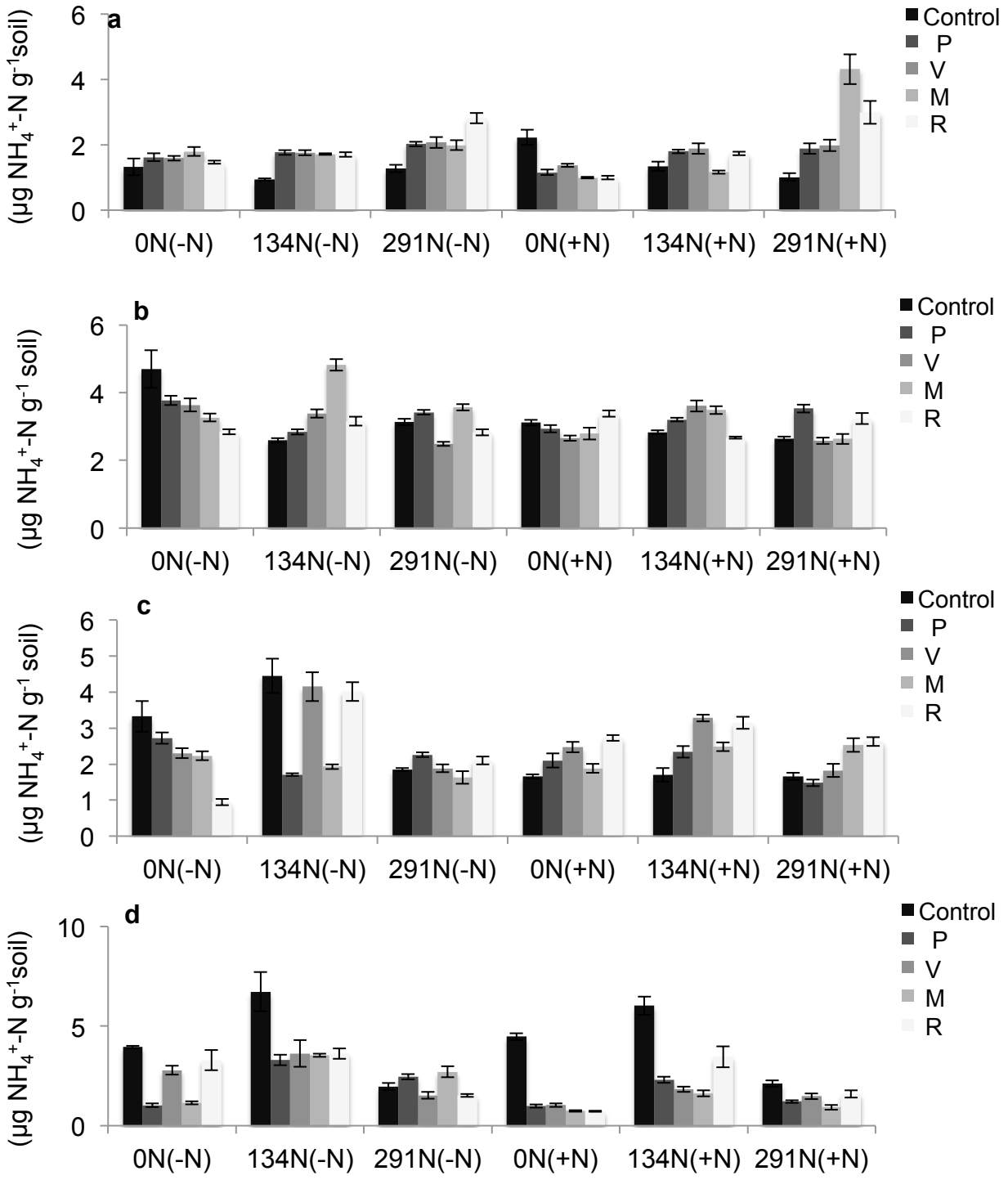

Figure 2.3. Soil ammonium (NH_4^+) measured at each soil sampling period (a) day 37 (b) day 110 (c) day 204 and (d) day 362, from sites receiving long-term N additions of 0, 134 or 291 kg N $ha^{-1}y^{-1}$ for 12 years with addition of cover crop residues (P=field pea, V=hairy vetch, M=Austrian pearl millet, R=annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

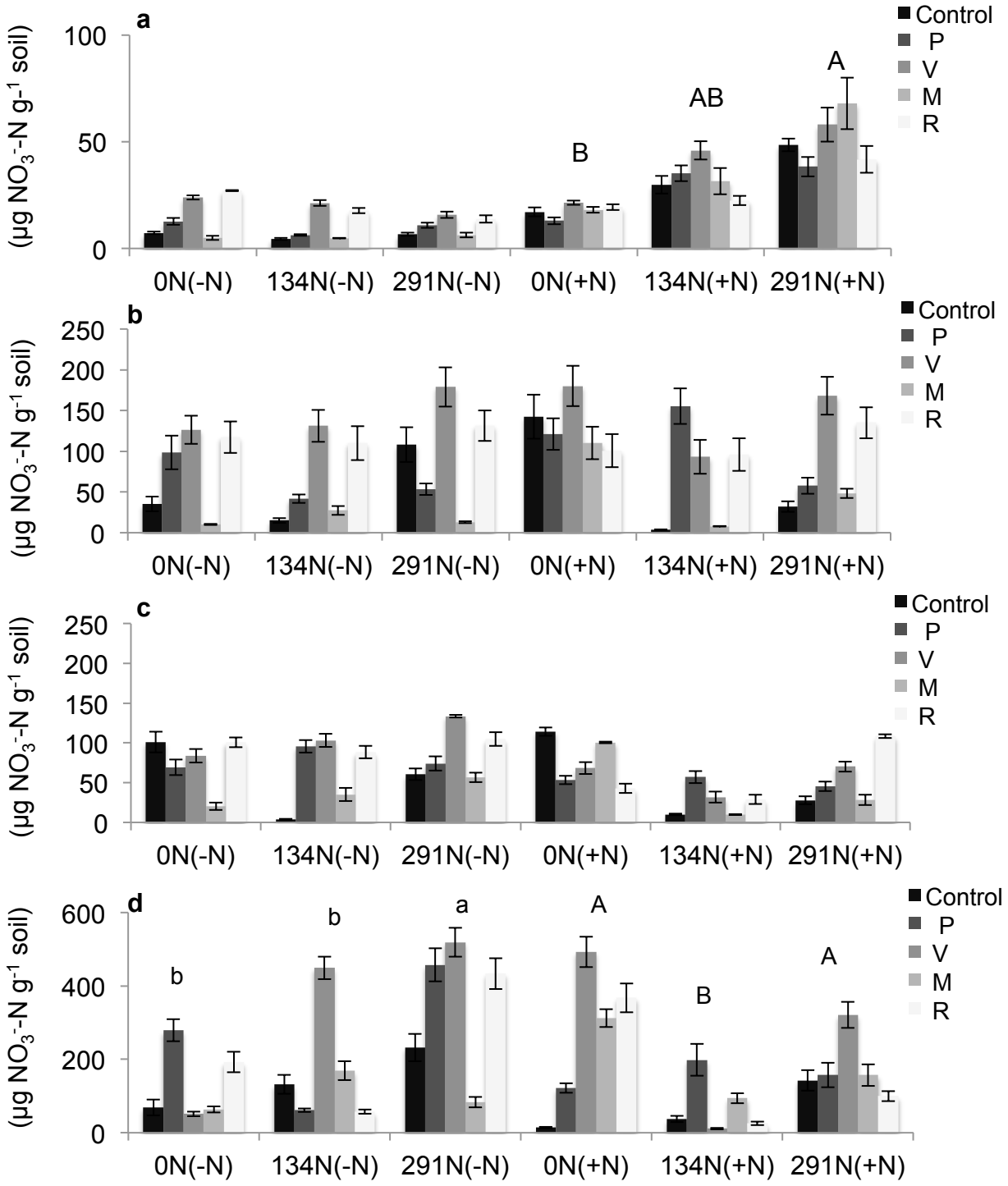

Figure 2.4. Soil nitrate (NO_3^-) at each soil sampling period (a) day 37 (b) day 110 (c) day 204 and (d) day 362, from sites receiving long-term N addition of 0, 134 and 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residues (P=field pea, V=hairy vetch, M=Austrian pearl millet, R=annual ryegrass). Different letters indicate significant differences between long-term (field) N treatment with or without short-term N addition. Bars represent means ± one standard error of the mean (n=4).

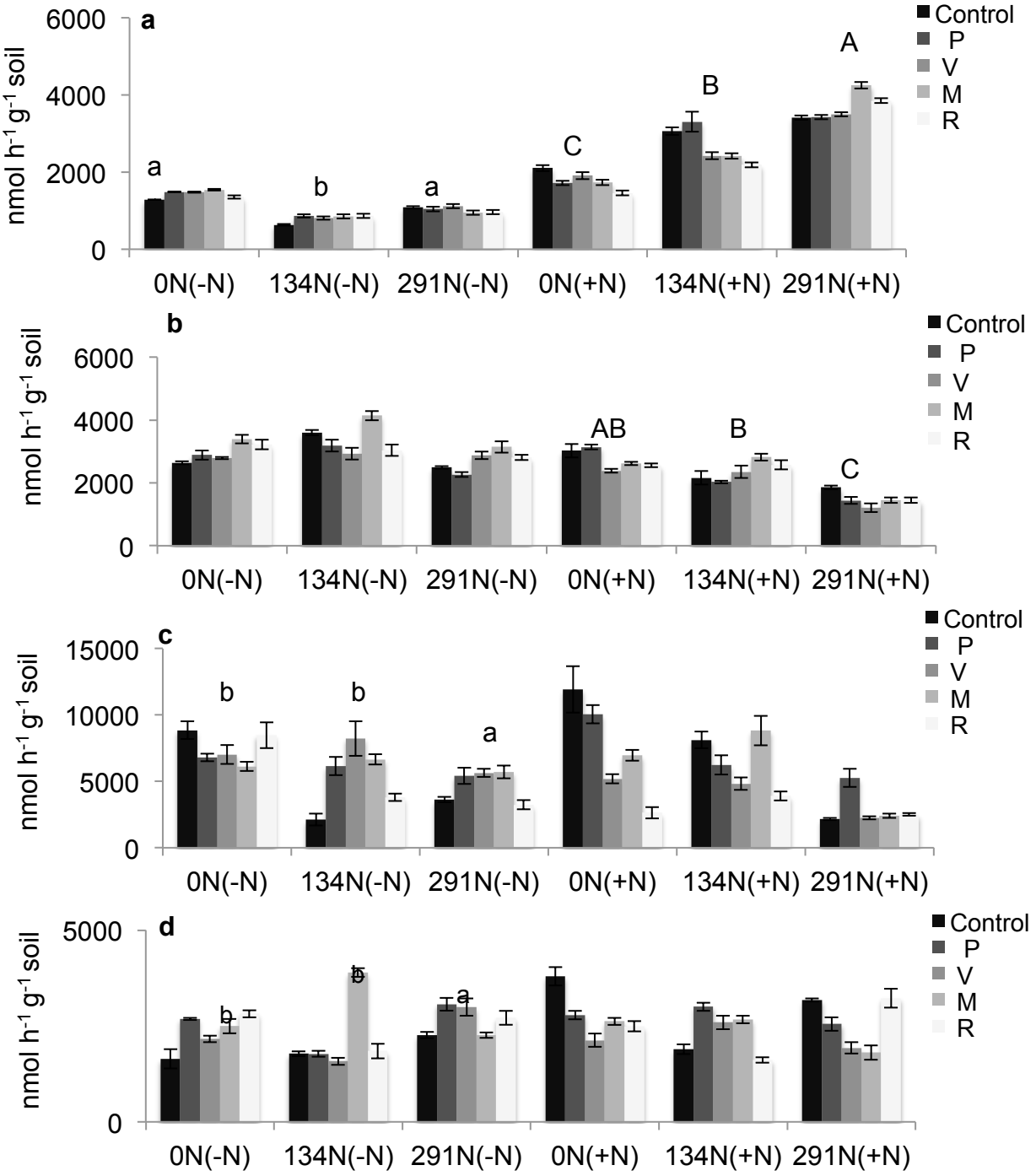

Figure 2.5. Cumulative extracellular enzyme activities (EEA): (a) β-1,4,-glucosidase (BG), (b) β -D-1,4-cellobiohydrolase (CBH), (c) phenol oxidase (PO) and (d) peroxidase (PER) during a 362-day laboratory incubation of soil from sites receiving long-term N addition of 0,134, and 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residues (P=field pea, V=hairy vetch, M=Austrian pearl millet, R=annual ryegrass). Different letters indicate significant differences between long-term (field) N treatment with or without short-term N addition. Samples with +N indicate short-term N addition. Bars represent means ± one standard error of the mean (n=4).

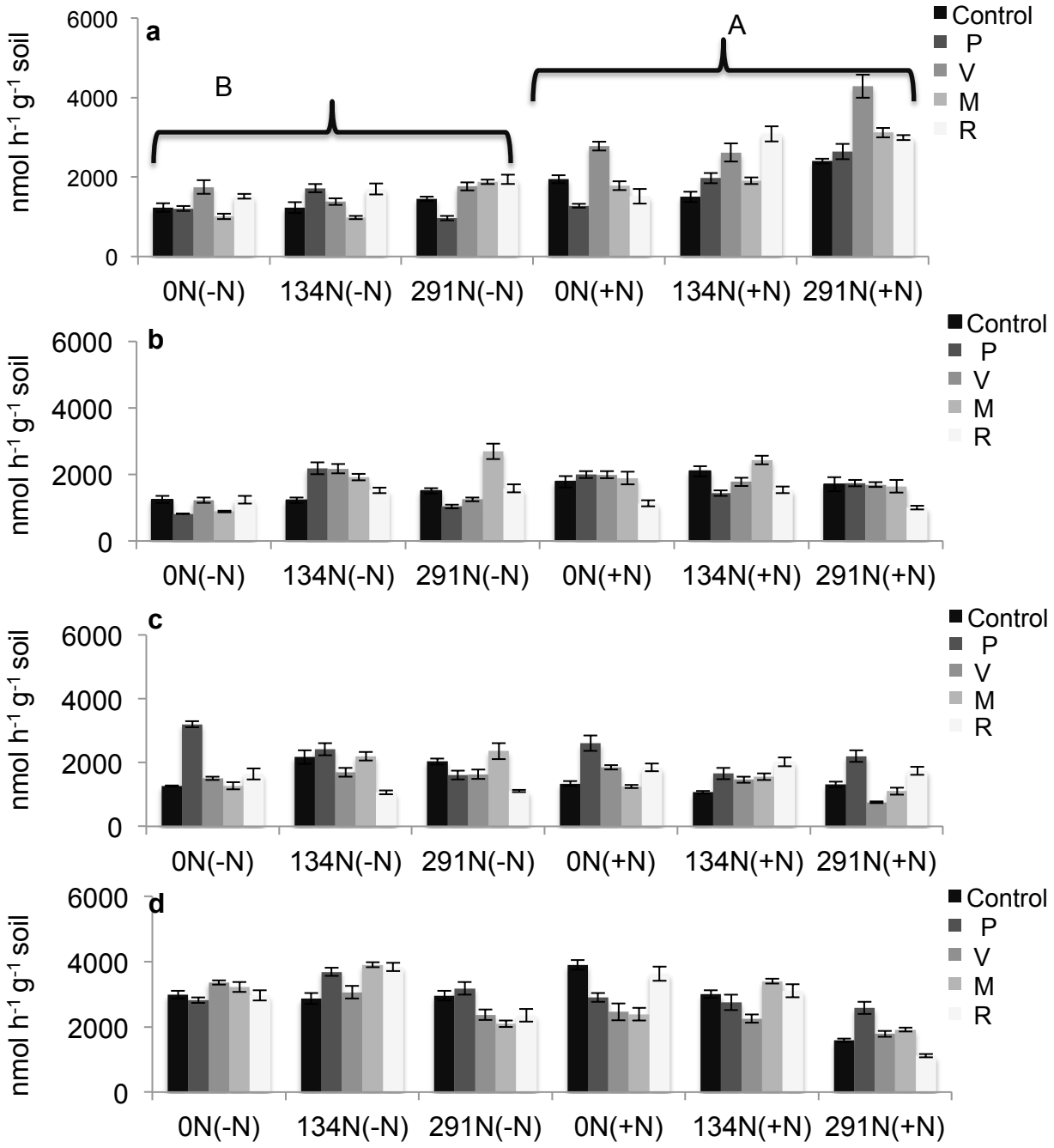

Figure 2.6. Cumulative extracellular enzyme activities (EEA): (a) β -1,4,-N-acetyl glucosaminidase (NAG), (b) Leucine-aminopeptidase (LAP), (c) Urease, (d) acid phosphatase (PHOS), during a 362-day laboratory incubation of soil from sites receiving long-term N addition of 0,134, and 291 kg N $ha^{-1}y^{-1}$ mixed with cover crop residues (P=field pea, V=hairy vetch, M=Austrian pearl millet, R=annual ryegrass). Different letters indicate significant differences between with or without short-term N addition treatment. Samples with +N indicate short-term lab N addition. Bars represent means ± one standard error of the mean (n=4).

LITERATURE CITED

LITERATURE CITED

Ajwa, H. A., Dell, C. J., & Rice, C. W. (1999). Changes in enzyme activities and microbial biomass of tall grass prairie soil as related to burning and nitrogen fertilization, 31, 769–777.

Alon, A., & Steinberger, Y. (1999). Effect of nitrogen amendments on microbial biomass, aboveground biomass and nematode population in the Negev Desert soil. Journal of Arid Environments 41: 429–441.

Bohme, L., Langer, U., & Bohme, F. (2005). Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. Agriculture, Ecosystems and Environment, 109(1–2), 141–152.

Bouwman, A. F., Boumans, L. J. M., & Batjes, N. H. (2002). Emissions of N2O and NO from fertilized fields: Summary of available measurement data. Global Biogeochemical Cycles, 16(4).

Bradley, K., Drijber, R. A., & Knops, J. (2006). Increased N availability in grassland soils modifies their microbial communities and decreases the abundance of arbuscular mycorrhizal fungi. Soil Biol Biochem 38:1583–1595

Butenschoen, O., Valentyna, K., Mark, M., Franca, M., Dorothee, S., & Stefan, S. (2014). Litter mixture effects on decomposition in tropical montane rainforests vary strongly with time and turn negative at later stages of decay. Soil Biology & Biochemistry 77:121e128

Carreiro, M. M., Sinsabaugh, R. L., Repert, D. A., & Parkhurst, D. F. (2000). Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81:2359–65

Chen, X., Wang, X., Liebman, M., Cavigelli, M., & Wander, M. (2014). Influence of residue and nitrogen fertilizer additions on carbon mineralization in soils with different texture and cropping histories. *PLoS ONE*, *9*(7), 1–11.

Chen, B., Liu, E., Tian, Q., Yan, C., & Zhang, Y. (2014). Soil nitrogen dynamics and crop residues. A review. Agronomy for Sustainable Development, 34(2), 429–442

Coq, S., Weigel, J., Butenschoen, O., Bonal, D., & Hättenschwiler, S. (2011). Litter composition rather than plant presence affects decomposition of tropical litter mixtures. Plant and Soil, 343(1–2), 273–286

Enrique, A. G., Bruno, C., Christopher, A., Virgile, C., & Steven, C. (2008). Effects of enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology, Ecological Society of America 81(9),pp. 2359–2365

Fontaine, S., Mariotti, A., & Abbadie, L. (2003). The priming effect of organic matter: a question of microbial competition? Soil Biology and Biochemistry, 35, 837–843.

Frey, S. D., Knorr, M., Parrent, J. L. & Simpson, R. T. (2004). Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. For. Ecol. Manage, 196, 159–171.

Geisseler, D., & Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms - A review. Soil Biology and Biochemistry, 75, 54–63.

Geisseler, D., Lazicki, P. A., & Scow, K. M. (2016). Mineral nitrogen input decreases microbial biomass in soils under grasslands but not annual crops. Applied Soil Ecology, 106, 1–10.

Grandy, A. S., Salam, D. S., Wickings, K., McDaniel, M. D., Culman, S. W., & Snapp, S. S. (2013). Soil respiration and litter decomposition responses to nitrogen fertilization rate in no-till corn systems. Agriculture, Ecosystems and Environment, 179, 35–40.

Grandy, A. S. & Neff, J. C. (2008). Molecular C dynamics downstream: the biochemical decomposition sequence and its impact on soil organic matter structure and function. Sci. Total Environ. 404, 297–307.

Hallin, S., Jones, C.M., Schloter, M., & Philippot, L. (2009). Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. The ISME Journal 3, 597e605.

Hobbie, S. E. (2005). Contrasting effects of substrate and fertilizer nitrogen on the early stages of litter decomposition. Ecosystems, 8(6), 644–656.

Hobbie, S. E. (2008). Nitrogen effects on decomposition: A five-year experiment in eight temperate sites. Ecology, 89(9), 2633–2644.

Hu, S., Grunwald, N. J., Van Bruggen, A. H. C., Gamble, G. R., Drinkwater, L. E., Shennan, C., & Demment, M. W. (1997). Short-Term Effects of Cover Crop Incorporation on Soil Carbon Pools and Nitrogen Availability. Soil Science Society of America Journal, 61(3), 901–911.

Keeler, B. L., Hobbie, S. E., & Kellogg, L. E. (2009). Effects of long-term nitrogen addition on microbial enzyme activity in eight forested and grassland sites: implications for litter and soil organic matter decomposition. Ecosystems, 12(1), 1–15.

Knorr, M., Frey, S. D., Curtis, P. S. (2005). Nitrogen Additions and Litter Decomposition: A Meta-Analysis. Ecology, 86(12), 3252–3257.

Lawson, A., Fortuna, A. M., Cogger, C., Bary, A., & Stubbs, T. (2013). Nitrogen contribution of rye-hairy vetch cover crop mixtures to organically grown sweet corn. *Renewable Agriculture and Food Systems*, *28*(1), 59–69.

Li, L. J., Han, X. Z., You, M. Y., Yuan, Y. R., Ding, X. L., & Qiao, Y. F. (2013). Carbon and nitrogen mineralization patterns of two contrasting crop residues in a Mollisol: Effects of residue type and placement in soils. *European Journal of Soil Biology*, *54*, 1–6.

Lupwayi, N. Z., Lafond, G. P., Ziadi, N., & Grant, C. A. (2011). Soil microbial response to nitrogen fertilizer and tillage in barley and corn. Soil and Tillage Research, 118, 139–146.

McDaniel, M. D., Grandy, A. S., Tiemann, L. K., & Weintraub, M. N. (2016). Eleven years of crop diversification alters decomposition dynamics of litter mixtures incubated with soil. Ecosphere, 7(8).

Nair, A., & Ngouajio, M. (2012). Soil microbial biomass, functional microbial diversity, and nematode community structure as affected by cover crops and compost in an organic vegetable production system. Applied Soil Ecology, 58, 45–55.

O'Connell, S., Shi, W., Grossman, J. M., Hoyt, G. D., Fager, K. L., & Creamer, N. G. (2015). Short-term nitrogen mineralization from warm-season cover crops in organic farming systems. Plant and Soil, 396(1–2), 353–367.

Prescott, C. E. (1995). Does nitrogen availability control rates of litter decomposition in forests? Plant Soil 168--169:83—88

Ramirez, K. S., Craine, J. M., & Fierer, N. (2012). Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. Global Change Biology, 18(6), 1918–1927.

Ramirez, K. S., Lauber, C. L., Knight, R., Bradford, M. A., & Fierer, N. (2010). Consistent effects of nitrogen fertilization on soil bacterial communities in contrasting systems. Ecology 91, 3463e3470

Sainju, U. M., Singh, B. P., & Whitehead, W. F. (2000). Cover crops and nitrogen fertilization effects on soil carbon and nitrogen and tomato yield. Can. J. Soil Sci. 80:523–532.

Saiya-Cork, K., Sinsabaugh, R., & Zak, D. (2002). The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biology and Biochemistry, 34(9), 1309–1315.

Sinsabaugh, R.L., Reynolds, H., Long, T.M. (2000.) Rapid assay for amidohydrolase (urease) activity in environmental samples. Soil Biology & Biochemistry 32, 2095e2097.

Sinsabaugh, R. L., Carreiro, M. M., & Repert, D. A. (2002). Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. 1–24.

Stark, C., Condron, L. M., Stewart, A., Di, H. J., & O'Callaghan, M. (2007). Influence of organic and mineral amendments on microbial soil properties and processes. Applied Soil Ecology, 35(1), 79-93. Soil Biology and Biochemistry, 40(6), 1352-1363.

Tiemann, L. K., & Billings, S. A. (2011). Changes in variability of soil moisture alter microbial community C and N resource use. Soil Biology and Biochemistry, 43(9), 1837–1847.

Treseder, K. K. (2008). Nitrogen additions and microbial biomass: A meta-analysis of ecosystem studies. Ecology Letters, 11(10), 1111–1120.

Venter, Z. S., Jacobs, K., & Hawkins, H. J. (2016). The impact of crop rotation on soil microbial diversity: A meta-analysis. Pedobiologia, 59(4), 215–223.

Waldrop, M. P., Zak, D. R., & Sinsabaugh, R. L. (2004). Microbial community response to nitrogen deposition in northern forest ecosystems. Soil Biology and Biochemistry, 36(9), 1443–1451.

Wang, J., Bu, W., Zhao, B., Zhao, X., Zhang, C., Fan, J., & Gadow, K. V. (2015). Effects of nitrogen addition on leaf decomposition of single-species and litter mixture in Pinus tabulaeformis forests. Forests, 6(12), 4462–4476.

Whittinghill, K. A., Currie, W.S., Zak, D. R., Burton, A. J., & Pregitzer, K. S. (2012). Anthropogenic N deposition increases soil C storage by decreasing the extent of litter decay: analysis of field observations with an ecosystem model. Ecosystems 15, 450–461.

Yevdokimov, I., Gattinger, A., Buegger, F., Munch, J. C., & Schloter, M. (2008). Changes in microbial community structure in soil as a result of different amounts of nitrogen fertilization. Biology and Fertility of Soils, 44(8), 1103–1106.

Zak, D. R., William, E. H., Matthew, J. T., Kurt, S. P., & Andrew, J. B. (2006). Microbial cycling of C and N in northern hardwood forests receiving chronic atmospheric NO_3 deposition. Ecosystems 9: 242–253.

Zhang, N., Shiqiang, W., Linghao, L., Jie, B., Mingming, Z., & Keping, M. (2008).

Impacts of urea N addition on soil microbial community in a semi-arid temperate steppe in northern China. Plant Soil 311:19–28.

Zhang, W., Chao, L., Yang, Q., Wang, Q., Fang, Y., & Wang, S. (2016). Litter quality mediated nitrogen effect on plant litter decomposition regardless of soil fauna presence. Ecology, 97(10), 2834–2843.

Zhong, W., Gu, T., Wang, W., Zhang, B., Lin, X., Huang, Q., & Shen, W. (2010). The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant and Soil, 326(1), 511–522.

Zhou, X., Chen, C., Wu, H., & Xu, Z. (2012). Dynamics of soil extractable carbon and nitrogen under different cover crop residues. J Soils Sediments 12:844–853

Zhu, X., Chen, H., Zhang, W., Huang, J., Fu, S., Liu, Z., & Mo, J. (2016). Effects of nitrogen addition on litter decomposition and nutrient release in two tropical plantations with N2-fixing vs. non-N2-fixing tree species. Plant and Soil, 399(1–2), 61–74.

CHAPTER THREE: HOW DOES NITROGEN ADDITION REGULATE SOIL MICROBIAL ACTIVITIES RELATED TO DECOMPOSITION RATE OF RESIDUE MIXTURES AND N MINERALIZATION?

ABSTRACT

The application of N fertilizer to agricultural systems has greatly increased over the past decades. It remains unclear how the addition of N fertilizers influences soil microbial activities and the soil processes they mediate. In a year-long laboratory incubation, I examined how long-term (12 years field N treatment) and short-term (N addition in the lab) influence soil microbial activities related to residue decomposition and plant available N. Three residue mixtures *Pisum sativum (*field pea) + *Pennisetum glaucum* (pearl millet) (PM), *Vicia villosa* (hairy-vetch) + *Lolium multiflorum* (ryegrass) (VR) and field pea + pearl millet+ hairy vetch + ryegrass (PMVR) were incubated for 362 days with soil from a 12-year field N experiment site involving a no-till rotational sequence of corn-soy-wheat. Soils were collected during the corn phase from three of the nine N addition rate 0, 134, or 291 kg N ha^{-1} y^{-1} in the F1, F5 and F9 plots respectively. In the lab, soils were either amended with or without N fertilizer at the rate of 0.013 g urea N per 100 g of dry soil. I found that residue mixtures had non-additive synergistic (NAS) effect on inorganic N at the initial (day 37) and final stage (day 362) of the incubation period. There was non-additive antagonistic (NAA) effect of residue mixtures on cellulase, total oxidase, β -1,4,-N-acetyl glucosaminidase (NAG) and NAS effect on Leucine-aminopeptidase (LAP), urease and acid phosphatase (PHOS) enzyme activities. The addition of residue mixtures likely stimulated microbial growth, but the NAA effect on C acquisition enzymes suggests C limitation. Both long- and short-term N additions had NAS effects on inorganic N and enzyme activities while short-term N had NAA effect on total oxidase but did not necessarily reduce the decomposition rate.

3.1 Introduction

Decomposition is a complex and important process in nutrient cycling. Decomposition of residues has been shown to regulate soil organic matter (S0M) formation and mineralization of nutrients for both plants and microorganisms (Wang et al., 2015). Since plant productivity largely depends on nutrient cycling, it is crucial we understand how residues influence soil microbial activities related to decomposition and N mineralization. Studies have shown that individual crop residues can differ in chemical composition (Bending et al., 2002; Gartner and Cardon, 2004). Therefore it is expected that mixtures of different crop residues would have more chemical complexity (Wickings et al., 2011). Residue characteristics have been reported as a major factor that controls the rate of decomposition (Aerts, 1997). Mixtures of residue may decompose differently from the individual residue types that make up the mixture due to the interaction between residues and chemical diversity of the mixtures. It has been observed that the decomposition rates of residue mixtures can differ from the expected rate based on the decomposition of individual residues (Wardle et al., 1997; Hoorens et al., 2003; Gartner and Cardon, 2004; Bonanomi et al., 2010). These non-additive effects on plant residue decomposition can be either non-additive synergistic (NAS, positive) or non-additive antagonistic (NAA, negative) (Hooren et al., 2003; Gartner and Cardon, 2004; Meier & Bowman, 2010). While the NAS mixture effect on decomposition has been reported to occur more frequently (>65%) (Gartner and Cardon, 2004; Liu et al., 2007; Meier and Bowman 2010; Cardinale et al., 2012) both have been observed (Butenschoen et al., 2014).

Several factors have been proposed to explain the non-additive mixture effects

on residue decomposition. Differences in nutrient concentrations of the residues in a mixture can influence decomposition (Wardle et al., 1997; Hättenschwiler and Vitousek, 2000; Makkonen et al., 2013). For example, a high nutrient content in one residue type can aid in the decomposition of a different, low nutrient residue in the mixture. Nutrients can be transferred from one residue type to another through the hyphae of fungi that connect the two residue types (Hooren et al., 2003; Gartner and Cardon, 2004; Bonanomi et al., 2014). In addition, a negative interaction may occur if one residue type contains secondary compounds. For example, phenolic secondary compound may complex with proteins that inhibit microbial growth and activity and consequently slow down decomposition rates of the entire residue mixture (Hättenschwiler and Vitousek 2000). In addition, mixing of residues with different chemical composition may alter the chemical environment where decomposition occurs thus influencing decomposition rate (Wardle, 2002; Gartner and Cardon, 2004). Studies on the decomposition rate of multi-species residue mixtures have been conducted largely in natural systems (Hobbie, 1996; Aerts, 1997; Hector et al., 2000; Hooren et al., 2003; Meier & Bowman, 2010; Zhang et al., 2016), but very few studies have been done in agroecosystems (McDaniel et al., 2016).

One of the important factors regulating residue decomposition is nutrient availability, especially N availability. For example, the addition of N could affect nutrient cycling and rates of residue decomposition by influencing microbial processes such as N mineralization and extracellular enzyme production (Hobbie, 2008; Tiemann and Billings, 2011; Zhou et al., 2012). Results on the effects of N addition on residue decomposition rates have been varied, apparently because the circumstances under

study are variable. In long-term studies N additions can change plant community composition (natural systems) and/or productivity (managed systems) such that the quantity and/or quality of organic material inputs in the form of aboveground residues, decaying roots and exudates is increased (Clark et al., 2007; Meier and Bowman, 2008; Tiemann and Billings, 2011; Geisseler and Scow, 2014). Long-term repeated N additions have been shown to result in changes in the soil microbial community and activities that mediate residue decomposition (Bohme et al 2005; Keeler et al., 2009; Zhong et al., 2010; Geisseler and Scow, 2014; Geisseler et al., 2016). For example, N addition in grassland systems increases primary productivity and changes plant community composition, which leads to an increase in cellulose degradation enzyme activity, and does not appear to reduce lignin degradation (Tiemann and Billings, 2011; Zeglin et al., 2007). In a meta-analysis from 64 long-term trials, Geisseler and Scow (2014) reported that N additions led to 15.1% increase in microbial biomass relative to control (unfertilized). These results suggest that the availability of N could alter soil microbial communities that mediate residue decomposition. Residue decomposition in agroecosystems with long-term N fertilizer follow the patterns seen in grassland systems, where even though the plant community doesn't change, the increase in productivity with N addition stimulates microbial biomass and activity (Geisseler and Scow, 2014).

Studies have also shown that microbial communities are affected directly by N addition in the short-term. For example, in a short-term N addition study (one-year soil incubation), Ramirez et al. (2012) showed that N addition reduced soil microbial biomass and respiration rates and altered community structure when isolated from

effects of C (i.e no plant residue) inputs. In another short-term N addition study, Stark et al. (2007) reported no change in microbial biomass or microbial activity, but changes in microbial community composition following urea N additions in a 91-day soil incubation period. These results suggest that N additions can have both immediate and long lasting effects on microbial community structure, but the direction and magnitude of changes in decomposition due to these changes in community structure are still relatively unknown.

As agricultural systems move towards increased diversity through addition of cover crops, it is uncertain how residue mixtures will influence decomposition and N availability. Another aspect that is unclear is if fertilizer N additions will impact decomposition of these diverse residue mixtures, particularly if they are N-rich residues (i.e. legumes and young grasses with low C:N ratios). In order to better understand how residue diversity affects the way microbes decompose residues and release plant available N, the objective of this study were to: 1) determine residue mixture effects on soil microbial activities related to decomposition and N mineralization; 2) investigate the effects of N addition on these residue mixture effects. I hypothesized that there would be a non-additive synergistic (NAS) mixture effect on decomposition and N mineralization and that NAS effect would be more pronounced with greater residue diversity. Because the residues used in this experiment all had relatively low C: N ratios, I further hypothesized that long-term N addition would reduce or eliminate synergistic effects on decomposition and N mineralization. In soils under both long- and short-term N addition, I hypothesized a similar reduction or elimination of NAS effects. However, in soils without the long-term N addition I expected short-term N addition would lead to

non-additive antagonistic (NAA) mixture effects on decomposition and N mineralization because microbes without the long-term N addition soils are not well adapted to cope with short-term N addition (Stark et al., 2007; Geisseler and Scow, 2014).

3.2 Material and Methods

3.2.1 Study Site

Soils for this research were collected from the Resource Gradient Experiment established in May 2005 at the Michigan State University W.K Kellogg Biological Station (KBS) Long-term Ecological Research (LTER) site. The mean annual rainfall and temperature are 890 mm and 9.7^{o}C respectively (KBS, 2015). The soils are classified as Kalamazoo (fine-loamy) and Oshtemo (coarse- loamy) mixed, mesic, Typic Hapludalfs (Alfisols) that developed on glacial outwash. The experiment is set-up in a corn-soy-wheat rotation that during the corn and wheat phases receives nine different N fertilization rate ranging from 0-291 kg N ha^{-1} y^{-1} (corn) and 0-180 kg N ha^{-1} y^{-1} (wheat). The experimental plots, which are 5 m x 30 m, are randomized across two sets of four blocks, with one set of blocks irrigated and the other not. A detailed description of the experiment can be found at www.lter.kbs.msu.edu.

3.2.2 Laboratory Soil Incubation Experimental Design

Treatments in this study were comprised of 3 CC residues mixtures (field pea+ millet, annual rye+ hairy vetch & all residue type), 3 field N rates (0, 134, & 291 N kg ha^{-1} y^{-1}) and 2 laboratory N addition levels (Yes or No). Soils for the laboratory incubation were collected in December 2015, after the soy phase of the rotation so that no N

fertilizer was applied to the site in 2015. Soils were collected from three of the nine-corn N addition rate (F1=0, F5=134, and F9=291N kg ha^{-1} y^{-1}) from the four irrigated block, while during the wheat phase N addition on these plots were F1=0, F5=60, and F9= 180N kg ha^{-1} y_{-1}). I collected soils from irrigated plots in order to reduce the effects of moisture variability, which is prevalent without irrigation. Five 2.5 cm (diameter) soil cores were randomly collected (0-15 cm depth) within each of the four blocks. The soils were arranged in coolers and transported to the laboratory at Michigan State University. Fresh soils were passed through 8mm mesh sieve, and a sub-sample was dried at 60 ^{o}C to determine gravimetric soil moisture. Using dried soils; I determined water-holding capacity (WHC) by saturating 5 g dry soil placed in Whatman #4 filter paper fitted into funnels. These were covered to prevent evaporative loss and allowed to drain overnight (Fierer and Schimel, 2002), to allow determination of 50 % WHC for the incubation study described below.

The amount of water added to maintain the soil at 50 % WHC was estimated from the gravimetric moisture content of the soil without residue and moisture content at 50 % WHC for each treatment. Cover crop residues were collected from farmer's field in late October, oven dried and then chopped to 2 mm size pieces. Four different CC residues were used including two grasses, ryegrass (R), pearl millet (M), and two legumes hairy vetch (V), and field pea (P). There were three CC residue mixtures, P+M, V+R, PMVR, which were made using equal weights of each residue (e.g in a four species mix, each species represent 25 % of the total 0.5 g added to 100 g dry soil). Duplicate sets of soils mixed with residues were placed in 10 cm long by 6.35 cm wide PVC cores, sealed at the bottom with course filter paper. These were then placed on

top of a layer of marbles in 950 ml Mason jars. A thin layer of water was added to the bottom of the jars to help maintain soil moisture. Soils were incubated at 22^{o}C with loosely placed lids on the jars that were removed weekly to allow for gas exchange.

Soils mixed with residues were placed in 10 cm long by 6.35 cm wide PVC cores, sealed at the bottom with course filter paper. These were then placed on top of a layer of marble in 950 ml Mason jars. A thin layer of water was added to the bottom of the jars to help maintain soil moisture. Soils were incubated at 22^{o}C with loosely placed lids on the jars that were frequently removed to allow for gas exchange. Two sets of the total soil samples were prepared, such that to one set, I added 0.013 g N (urea) per 100 g of dry soil to observe the impact of short-term N additions. Approximately 10g of soil were sub-sampled from each jar using a spatula on days 37, 110, 204, and 362 of the incubation.

3.2.3 Soil total organic C and Total Nitrogen

Total organic C (TOC) and total nitrogen (TN) were measured on day 1 after the addition of residue treatments and at the end of the incubation period (day 362). Subsamples were oven dried, sieved through a 2 mm mesh and finely grounded prior to analysis on an elemental analyzer (Costech ECS 4010; Costech Analytical Technologies Inc., Valencia, CA, USA).

3.2.4 Extracellular enzyme activity and inorganic nitrogen

Soil extracellular enzyme activities (EEA) and inorganic N were measured at each point of subsampling throughout the incubation period. Soil extracts for inorganic

N analysis were obtained by shaking 8 g of fresh soil with 40 ml of 0.5 M K_2SO_4 on orbital shaker for 1 hr. The resulting slurries were filtered with Whatman #1 filter paper and stored at 4 °C prior to analysis. The soils were assayed for eight enzymes involved in decomposition and nutrient cycling as described by Saiya-Cork et al., (2002) with slight adjustment. Five hydrolytic enzymes including β-D-1, 4-cellobiohydrolase (CBH), β-1,4,-N acetylglucosaminidase (NAG), β-1,4-glucosidase (BG), acid phosphatase (PHOS), Leucine aminopeptidase (LAP), two oxidative enzymes, phenol oxidase (PO) and peroxidase (PER), and urease enzyme were measured. Soil slurries were made with 1 g of soil frozen at -20 °C and 125 ml of a 50 mM acetate buffer adjusted to a pH of 6.5. The mixture was homogenized for 30 seconds with an immersion blender. The soil slurries were continuously stirred with a magnetic stir bar while 200 µl aliquots were dispensed into 96-well microplates using a multi-channel pipette. All assays, except PO, PER, and urease, used fluorescently labeled substrates, with the fluorescent molecule, 4-methylumbelliferone or 7-amino-4-methylcoumarin, fluorescing when cleaved from the target substrate by the enzyme of interest. The oxidative enzymes, PO and PER, received 50 µl of 25 mM L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate, with PO assays additionally receiving 10µl of 0.3% H_2O_2. Urease plates received 10ul of 20mM urea as substrate. I incubated soil slurries with the labeled substrates at 22^0C for 18 hrs. After the incubation, hydrolytic enzyme plates received 10 µl of 0.5 M NaOH to increase pH to a level optimal for MUB and MC fluorescence before plates were read on a Synergy HT1 plate reader (BioTek Instruments, Inc., Winooski, VT USA) at 355 nm excitation and 450 nm emission wavelengths. Color change in the L-DOPA substrate for PO and PER plates was assessed spectrophotometrically at 460 nm.

Determination of ammonium concentrations after addition of urea was used to assess urease activity and soil extractable ammonium was done using the same colorimetric method (Sinsabaugh et al., 2000). Each well in a 96-well plate containing either soil slurry (for urease) or soil extract received 40 µl of both ammonia salicylate and ammonia cyanurate solutions prepared per manufacturer's instructions (Hach, Loveland, CO, USA). Color change related to ammonium concentration was determined at 610 nm on the Synergy HT1 plate reader. To determine concentration of nitrate in soil extracts, I used a 96-well plate colorimetric assay based on Doane and Horwath (2003) with one change. Instead of using a chemical reduction agent to convert nitrate to nitrite, I used nitrate reductase (AtNaR2; Nitrate reductase from Arabidopsis thaliana, EC 1.7.1.1; NECi, Lake Linden, MI, USA). Color change related to final nitrite concentrations was determined spectrophotometrically on the Synergy HT1 plate reader at an absorbance wavelength of 540 nm.

3.2.5 Mixture Effect index (MEI)

To determine the effect of residue mixtures on the soil inorganic N and extracellular enzyme activities, I used an index called mixture effect index (MEI). The MEI was calculated as reported by (Meier and Bowman, 2010; McDaniel et al., 2016)

$$MEI = \frac{V_{obs} - V_{exp}}{V_{exp}}$$

Where V_{obs} is the observed response of the mixed residues and V_{exp} is the expected response of the mixed residues that is: the average response of each individual residue within the mixture, see chapter 2. A positive MEI value represents a non-additive synergistic (NAS) mixture effect, a negative MEI value represents a non-additive

antagonistic (NAA) mixture effect and a near zero value represent additive effect. To assess mixture effects on microbial activities, I measured the response of each soil extracellular enzyme activities (EEA) to both single residue (chapter 2) and mixed residue additions on multiple sampling dates and then calculated the cumulative EEA of each enzyme for the entire 362-day incubation: $EEA_{cum} = (EEA_{hourly}) * 24 * (T_{1days})$. The cumulative EEA response to single residues and mixed residue additions were used to determine the MEI, where the average response of each individual residue within the mixture = V_{exp} and the observed response of mixed residue = V_{obs}. The cellulase degrading enzymes - β-1,4,-glucosidase (BG) and β -D-1,4-cellobiohydrolase (CBH) were reported as cellulase and the oxidase enzymes - phenol (PO) and peroxidase (PER) were summed up as total oxidase (TOX) because enzymes within each group show similar response to residue mixture effect at different sampling dates.

3.2.6 Statistical analysis

Statistical analyses were accomplished using SAS (SAS Institute, Cary, SC). PROC GLIMMIX three-way ANOVA was used to analyze the main effects and interactions of long-term (field) N addition, short-term (laboratory) N applications and residue addition treatments on soil microbial functions, residue decomposition rates and plant N availability. Normality of residuals was checked using normal probability and boxplot. Any variables that were non-normal or showed low heterogeneity were appropriately transformed (square root transformation) to meet variance analysis assumptions. Homogeneity of variances was checked using Levene's test. Where interactions were not significant, pairwise comparisons were done within significant

main effects using LS means tables. When interactions were significant, we used the slice function within SAS to compare difference among treatments. The results are reported statistically significant at α= 0.05.

3.3 Results

Prior to residue additions, there were no differences in SOC or TN due to long-term N addition (Table 2.2). Although I added the same amount of residue per gram of soil (5 mg dry weight) for each of the residue mixtures, residue C:N ratios varied (Table 2.1) so that after residue additions (day 1), total soil organic C ranged from 13.9 - 17.7 mg C g^{-1} soil and total soil N (TN) ranged from 1.1 - 1.7 mg N g^{-1} soil. There was a significant effect of long-term N at day 1 after the addition of residue mixtures (Table 3.1) such that soil that received 291N showed greater total SOC while 134N had lower TN. As with single residue additions (Chapter 2), after 362 days there were no differences in total SOC or TN between residue addition and non-residue addition (control) soils (Table 3.1; Fig. 3.1). Further, neither long- and short-term N additions had any effect on the remaining SOC and TN at the end of the incubation under any of the different residue treatments (Table 3.1; Fig 3.1).

3.3.1 Residue Mixture Effects

To assess residue mixture effects on extracellular enzyme activities and soil inorganic N, I used the mixture effect index (MEI). I found no clear trends in non-additive effects of residue mixtures on EEA (Figs. 3.2 and 3.3). When looking solely at data without N addition (no short- or long-term N), I found NAA (MEI < 0) residue effects on cellulase activities (Fig. 3.2 a) and NAS (MEI > 0) effects on PHOS, LAP and UR (Fig.

3.2c & Figs. 3.3b & c respectively). Mixture effects on oxidase were determined by residue type; PM showed NAS effects while VR and PMVR showed NAA effects on total oxidase (TOX) (Fig. 3.2 b).

Short-term N additions had NAS effects on cellulase, PHOS (Figs. 3.2a & c), LAP and UR (Figs. 3.3b & c) activities and NAA effects on TOX activities (Fig. 3.2 b). Long-term N additions had NAS effects on TOX and PHOS (Fig. 3.2 b & c) activities and NAA effects on cellulase (Fig. 3.2 a), NAG & LAP activities (Figs. 3.3a & b). The widest range of MEI values was found in TOX activities, which ranged from a NAS effect of 1.62 in soil without short-term N addition to a NAA effect of -0.71 in soil with short-term N additions. For the C acquisition enzyme activities, I found no significant treatment effects on cellulase MEI (Table 3.2). While N addition influenced oxidase activities, there was also a significant 3-way interaction between short-term N, long-term N and residue type such that with no short-term N, both VR and PMVR residue mixture in the 134N and 291N soils had greater NAS effects than in 0N soils ($P= 0.011$ and $P=0.002$; Fig 3.2 b). There were no significant treatment effects on PHOS MEI and N acquisition enzyme activities except on LAP (Table 3.2). Residue type had a significant effect on LAP activity with greater NAS effects in VR mixture ($p<0.0001$) compared to PM and PMVR mixtures (Fig. 3.3b).

The MEI of soil inorganic NH_4^+ and NO_3^- showed similar response to residue mixture effect over the one-year laboratory incubation (Fig. 3.4 & 3.5). For soil NH_4^+ there were NAS effects on day 37 and 362 (Fig 3.4a & d) but NAA effects on day 110 and 204 for almost all treatments (Fig. 3.4b &c). I found significant treatment effects of long-term N addition on the NH_4^+ MEI only on day 362 (Table 3.3); the 134N long-term

N treatment had a greater NAS effect on NH_4^+ than 291N and 0N treatments, regardless of short-term N or residue type (Fig. 3.4d).

Similar to the NH_4^+ MEI I found synergistic effects on soil NO_3^- but NAS effects were greater on days 37 and 362 (Fig 3.5a & d). However, in contrast to NH_4^+ MEI, there was a significant interaction of long- and short-term N additions on NO_3^- MEI on day 37 (Table 3.3). With short-term N additions there were greater NAS effects on NO_3^- in soils with the highest long-term N addition, 291N compared to 0N and 134N ($p<.0001$) (Fig. 3.5a). On day 110, VR residues exhibited greater NAS effects on soil NO_3^- compared to PM and PMVR residues ($p=0.0002$) (Table 3.3). On day 362, there was a significant treatment interaction between long- and short-term N additions on NO_3^- MEI (Table 3.3). Without short-term N addition, NAS effect on NO_3^- MEI was greatest in 291N treatment compared to 0N and 134N ($p=0.0006$), while with short-term N additions, NAS effect on NO_3^- was greatest in 0N compared to 134N and 291N long-term N treatments ($p=0.001$) (Fig. 3.5d).

3.3.2 Effects of N additions and residue mixtures on soil inorganic N

There were no effects of residue mixture type on soil NH_4^+ on any sampling date. On days 37 and 362, there was a significant although opposite interaction between short- and long-term N additions (Table 3.4). On day 37, in soils with short-term N addition, the 291N long-term N treatment had greater NH_4^+ than 0N and 134N soils ($p=0.03$) (Fig 3.6a). In contrast, on day 362, in soil with short-term N, the 291N long-term N treatment had less NH_4^+ compared to 0N and 134N treatments ($p=0.0001$) (Fig 3.6d).

Soil NO_3^- was influenced by different factors on different sampling dates (Table 3.4). On day 37, there was an interaction of short- and long-term N addition such that with short-term N addition, soil NO_3^- increased as long-term N addition rate increased and was greatest in 291N ($p<.0001$) (Fig. 3.7a). On day 110, I found that VR had greater NO_3^- than PM and PMVR residue mixtures ($p<.0001$) (Fig. 3.7b). On day 204 there was a significant interaction effect between short- and long-term N additions (Table 3.4). With short-term N addition NO_3^- was less in 291N compared to without short-term N addition ($p=0.005$) (Fig. 3.7c). Finally, on day 362, there was a significant interaction between short-term N and residue type (Table 3.4); without short-term N addition, PMVR had greater NO_3^- than VR and PM residue treatments ($p=0.0046$) (Fig. 3.7d).

3.3.3 Effects of N additions and residue mixtures on extracellular enzyme activities

On day 37, soil with short-term N addition had greater cellulase activity in 0N ($p=0.0002$) and 291N ($p=0.0006$) compared to 134N long-term N levels (Table 3.5, Fig. 3.8a). With 291N long-term N additions, PM residue mixture had greater cellulase activities than VR and PMVR residue types ($p=0.028$) (Table 3.5, Fig.3.8a). On day 110, cellulase activity was greater in 0N long-term N treatment without short-term N addition than in 0N treatment with short-term N addition ($p=0.006$) (Fig. 3.8b). On day 204, soil with short-term N addition had greater cellulase activity than soil without ($p<.0001$) (Fig. 3.8c). There were no significant treatment effects on cellulase activity on day 362 (Table 3.5; Fig 3.8d). There was an interaction effect of long-term N addition

and residue types only on day 204 (Table 3.5). The TOX was lowest in 291N soil mixed with PM (p=0.017) and in 0N soil mixed with RVMP (P=0.02) (Fig. 3.9c).

There was a significant interaction effect of short- and long-term N additions on PHOS activity on day 37 (Table 3.5); such that without short-term N addition PHOS activity was greater in 0N compared to 134N and 291N long-term N treatments (p=0.047) (Fig.3.10a). PHOS activity was greater in VR and PM residue mixtures compared to PMVR residue mixture (p=0.01; p=0.001) (Fig. 3.10a). On day 110 and 204, there were significant main effects of short-term N addition on PHOS activity (Table 3.5); such that PHOS activity was greater in soils without short-term N addition (Fig. 3.10b & c).

There were no effects of residue mixture type on NAG activity on any sampling date and no significant treatment effects on day 37 (Table 3.5). On day 110 I found a significant main effect of short-term N addition such that NAG was greater in soils without short-term N addition than in soils with short-term N addition (p=0.0035) (Fig. 3.11b), while on day 204 NAG was greater in soils with short-term N addition than in soils without (p<.0001) (Fig. 3.11c). On day 362, there was a two-way interaction effect of short- and long-term N additions and a three-way treatment interaction (Table 3.5). Soil with short-term N addition had greater NAG activity in 0N than in soil without short-term N addition (p=0.007) (Fig. 3.11d). Residue mixture PMVR had greater NAG activity in 0N long-term treatment with short-term N addition (p=0.02) and in 134N treatment without short-term N addition (p=0.04) (Fig. 3.10d). There were no significant treatment effects on LAP activity at any sampling period. I found a significant interaction between short-term N addition and residue type on urease activity only on day 362 (Table 3.5);

such that with short-term N addition, urease activity is significantly lower in PMVR residue mixture (p=0.02) (Fig 3.13d).

Cumulatively, short-term N addition had greater impact on cellulase, TOX and PHOS activities than either long-term N and residue type (Table 3.6). Generally, short-term N addition increased cellulase activity (Fig 3.14a), and decreased TOX and PHOS activities (Fig 3.14b & c). There was a three-way interaction of short-term N, long-term N and residue mixture on NAG activity (Table 3.6). The NAG activity was significantly greater in PM residue mixture in 291N treatment with short-term N addition compared to VR and PMVR (p=0.003) (Fig. 3.15a).

3.3.4 Relationship between soil inorganic N and EEA

A Pearson's correlation was conducted between soil inorganic N and extracellular enzyme activities at each sampling dates. I observed a significant correlation between inorganic NO_3^- and EEA (Table 3.7). There were no significant correlations between inorganic NO_3^- and cellulase activity on any sampling dates other than day 110. Cellulase activity showed a strong positive relationship with inorganic NO_3^- ($p<.0001$) (Table 3.7). On day 110, there was a negative correlation between TOX activity and soil NO_3^- content ($r=-0.239$; $p=0.042$) (Table 3.7). There were no significant correlations between soil inorganic N and LAP & NAG activities on any sampling dates. On day 362, I found a positive correlation between soil NO_3^- and urease activity (Table 3.7). There was also a positive correlation between soil NO_3^- and PHOS activity on day 204 ($p=0.009$) (Table 3.7).

3.4 Discussion

3.4.1 Residue Mixture Effects

The difference in the chemical complexity of residue mixtures may influence the rates of decomposition and the release of N (Wickings et al., 2012) in either additive or non-additive (synergistic and antagonistic) relationships (Gartner and Cardon, 2004; Meier and Bowman, 2008). Residue mixtures can increase the chemical complexity of the organic material entering a soil, which provides a wider suite of carbon resources for different microbes to choose from. This can lead to an increase in microbial diversity and activity such that measures of the decomposition of residue mixtures often display NAS effects. I hypothesized that mixtures of cover crop residues, specifically mixtures of grass and legume species, would result in NAS decomposition as assessed via net N mineralization and EEA at various stages during a year-long incubation of soils and residues.

When isolating the cover crop residue mixture effect (no short- and long-term N additions), I found NAS effects on soil inorganic N (Figs 3.4 & 3.5). The mixture effect index (MEI) of soil inorganic NH_4^+ and NO_3^- showed NAS effects at the early (day 37) and late (day 362) stages of the incubation. This result supports my hypothesis that there would be NAS mixture effects on soil inorganic N. In addition, I found increased activities of PHOS, LAP and urease in soils with more diverse residue mixtures (Figs 3.2c, 3.3b & c) suggesting that the availability of greater diversity of substrates stimulated microbial activities. This also supports the idea that easy accessibility of labile C from the residues promoted nutrient mineralization at the early stages of decomposition and EEA. Previous studies have found that synergistic effects become

stronger as decomposition progresses, probably due to cumulative effects of increased microbial abundance and activity by the end of the incubation (Liu et al., 2007). McDaniel at al. (2016) reported NAA effects on soil NO_3^- at day 30 but NAS by day 90 through day 360 (McDaniel et al., 2016). I also observed the highest NAS related to N availability (NO_3^-; Fig 3.5) on day 362. These results show that the addition of high quality (low C:N) cover crop residue mixtures resulted in increased decomposition rates that increased the N availability in the soil.

The fact that plant available N was increased over the course of the incubation suggests microbes were not N limited. In fact, my data suggests that microbes were actually more C than N limited. While mixture effects on EEA were varied, when I looked at just the mixture effects (no long- or short-term N addition treatments) across all enzymes I saw general patterns of C acquisition EEA showing NAA effects and nutrient acquisition EEA (N & P) showing NAS effects. Also, C acquisition EEA rates were generally greater than N acquisition EEA rates. Because the residue additions likely stimulated microbial growth and competition for C for energy and biomass building, it is not surprising that we would see NAA effects of residue mixtures on C acquisition. The EEA results also suggest that residue complexity is important for increasing nutrient availability, particularly in high nutrient content residues such as those used in the current study. The suggestion that NAA effects on C acquisition enzymes signifies competition for C is supported by excess inorganic N and NAS effects on N-mineralization EEA described above. Consistent with my results are previous studies showing high nutrient concentrations with diverse residue mixtures, particularly when the mixtures include high quality (low C:N) residues (Zak et al., 2003;

Oelmann et al., 2007; McDaniel et al., 2016). Similarly, other studies have shown synergistic effects of residue mixtures on PHOS and N acquisition EEA in N rich soils (Gartner and Cardon 2004; Meier and Bowman, 2010; Wang et al. 2015; McDaniel et al., 2016). Taken together my results and these studies show that residue diversity, especially with high quality cover crop residues, can increase N availability, thus increasing soil microbial activity and competition for energy (i.e. C).

Contrary to my hypothesis that the most diverse residue mixtures would have the greatest NAS effects due to the increased diversity of microbial substrates that may allow a more diverse microbial communities, I observed that soil samples with the VR residue mixture had the strongest NAS effects on soil inorganic NO_3^- and EEA, particularly N acquisition enzymes, compared to PM and PMVR. In addition, when looking at non-MEI data (data not transformed to MEI), I also observed consistently greater soil NO^-_3 throughout the incubation with VR compared to PMVR or PM residue mixtures. McDaniel et al. (2016) also reported greater NAS effects on soil NO_3^- with one mixture (corn+soy+wheat) compared to other similar mixtures (e.g. corn+soy+wheat+redclover or corn+soy). This suggests that non-additive effects observed in residue decomposition studies likely go beyond simple indices of residue quality such as C:N ratios. Indeed, it has been noted previously that more detailed chemical analyses of plant residues are likely needed to understand decomposition dynamics with residue diversity (Meier and Bowman, 2008; 2010). Residues used in this study had similar C:N ratios but did have some other differences in chemistry that may be important (Table 2.1). My results support the idea that we need to move beyond C:N

ratios and further suggest that even subtle differences in residue chemistry can be important determinants of residue decomposition and subsequent plant N availability.

3.4.2 Effects of long-term N addition on the decomposition of residues and N mineralization

In addition to the quantity and quality of plant residues, N additions across multiple years have been reported to strongly influence residue decomposition rates (Gartner and Cardon, 2004; Hobbie, 2008; Keeler et al., 2009; Tiemann and Billings, 2011; Geisseler and Scow, 2014). Long-term N additions can increase plant productivity thus increasing residue quantity. Repeated N addition could result in increase in residue N content (narrower C:N ratios) therefore are likely to decompose faster and less efficiently (Knorr et al., 2005; Tiemann and Billings, 2011). For this experiment, I collected soils from a long-term N addition experiment where presumably microbial communities in high N addition plots have adapted to processing greater quantities and quality residues. Because residues used in my study are all relatively high quality, I hypothesized that long-term N additions would reduce or eliminate synergistic effects on residue decomposition and N mineralization.

In contrast with my hypothesis, I generally observed no significant effects of field N addition rates on decomposition. I found no difference in EEA rates and generally all NAS effects on EEA across all long-term N addition treatments, especially at the early (day 30) and late (day 362) sampling dates. For plant available N (NH_4^+ or NO_3^-) there were no clear patterns in concentration or MEI associated with specific long-term N treatments (Figs. 3.4 & 3.5). When looking at long-term N-addition effects on EEA,

significant results again did not support my hypothesis, although I did observe a general trend of increasing MEI with field N addition level for C acquisition EEA (Fig. 3.2). There were no consistent mixture effect trends for N (NAG, LAP, UR) or P acquisition EEA with long-term N addition rates (Fig. 3.3). The MEI calculated for EEA varied greatly across enzymes and residue types, although there were very few significant differences. Even though these sites see different levels of N and presumably crop productivity this has not translated into differences in the way microbial communities process residues. In fact, after 12 years there was no difference in total C stocks between the treatments (Table 2.2) suggesting the same – that all residues are processed similarly at this site regardless of N addition rate. This is likely due to microbial C rather than N limitation as discussed above. When the high-quality residues were added in the soil, there was increased microbial activity related to C acquisition that was unrelated to the history of N addition rates. The soils in this study are relatively sandy, such that when coupled with regular tillage the soils cannot maintain structure necessary to contain soil organic C stocks adequate for supporting microbial communities (Kazanski et al., 2019; Paul, 2016; de Andrade Bonetti et al., 2017).

3.4.3 Effects of short-term N addition on the *decomposition of residue*s *and N mineralization*

The immediate availability of N is one of the critical factors that influences rates of residue decomposition (Knorr et al., 2005; Kazanski et al., 2019). Addition of N to soils incubated in lab studies is quite common and has produced a range of results with these short-term N additions directly affecting decomposition rates by influencing

microbial production of biomass and/or enzymes (Stark et al., 2007; Treseder, 2008; Kazanski et al., 2019). To better understand the direct effects of short-term N addition on N mineralization and EEA, I added urea N fertilizer to soils mixed with residues. Here I will focus on these short-term (lab) N additions by looking at short-term N addition where there was no field N added (i.e. 0 kg N ha^{-1} treatment) or by averaging across the long-term field N treatments where there are no significant interactions between long- and short-term N additions.

When looking at effects of short-term N addition I observed increased soil NO_3^- and NAS effects on soil NH_4^+ at the initial and final stages of the incubation and NAS effects on soil NO_3^- throughout the incubation (Figs. 3.4 & 3.5). This result is contrary to my hypothesis that short-term N addition will result in NAA mixture effects on soil inorganic N. One explanation could be that the C limitation, relative to N, coupled with rapid decomposition of residue mixtures released excess N in addition to N fertilizer that was added led to these synergistic effects on inorganic N.

The mixture effect response of EEA to short-term N additions included NAS effects on cellulase, PHOS, NAG, LAP and urease activities and NAA effects on oxidase (Figs. 3.2 & 3.3). The negative effect of residue mixtures on oxidase with short-term N is not surprising given that correlations between lignin degradation and N addition are generally negative (Carriero et al., 2000; Sinsabaugh et al., 2002; Knorr et al., 2005; Enrique et al., 2008; Keeler et al., 2009; Zhou et al., 2012; Tu et al., 2014). This reduction in oxidase activity did not necessarily reduce the overall decomposition rates in my study because the residues used in my study had relatively low lignin content (Table 2.2). Additionally, in the non-MEI EEA, short-term N addition increased

the activities of cellulase and NAG while decreasing PHOS and TOX but had no significant effect on LAP (Table 3.6). Zhou et al. (2012) reported increased alkaline phosphatase, decreased urease and no effect on oxidase activities with N addition. Another study Ramirez et al. (2012) reported significant decrease in BG, PHOS, LAP, PER activities with N addition in one-year incubation study, which was attributed to a shift in soil microbial communities. Overall, my results support other studies that have shown short-term N additions can directly influence soil microbial enzyme and biomass production and even community structure, with implications for residue decomposition and N mineralization.

3.4.4 Interactive effects of short- and long-term N additions on the decomposition of residues and N mineralization

Most studies have reported the effects of either long-term N (Treseder, 2008; Ladha et al., 2011; Geisseler and Scow, 2014) or short-term N (Ramirez et al., 2012; Geisseler et al., 2016) addition on soil microorganisms and N mineralization. To my knowledge this is the first study to assess how microbial processes respond to the interaction of short- and long-term N addition with regards to residue decomposition. To better understand the direction of residue mixture effects as influenced by both short- and long-term N additions on microbial processes, I added urea N fertilizer (short-term) to soil mixed with residues that had received different N addition rates for 12 years (long-term). I hypothesized that there would be a reduction of NAS effects on soil inorganic N and EEA related to decomposition at the highest levels of combined N addition (i.e. urea added to 291 kg N ha^{-1} y^{-1} soils).

At the early and final stages of the incubation and residue decomposition, my hypothesis was not supported; I found that the MEI for NH_4^+ and NO_3^- (Fig. 3.4 & 3.5) as well as concentrations of NH_4^+ and NO_3^- (Figs 3.6 & 3.7) increased as field N addition rate increased with short-term N addition. However, at the later stage of the incubation I found some support for my hypothesis, with lowest NAS effects on NH_4^+ and NO_3^- MEI on day 204. On day 362, soil with short-term N addition had greater inorganic N concentrations in the highest field N addition rate. There were no consistent trends in interactions between short- and long-term N addition for the activity rates or MEI of any EEA. The soil inorganic N results controlled by the interaction between short and long-term N additions showed increasing inorganic N as long-term N addition rate increased in the presence of short-term N treatment. With faster decomposition of residue mixtures and C limited microbes at early stages of decomposition, there is an excess of N released and the long- and short-term N treatments act cumulatively to increase N content and NAS. By the end of the incubation, microbes are relatively less C limited (more N limited) and these effects are reversed. Other studies, both field and lab-based, have reported that N additions accelerate N mineralization and increase soil N availability (Hobbie, 2005; Zhang et al., 2008; Zhou et al., 2012; Grandy et al., 2013; Chen et al., 2014 and Zhu et al., 2016). Collectively, these results show that increasing N additions can accelerate N mineralization when microbes are relatively C limited.

3.5 Conclusions

Reliance on high doses of inorganic N fertilizers has raised questions about how N additions influence soil microbial activities and the soil processes they mediate. In a

year-long laboratory incubation experiment study, I provided insight on how long- and short-term N additions influence soil microbial activities related to decomposition of residue mixtures and plant available N. When isolating only the mixture effects, my results showed NAS effects on soil inorganic N at the initial (day 37) and final stage (day 362) of the incubation period. Mixture effects on enzyme activities showed NAA effects on C acquisition enzymes and NAS effects on N and P acquisition enzymes. The addition of residues likely stimulated microbial growth and competition for C resulting in C limitation. Increases in both C and N acquisition enzyme activities could be due to multipurpose activity of some enzymes (e.g β-1,4,-N acetylglucosaminidase responsible for degrading chitin). Overall, the controls on microbial decomposition of residue mixtures appear to be governed more by microbial stoichiometry (Sinsabaugh et al. 2014), than residue biochemistry. That is, energy or nutrient limitations regulate enzyme production and the mineralization or immobilization of nutrients to a much greater extent than residue chemistry.

APPENDIX

Table 3.1. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N, residues and their interaction on total soil carbon and total nitrogen of soil from the W. K. Kellogg Biological Station Nutrient Gradient Experiment on the initial stage (day 1) and final stage (day 362) of laboratory soil incubation.

	Treatment effects	Total carbon	Total nitrogen
Day 1	Field N (FN)	<.0001	0.0001
	Lab N (LN)	-	-
	Residue (R)	-	-
	FN*LN	-	-
	FN*R	-	-
	LN*R	-	-
	FN*LN*R	-	-
Day 362	Field N (FN)	-	-
	Lab N (LN)	-	-
	Residue (R)	-	-
	FN*LN	-	-
	FN*R	-	-
	LN*R	-	-
	FN*LN*R	-	-

† ANOVA significant at $p<0.05$, (-) means not significant.

Table 3.2. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N, residues and their interactions on mixture effect index (MEI) of cumulative extracellular enzyme activity of soils from the W. K. Kellogg Biological Station Nutrient Gradient Experiment the one-year of soil incubation. (362 days).

Treatments	Cellulase	TOX	PHOS	NAG	LAP	Urease
Field N	-	0.0298	-	-	-	-
Lab N	-	0.0023	-	-	-	-
Residue	-	-	-	-	0.0175	-
FN*Lab N	-	-	-	-	-	-
FN*R	-	-	-	-	-	-
Lab N*R	-	-	-	-	-	-
FN*Lab N*R	-	0.0243	-	-	-	-

† Significant treatment effect at $p \leq 0.05$, (-)=not significant. Extracellular enzyme activities include cellulase (BG +CBH), Total oxidase (TOX), PHOS=Phosphatase, NAG= β -1,4,-N-acetyl glucosaminidase, LAP= Leucine-aminopeptidase.

Table 3.3. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N, residues and their interactions on mixture effect index (MEI) of soil inorganic N of soils from the W. K. Kellogg Biological Station Nutrient Gradient Experiment at different sampling points – day 37, 110, 204 & 362 over the one-year of soil incubation.

	Treatment	Day 37	DAY 110	DAY 204	DAY 362
NH_4^+	Field N (FN)	-	-	-	0.0094
	Lab N	-	-	-	-
	Residue (R)	-	-	-	-
	FN*Lab N	-	-	-	-
	FN*R	-	-	-	-
	Lab N*R	-	-	-	-
	FN*Lab N*R	-	-	-	-
NO_3^-	Field N (FN)	-	-	-	**-**
	Lab N	<.0001	-	-	**-**
	Residue (R)	-	0.0023	-	**-**
	FN*Lab N	0.016	-	-	<.0001
	FN*R	-	-	-	**-**
	Lab N*R	-	-	-	**-**
	FN*Lab N*R	-	-	-	**-**

† Presented are p-values of treatment effects across each sampling time; day 37, day 110, day 204, and 362 day. Significant treatment effect at $p \leq 0.05$, (-)=not significant.

Table 3.4. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N, residues and their interaction on soil inorganic N inorganic NH^{4+} & NO_3^- of soils from the W. K. Kellogg Biological Station Nutrient Gradient Experiment at different sampling points over the one-year of soil incubation.

Variables	Treatment effects	Day 37	Day 110	Day 204	Day 362
NH_4^+	Field N (FN)	-	-	-	-
	Lab N (LN)	-	-	-	0.014
	Residue (R)	-	-	-	-
	FN*LN	0.035	-	-	0.003
	FN*R	-	-	-	-
	LN*R	-	-	-	-
	FN*LN*R	-	-	-	-
NO_3^-	Field N (FN)	-	-	-	-
	Lab N (LN)	<.0001	-	-	-
	Residue (R)	-	0.001	-	-
	FN*LN	0.042	-	0.011	-
	FN*R	-	-	-	-
	LN*R	-	-	-	0.040
	FN*LN*R	-	-	-	-

† Presented are p-values of treatment effects across each sampling time; day 37, day 110, day 204, and 362 day. Significant treatment effect at $p \leq 0.05$, (-)=not significant.

Table 3.5. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N, residues and their interaction on extracellular enzyme activity (EEA) of soils from the W. K. Kellogg Biological Station Nutrient Gradient Experiment at different sampling points over the one-year of soil incubation.

Day 37	Cellulase	TOX	PHOS	NAG	LAP	UREASE
Field N (FN)	-	-	-	-	-	-
Lab N (LN)	0.0004	-	-	-	-	-
Residue (R)	-	-	0.0072	-	-	-
FN*LN	0.0063	-	0.0439	-	-	-
FN*R	0.017	-	-	-	-	-
LN*R	-	-	-	-	-	-
FN*LN*R	-	-	-	-	-	-
Day 110						
Field N (FN)	-	-	-	-	na	-
Lab N (LN)	-	-	0.0292	0.0035	na	-
Residue (R)	-	-	-	-	na	-
FN*LN	0.033	-	-	-	na	-
FN*R	-	-	-	-	na	-
LN*R	-	-	-	-	na	-
FN*LN*R	-	-	-	-	na	-
Day 204						
Field N (FN)	-	-	-	-	na	-
Lab N (LN)	<.0001	0.001	<.0001	<.0001	na	-
Residue (R)	-	-	-	-	na	-
FN*LN	-	-	-	-	na	-
FN*R	-	0.012	-	-	na	-
LN*R	-	-	-	-	na	-
FN*LN*R	-	-	-	-	na	-
Day 362						
Field N (FN)	-	-	-	-	-	-
Lab N (LN)	-	-	-	-	-	-
Residue (R)	-	-	-	-	-	-
FN*LN	-	-	-	0.011	-	-
FN*R	-	-	-	-	-	-
LN*R	-	-	-	-	-	0.044
FN*LN*R	-	-	-	0.0128	-	-

† Significant treatment effect at $p \leq 0.05$, (-)=not significant and (na)=not applicable. Extracellular enzyme activities include cellulase (BG +CBH), Total oxidase (TOX), PHOS=Phosphatase, NAG= β -1,4,-N-acetyl glucosaminidase, LAP= Leucine-aminopeptidase,.

Table 3.6. Three-way ANOVA analyses of long-term (field) N, short-term (lab) N, residues and their interaction on the cumulative extracellular enzyme activity (EEA) of soils from the W. K. Kellogg Biological Station Nutrient Gradient Experiment at different sampling points over the one-year of soil incubation.

Treatments	Cellulase	TOX	PHOS	NAG	LAP	UREASE
Field N	-	-	-	-	-	-
Lab N	<.0001	0.0075	0.0023	<.0001	-	-
Residue	-	-	-	-	-	-
FN*LABN	-	-	-	-	-	-
FN*R	-	-	-	-	-	-
LABN*R	-	-	-	-	-	-
FN*LABN*R	-	-	-	0.010	-	-

† Significant treatment effect at $p \leq 0.05$, (-)=not significant. Extracellular enzyme activities include cellulose (BG +CBH), Total oxidase (TOX), PHOS=Phosphatase, NAG= β -1,4,-N-acetyl glucosaminidase, LAP= Leucine-aminopeptidase.

Table 3.7. Correlation between soil inorganic N and extracellular enzyme activities at each sampling points day 37, 110, 204 and 362

	Variables	Cellulase	TOX	NAG	LAP	Urease	PHOS
Day 37	NO_3^-	ns	ns	ns	ns	ns	ns
	NH_4^+	ns	ns	ns	ns	ns	ns
Day 110	NO_3^-	0.546^{***}	-0.239^{*}	ns	ns	ns	ns
	NH_4^+	ns	ns	ns	ns	ns	ns
Day 204	NO_3^-	ns	ns	ns	ns	ns	0.304^{*}
	NH_4^+	ns	ns	ns	ns	ns	ns
Day 362	NO_3^-	ns	ns	ns	ns	0.246^{*}	ns
	NH_4^+	ns	ns	ns	ns	ns	ns

† Significant treatment effect at $^{*}p \leq 0.05$, $^{**}p \leq 0.001$, $^{***}p \leq .0001$, ns=not significant. Extracellular enzyme activities include cellulase (BG &CBH summed), Total oxidase (TOX), NAG= β -1,4,-N-acetyl glucosaminidase, LAP= Leucine-aminopeptidase, PHOS=Phosphatase.

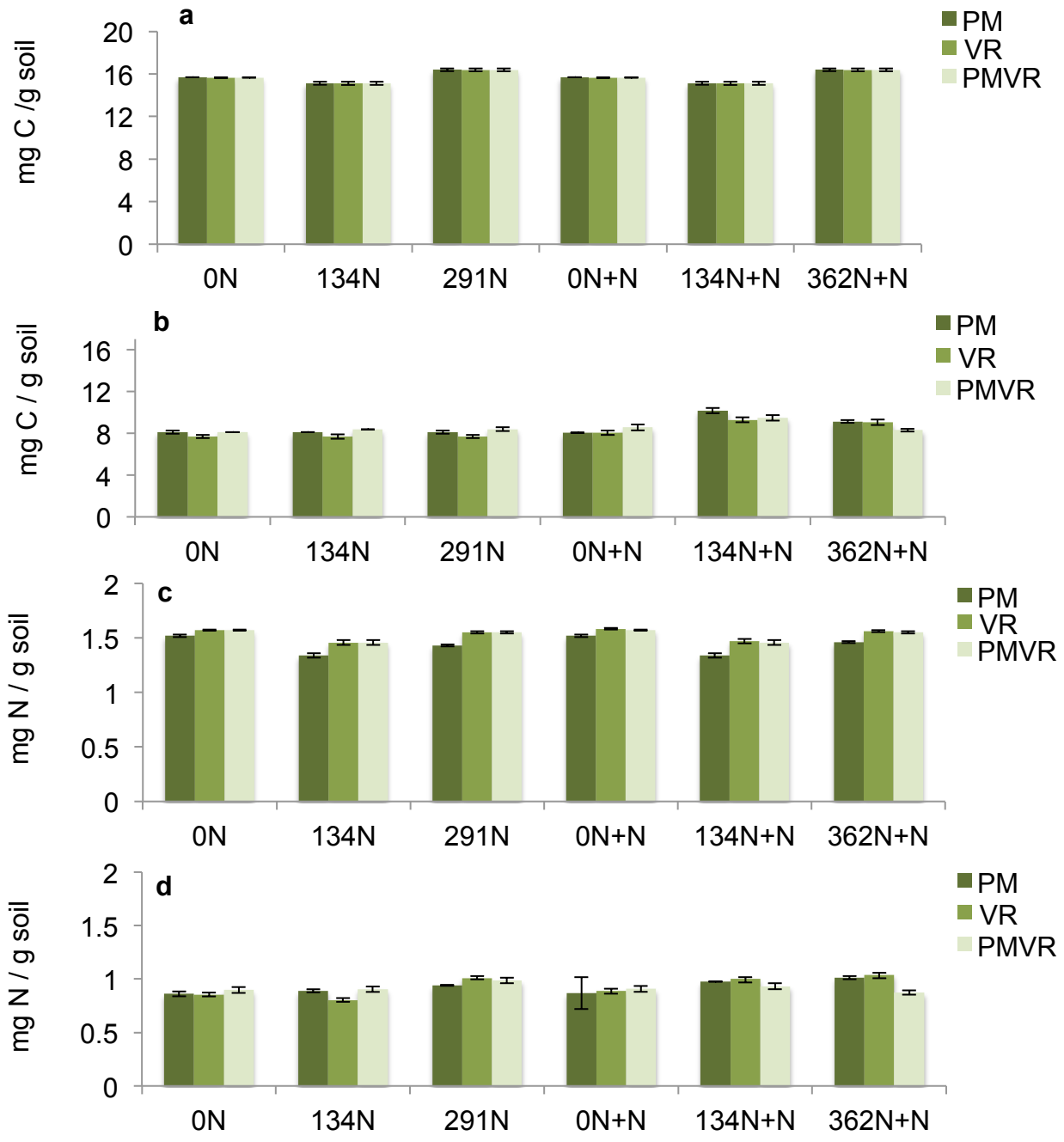

Figure 3.1. The initial (day1; a) and final (day 362; b) total soil organic C (including extant and residue C); and initial (c) and final (d) total soil N (including extant and residue N) in soils mixed with residues that received long-term N additions of 0, 134 or 291 kg N $ha^{-1}y^{-1}$ for 12 years with cover crop residue mixtures (PM= field pea and pearl millet, VR= hairy vetch and annual ryegrass, PMVR= field pea+ pearl millet+ hairy vetch + annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

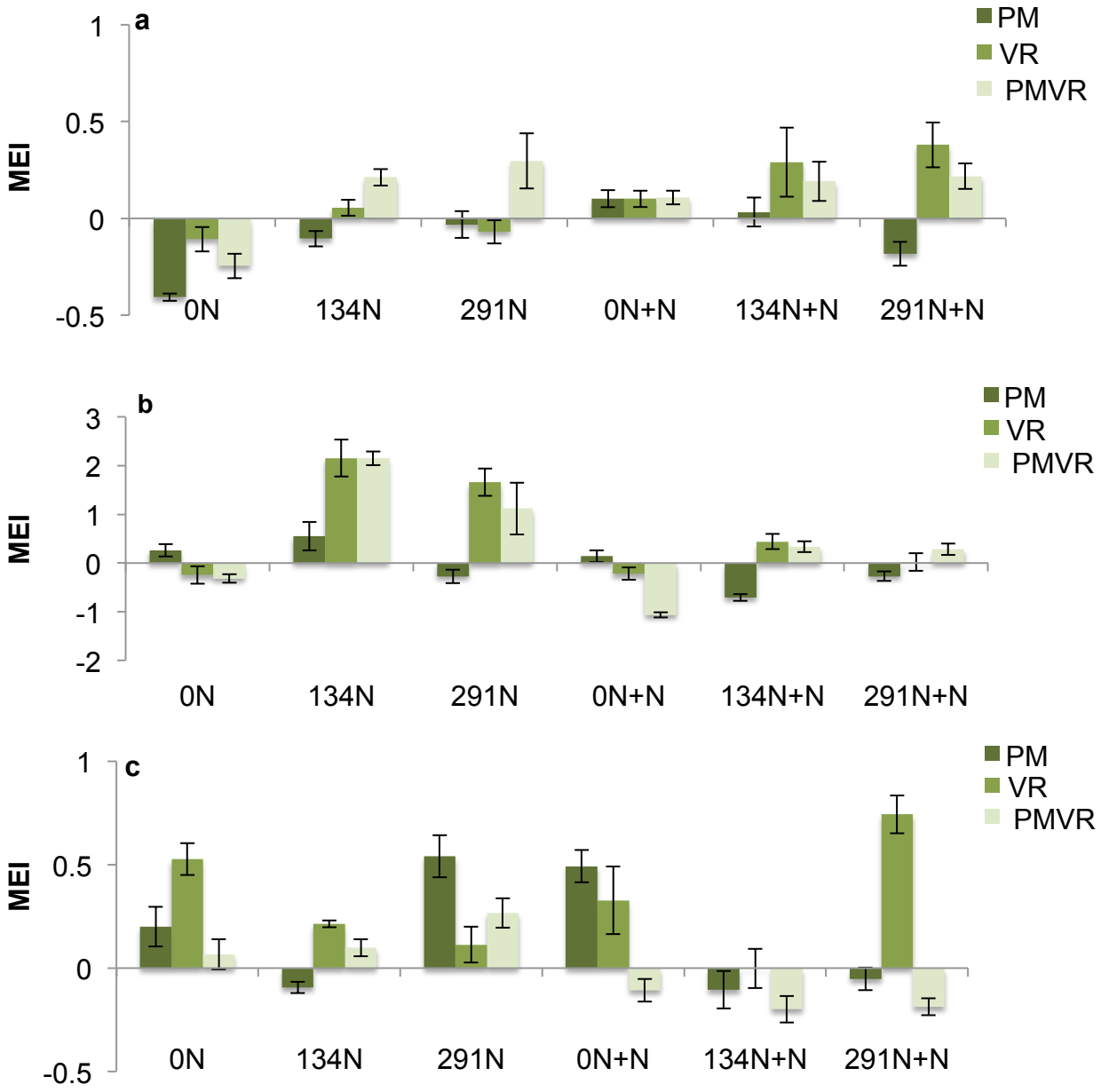

Figure 3.2. Mixture effect index (MEI) of cumulative extracellular enzyme activities; (a) cellulase, (b) total oxidase, (c) PHOS (acid phosphatase) during a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

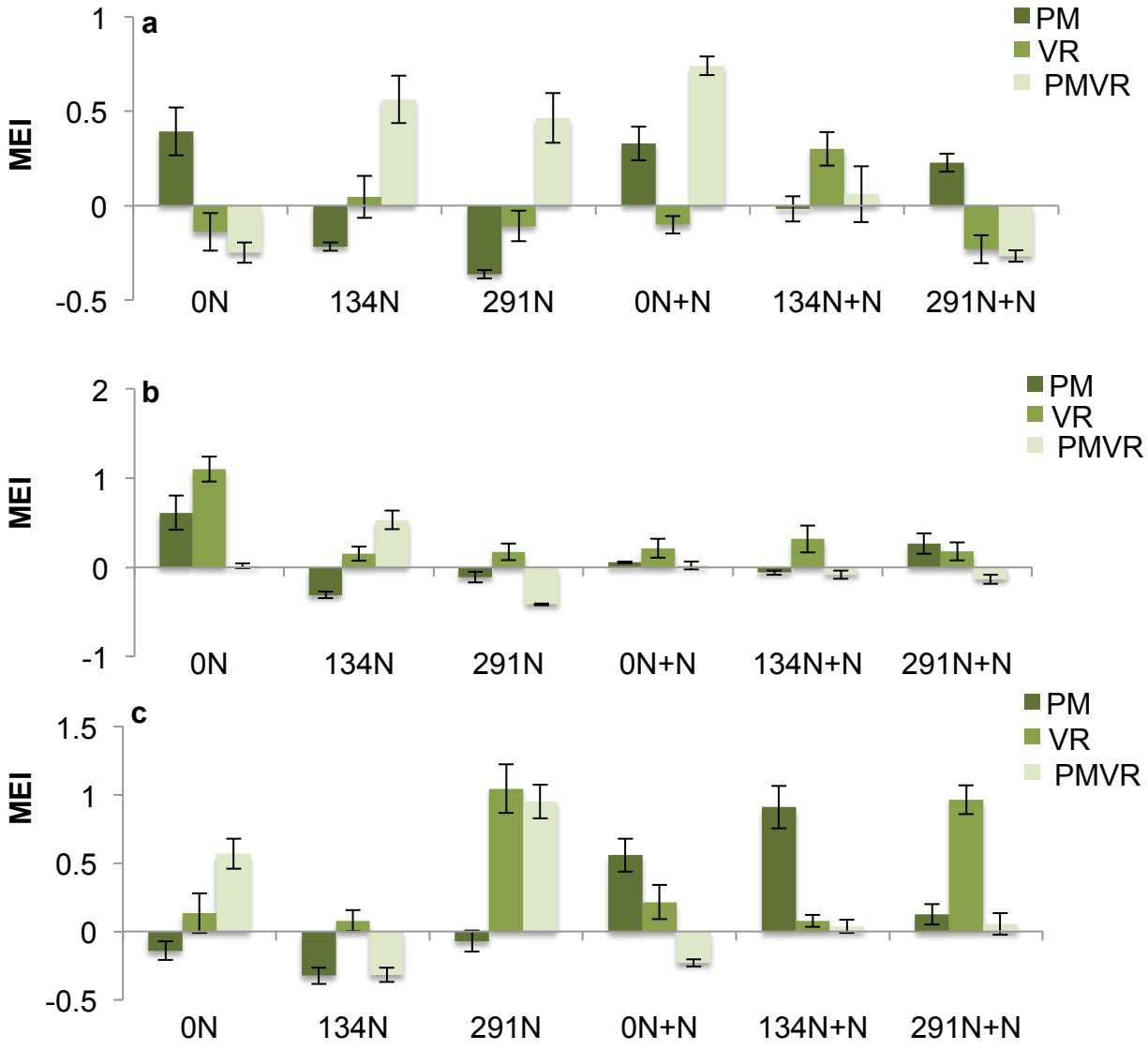

Figure 3.3. Mixture effect index (MEI) of cumulative extracellular enzyme activities; (a) β-1,4,-N-acetylglucosaminidase (NAG), (b) Leucine-aminopeptidase (LAP), (c) Urease, during a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

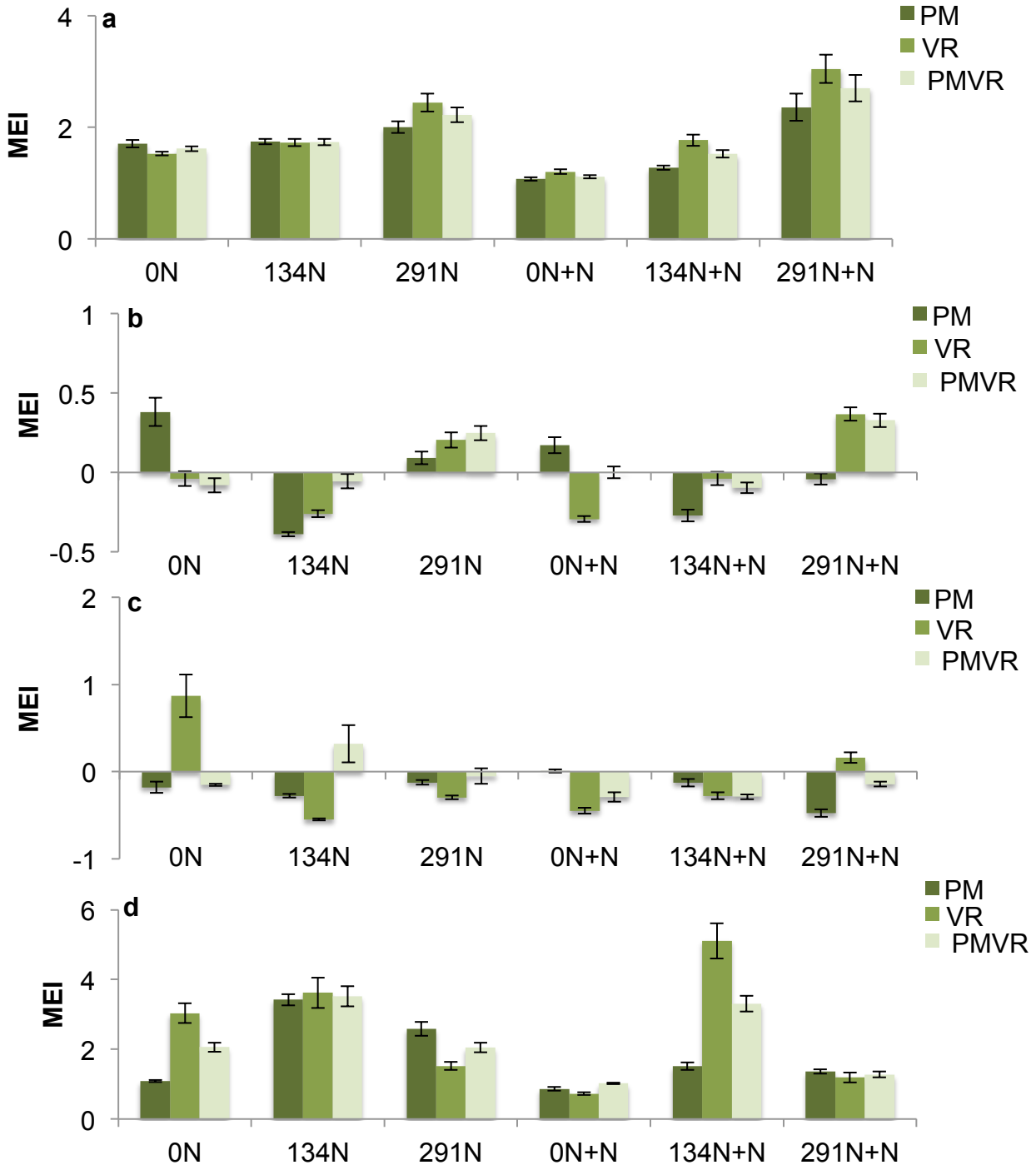

Figure 3.4. Mixture effect index (MEI) of soil ammonium (NH_4^+) measured at day (a) 37, (b) 110, (c) 204, (d) 362, throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

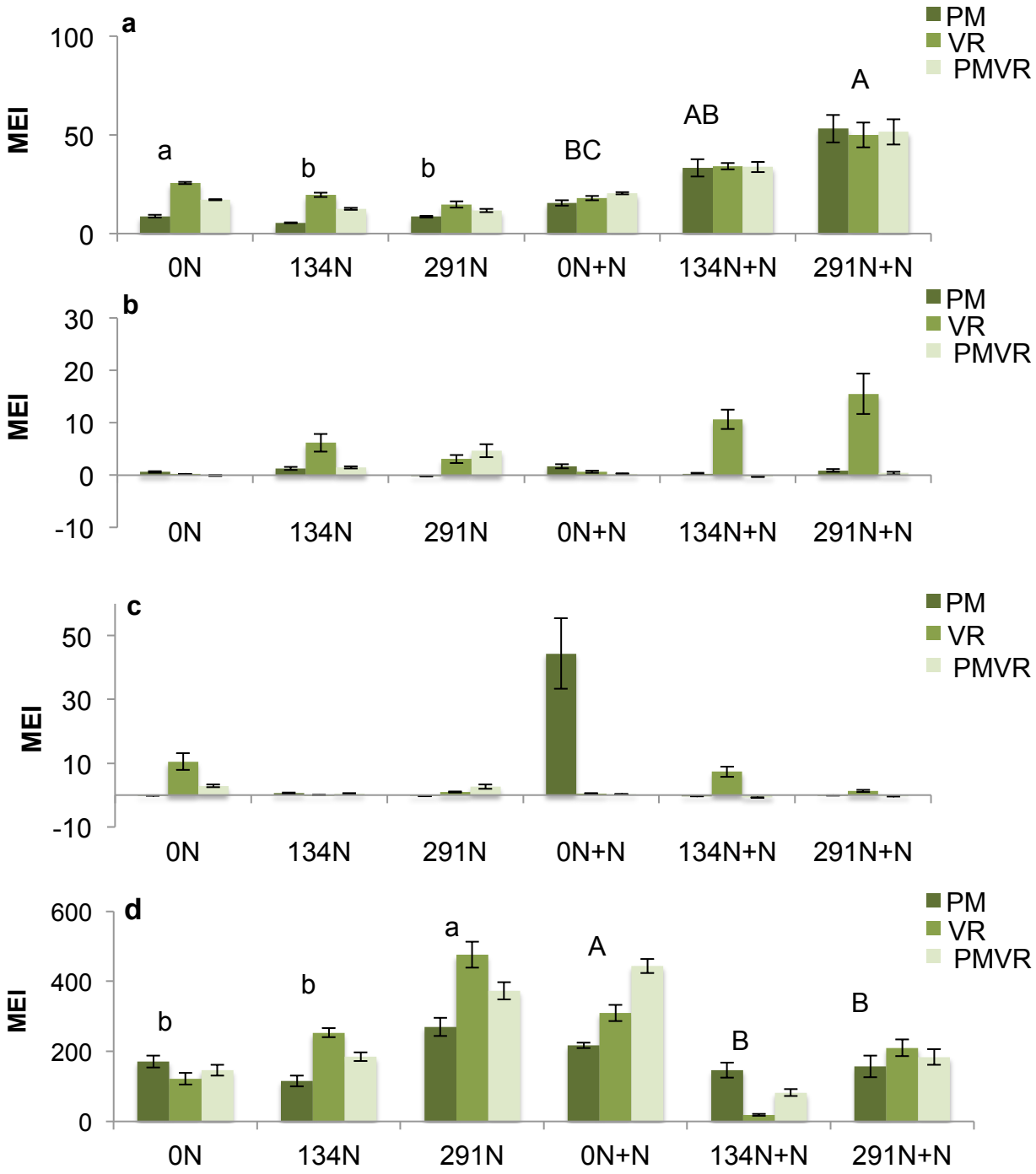

Figure 3.5. Mixture effect index (MEI) of soil nitrate (NO_3^-) measured at day a) 37, (b) 110, (c) 204, (d) 362 throughout during a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and different letters indicate significant differences between long-term N levels, with or without short-term N.

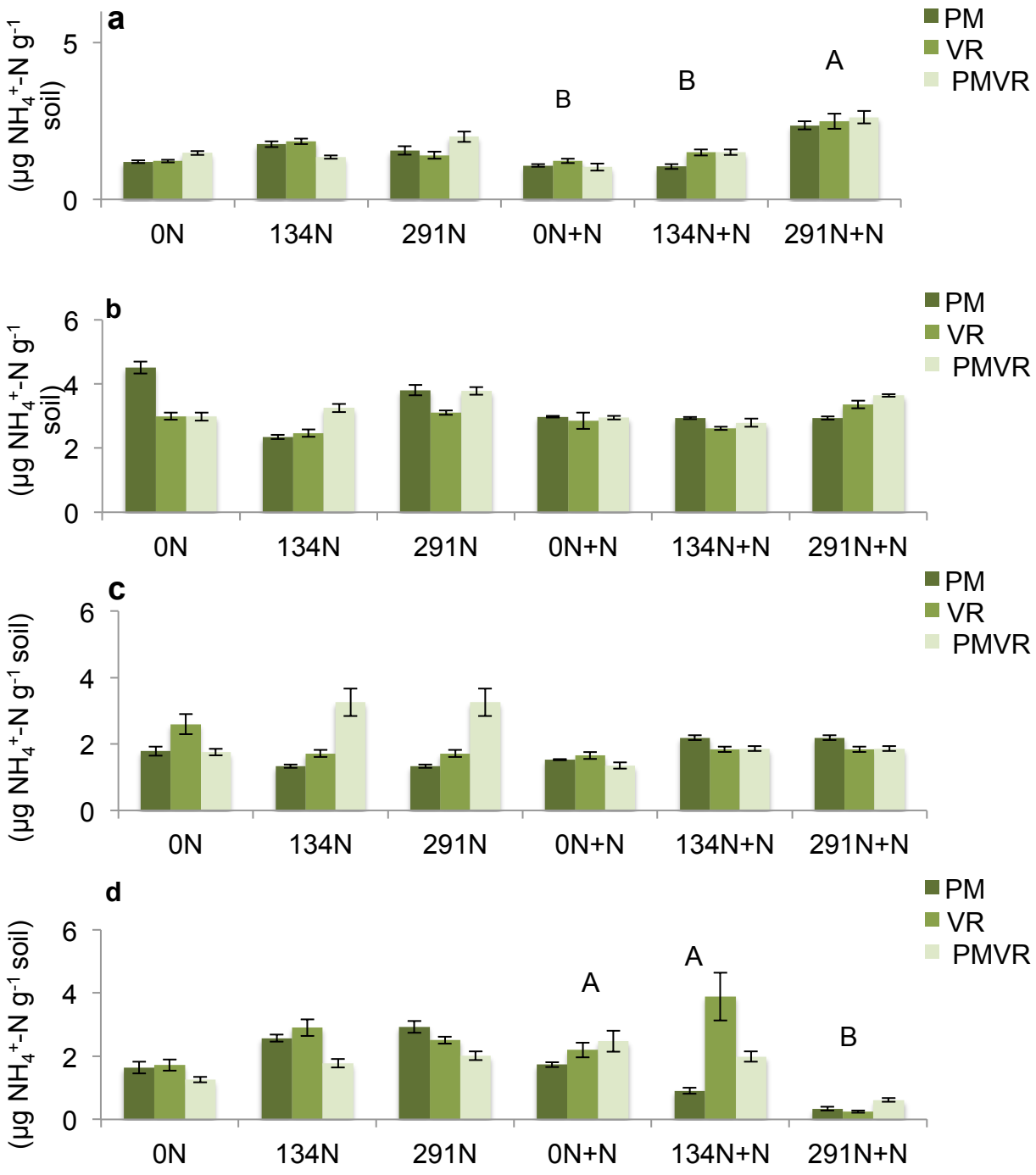

Figure 3.6. Soil inorganic ammonium (NH_4^+) measured at each sampling period (a) day 37, (b) 110, (c) 240 and (d) 362 throughout one year of laboratory incubation of soil from site receiving long-term N addition of 0, 134, and 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between long-term N addition treatments with short-term N addition.

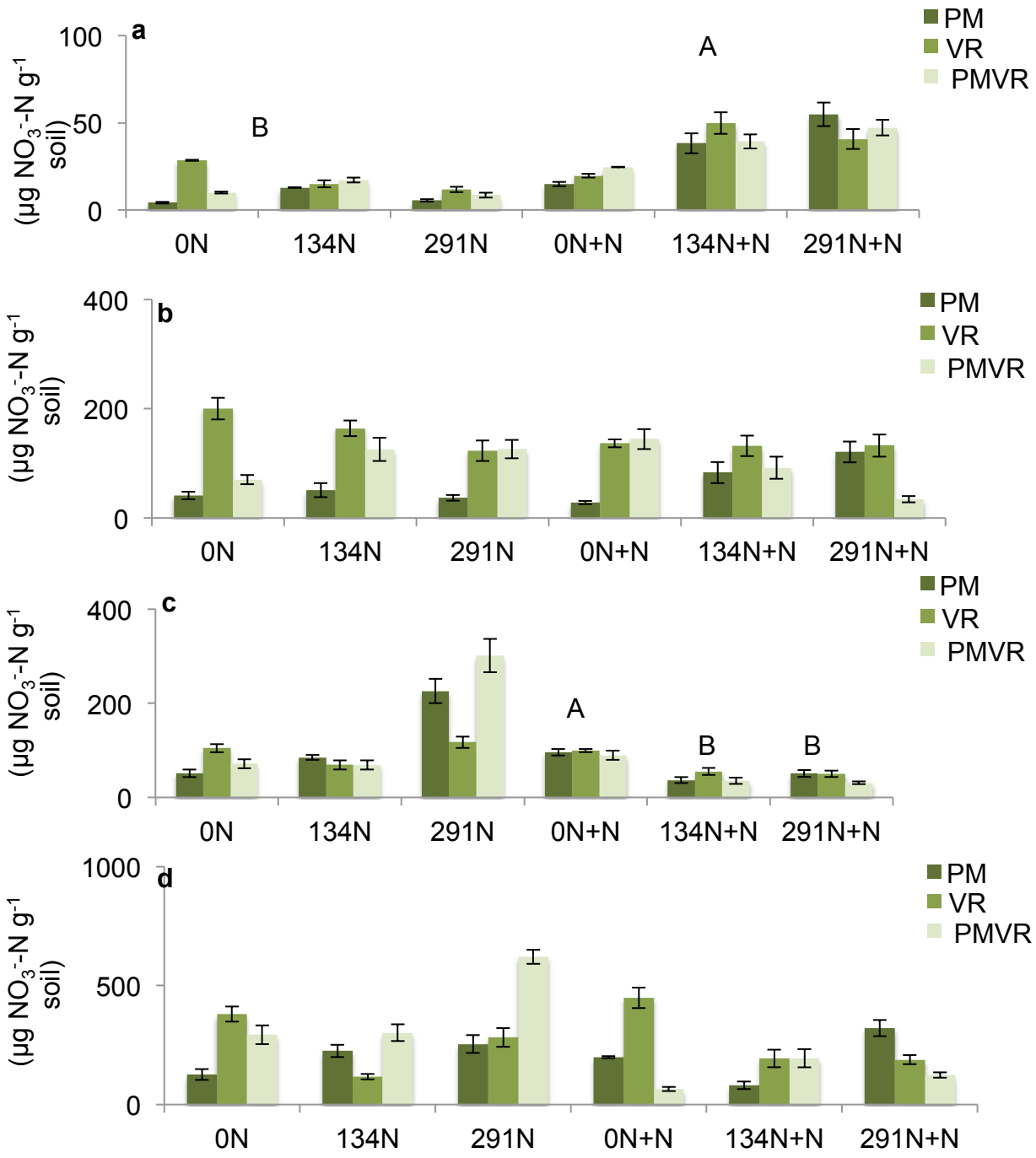

Figure 3.7. Soil nitrate (NO_3^-) measured at each sampling period (a) day 37, (b) 110, (c) 240 and (d) 362 throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between long-term N addition treatments with short-term N addition.

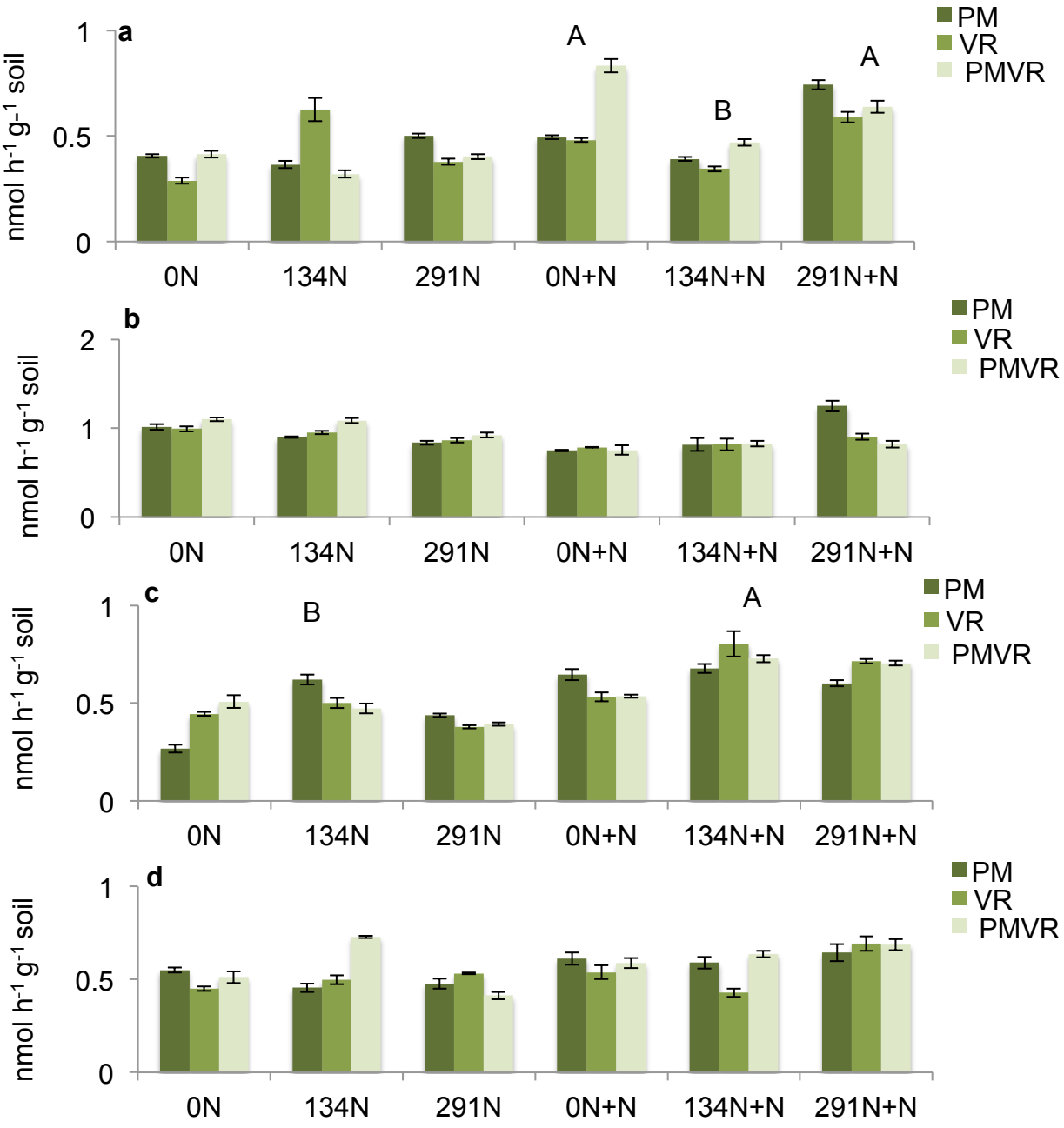

Figure 3.8. Cellulase activity measured on day (a) 37, (b) 110, (c) 204, (d) 362 throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between long-term N addition treatments with short-term N addition.

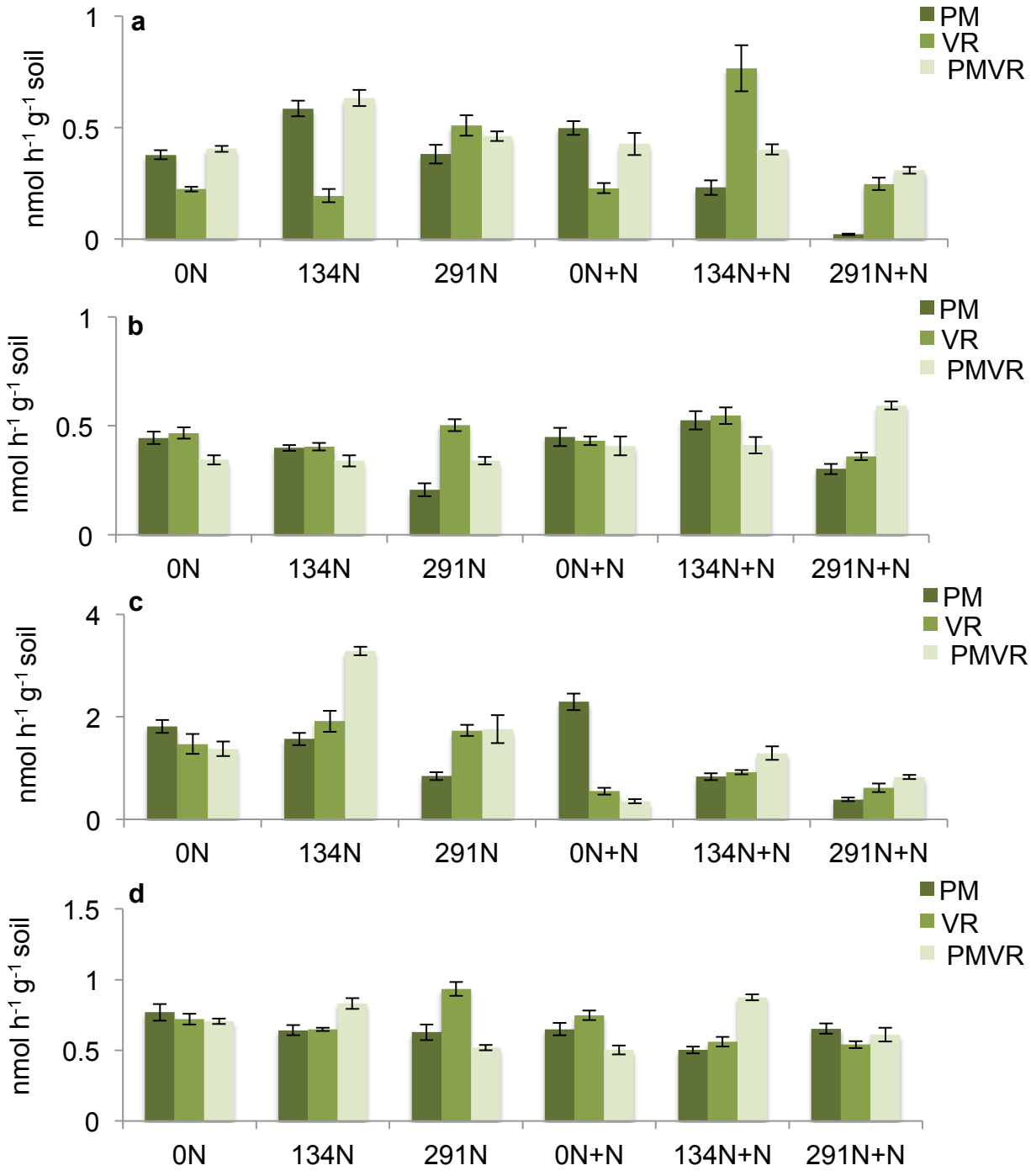

Figure 3.9. Total oxidase (TOX) activity measured on day (a) 37, (b) 110, (c) 204, (d) 362 throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

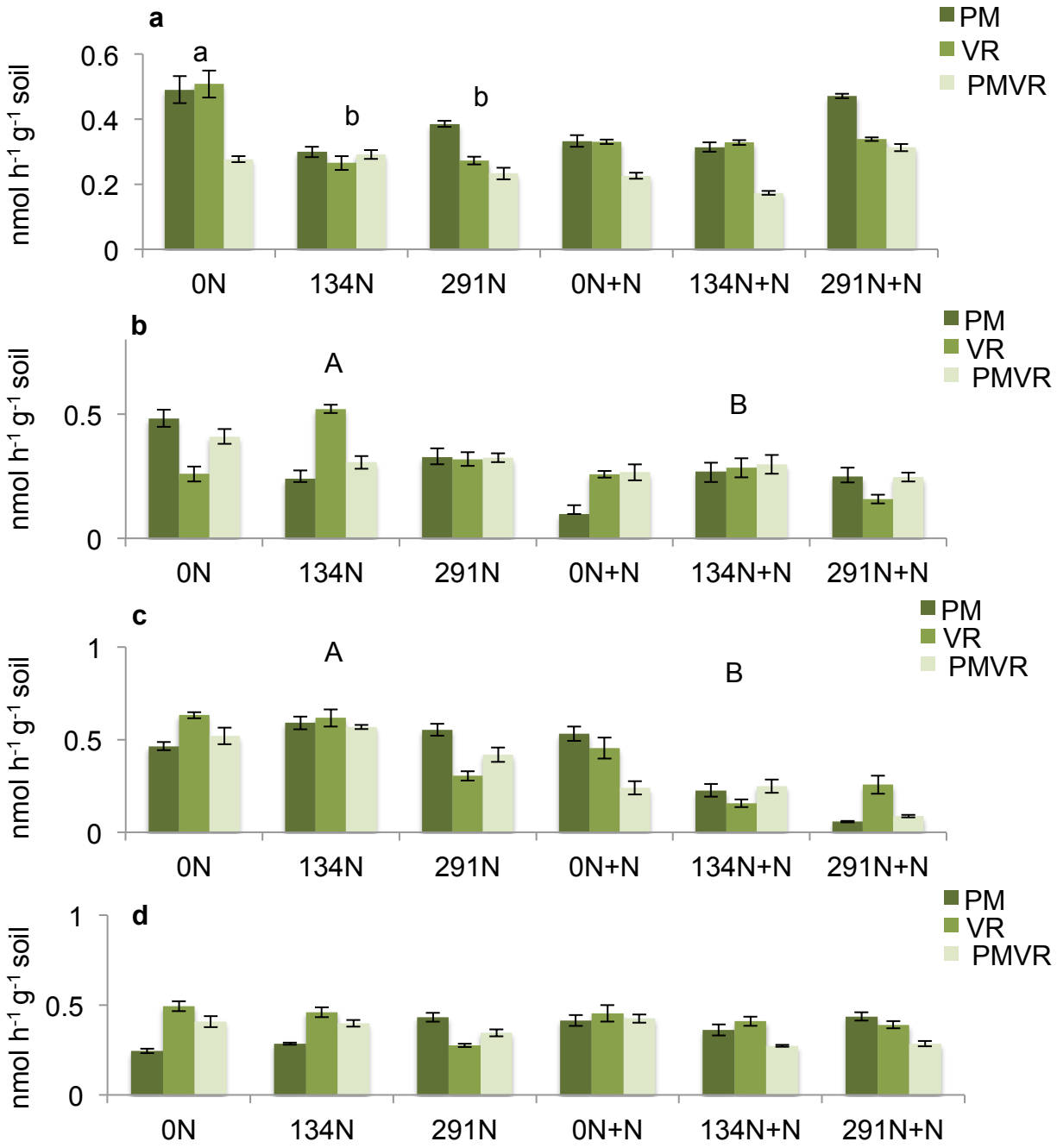

Figure 3.10. Phosphatase activity (PHOS) measured on day (a) 37, (b) 110, (c) 204, (d) 362 throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between long-term N addition treatments without short-term N addition or between short-term N addition treatments.

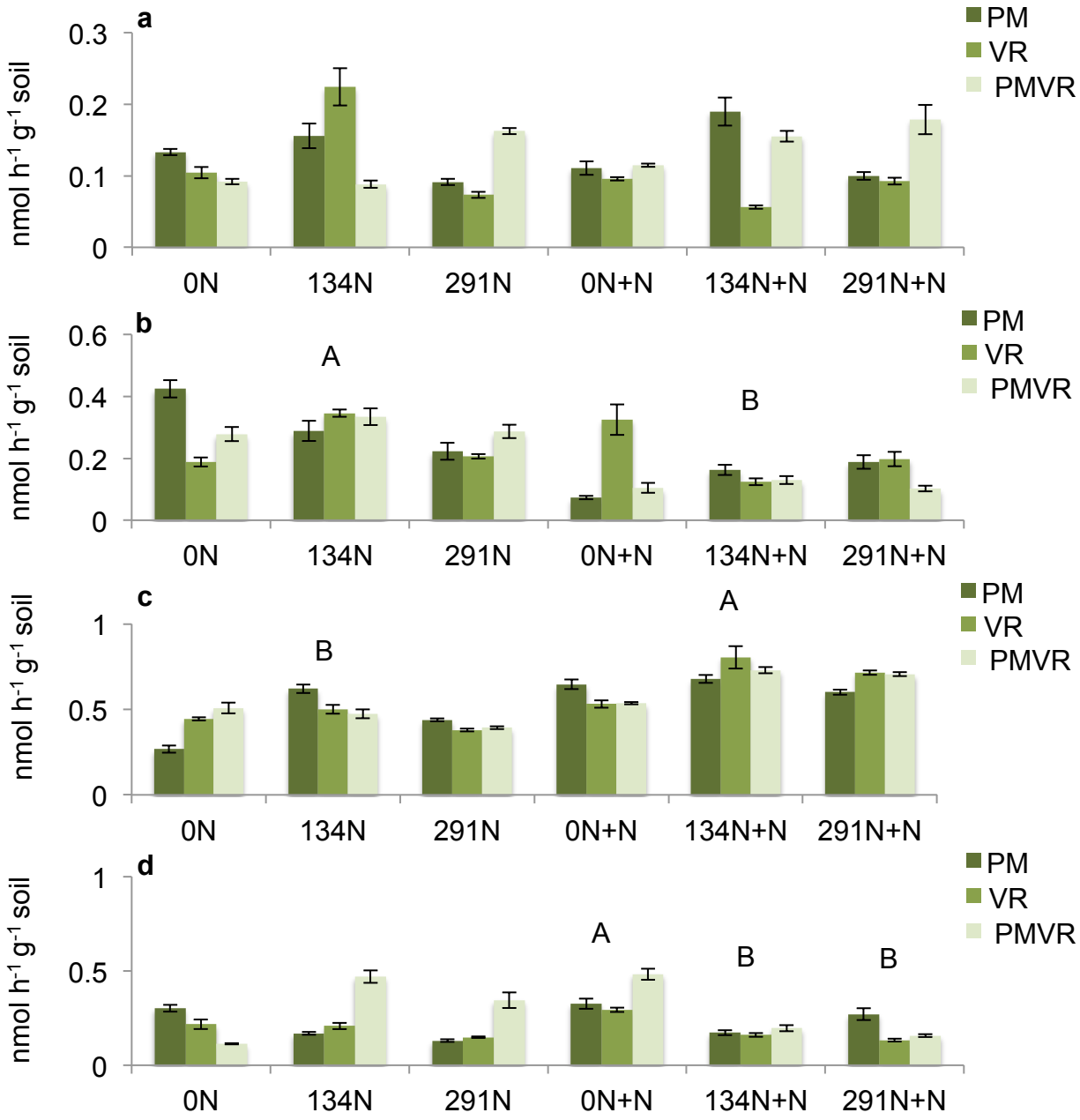

Figure 3.11. The β -1,4,-N-acetyl glucosaminidase (NAG) activity measured on day (a) 37, (b) 110, (c) 204, (d) 362 throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between short-term N addition treatments (b & c) or long-term N addition treatments with short-term N addition (d).

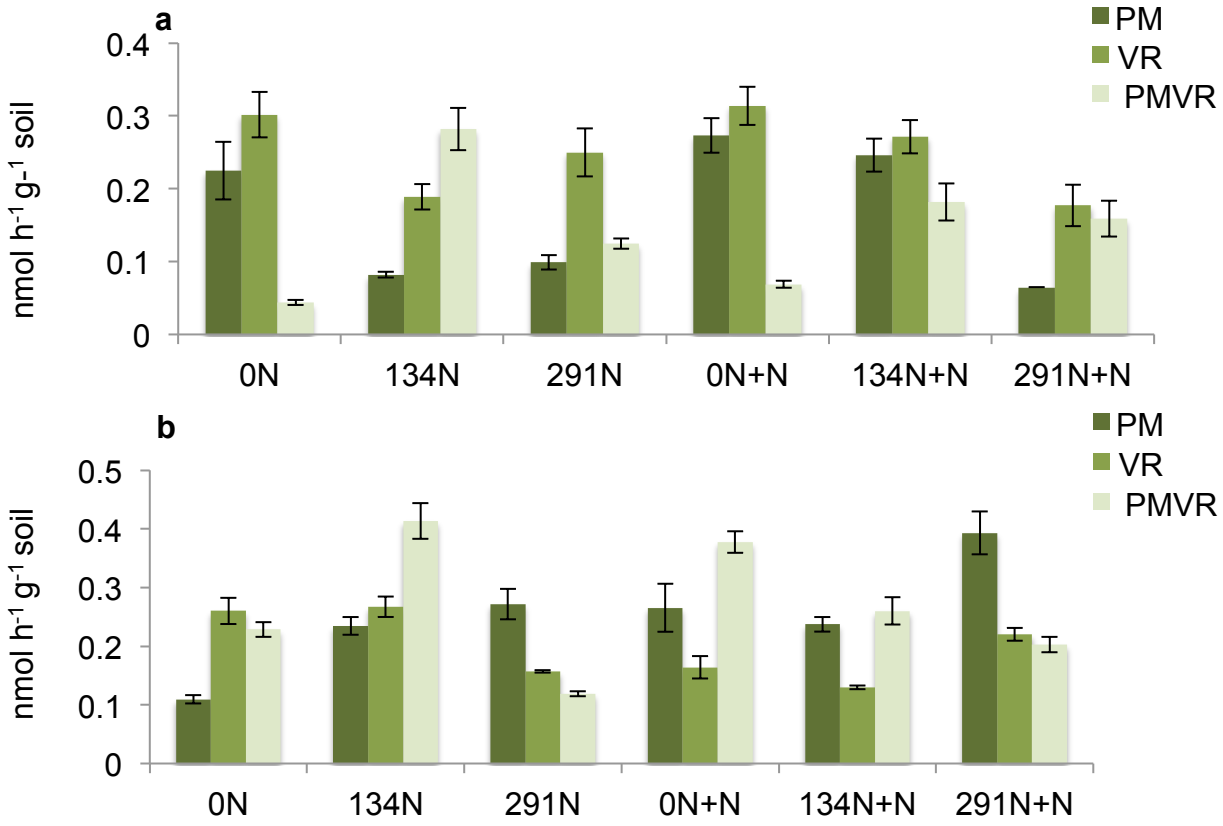

Figure 3.12. Leucine-aminopeptidase (LAP) activity measured on day (a) 37 and (b) 110 during a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

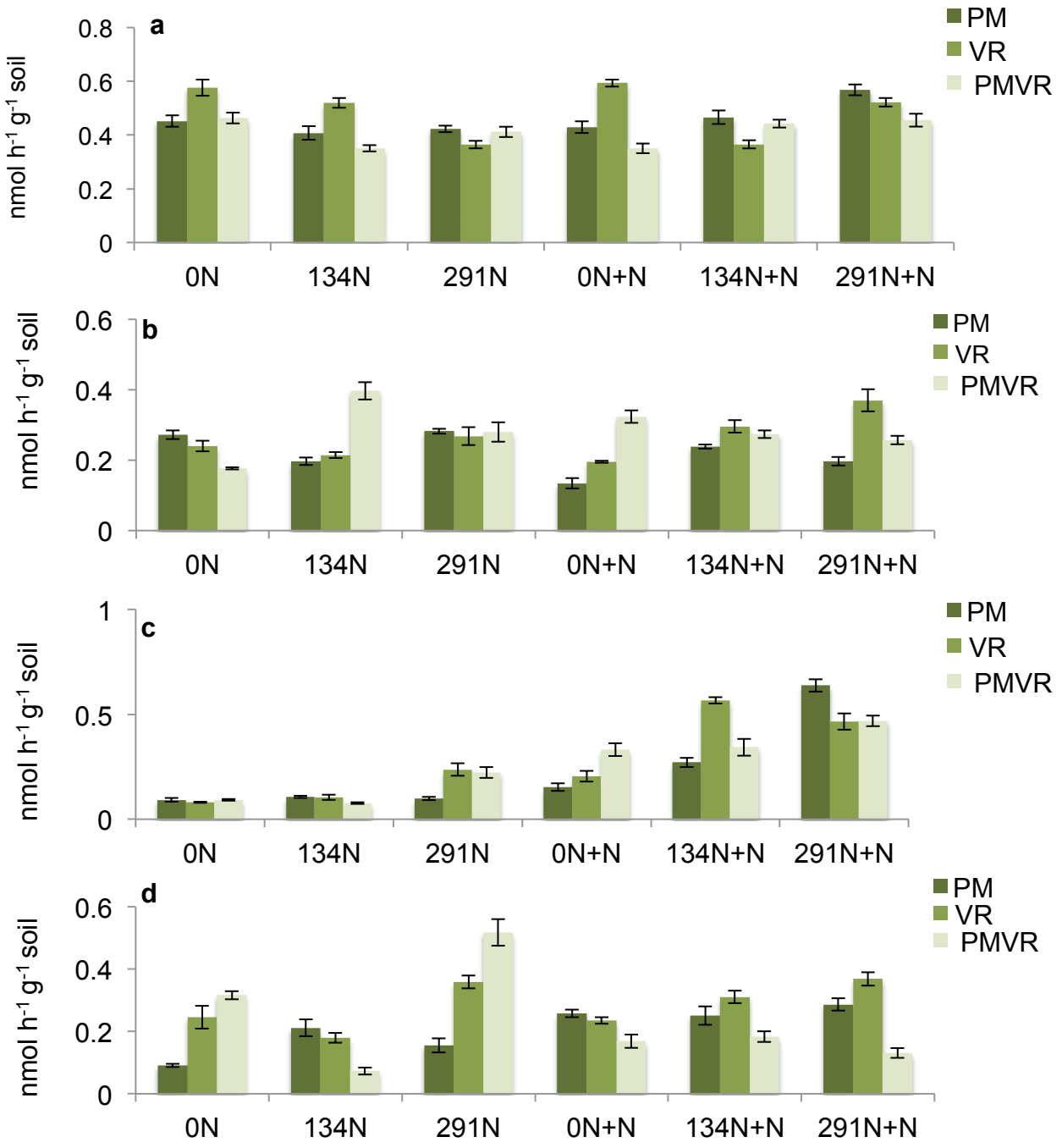

Figure 3.13. Urease activity measured on day (a) 37, (b) 110, (c) 204, (d) 362 throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4).

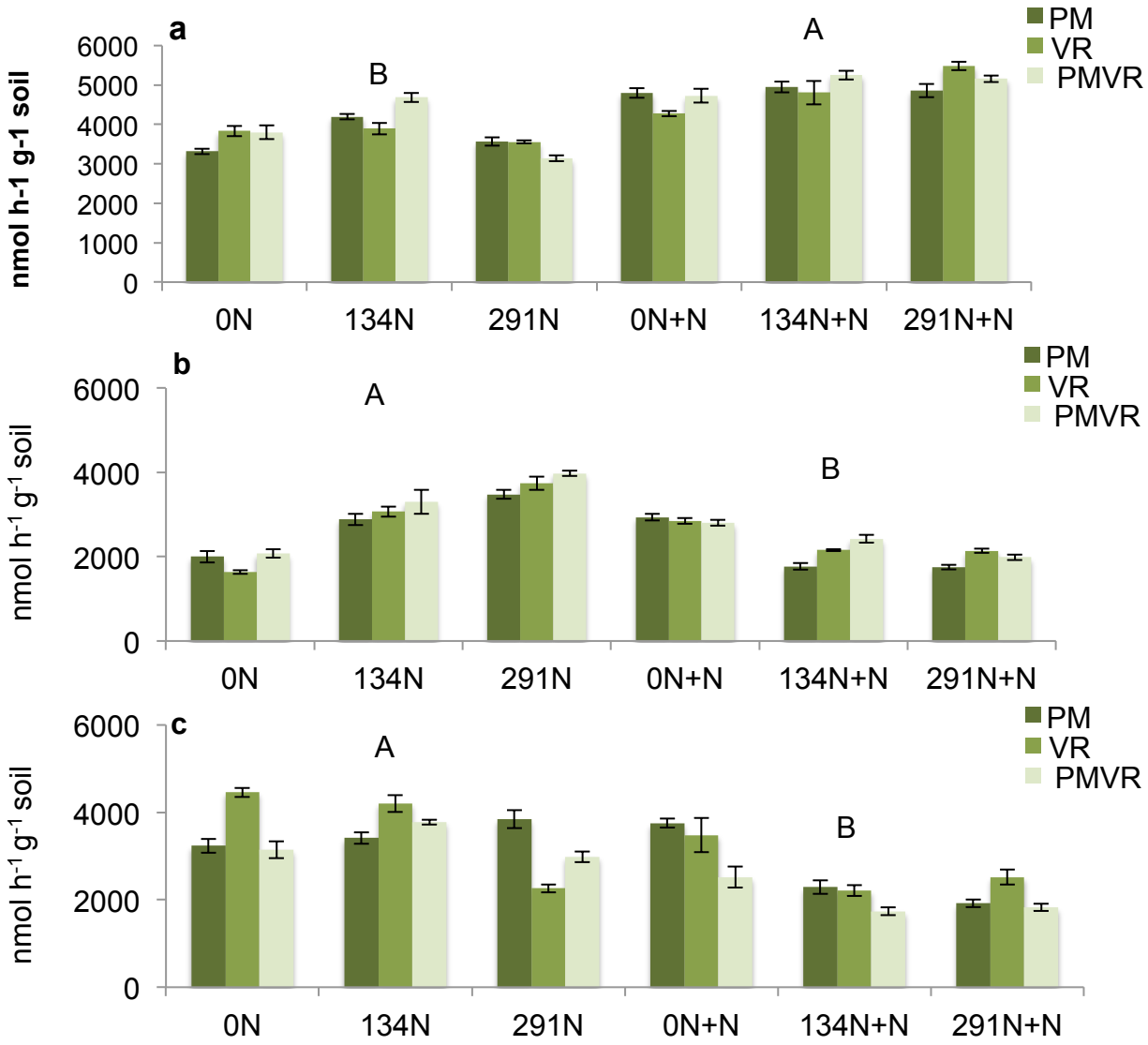

Figure 3.14. Cumulative extracellular enzyme activities of (a) cellulase (BG+CBH), (b) total oxidase (TOX), and (c) acid phosphatase (PHOS), throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between short-term N addition treatments.

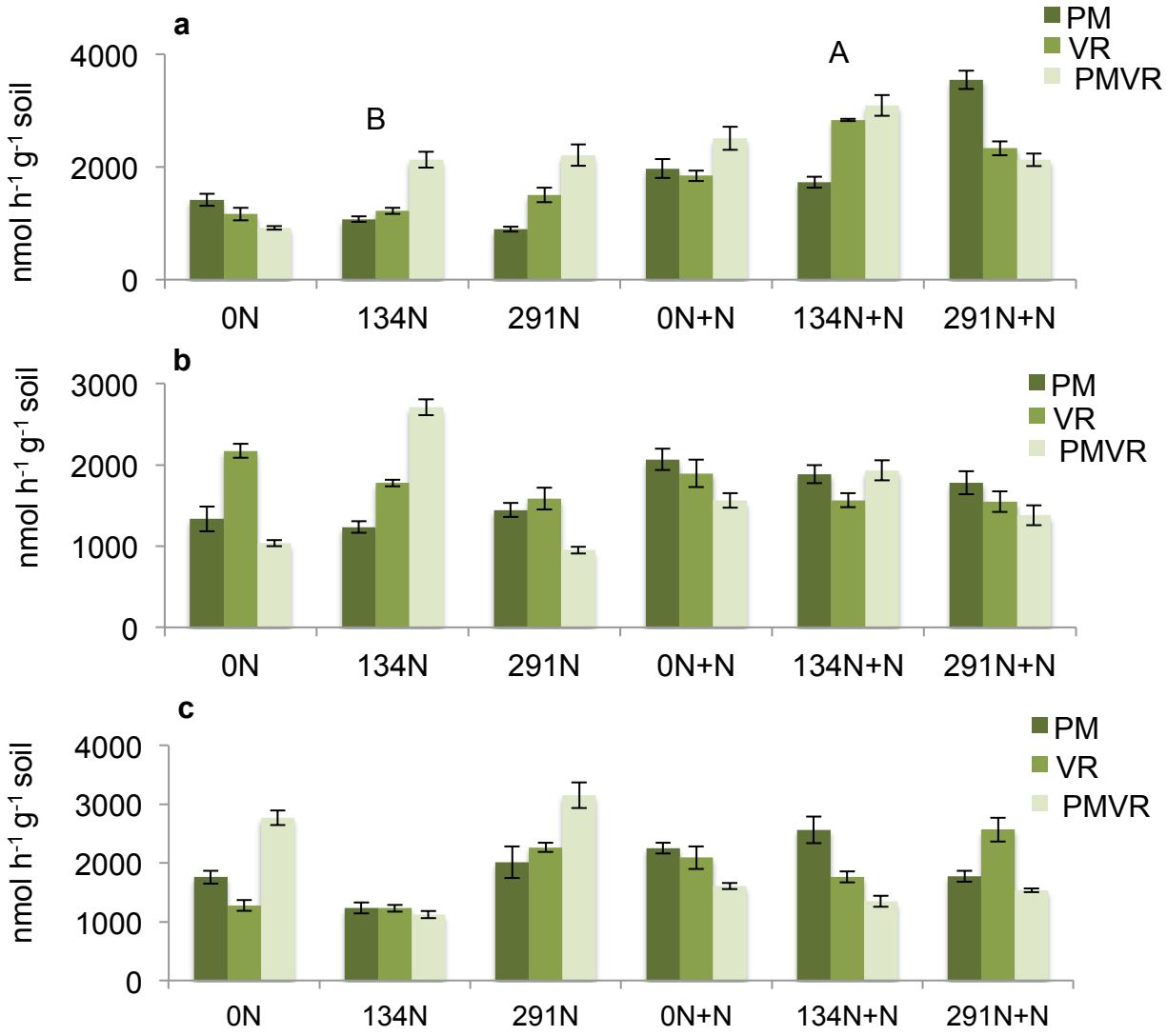

Figure 3.15. Cumulative extracellular enzyme activities (a) β -1,4,-N-acetyl glucosaminidase (NAG), (b) Leucine-aminopeptidase (LAP) and (c) Urease, throughout a year-long (362 days) laboratory incubation of soils that received long-term N additions of 0,134, or 291 kg N $ha^{-1}y^{-1}$ for 12 years mixed with cover crop residue mixtures (PM=field pea + Austrian pearl millet, VR=hairy vetch+ annual ryegrass, PMVR= field pea + Austrian pearl millet + hairy vetch+ annual ryegrass). Samples with short-term N addition are indicated with +N. Bars represent means ± one standard error of the mean (n=4) and letters indicate significant differences between short-term N addition treatments.

LITERATURE CITED

LITERATURE CITED

Aerts, R., & De Caluwe, H. (1997). Nutritional and plant-mediated controls on leaf litter decomposition of Carex species. Ecology 78:244–260.

Bending, G. D., & Turner, M. K. (1999). Interaction of biochemical quality and particle size of crop residues and its effect on the microbial biomass and nitrogen dynamics following incorporation into soil. Biology and Fertility of Soils, 29(3), 319–327.

Bohme, L., Langer, U., & Bohme, F. (2005). Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. Agriculture, Ecosystems and Environment, 109(1–2), 141–152.

Bonanomi, G., Manuela, C., Guido, I., & Stefano, M. (2014). Nitrogen transfer in litter mixture enhances decomposition rate, temperature sensitivity, and C quality changes. Plant Soil 381:307–321

Butenschoen, O., Valentyna, K., Mark, M., Franca, M., Dorothee, S., & Stefan, S. (2014). Litter mixture effects on decomposition in tropical montane rainforests vary strongly with time and turn negative at later stages of decay. Soil Biology & Biochemistry 77:121e128

Cardinale, B. J., Matulich, K. L., Hooper, D. U., Byrnes, J. E., Duffy, E., Gamfeldt, L., Balvanera, P., O'Connor, M. I., Gonzalez, A. (2011). The functional role of producer diversity in ecosystems. Am. J. Bot. 98, 572e592.

Carreiro, M. M., Sinsabaugh, R. L., Repert, D. A., & Parkhurst, D. F. (2000). Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81:2359–65.

Clark, C. M., Cleland, E. E., Collins, S. L., Fargione, J. E., Gough, L., Gross, K. L., Pennings, S. C., Suding, K. N., & Grace, J. B. (2007). Environmental and plant com- munity determinants of species loss following nitrogen enrichment. Ecology Letters 10, 596e607.

De Andrade Bonetti, J., Anghinoni, I., de Moraes, M. T., & Fink, J. R. (2017). Resilience of soils with different texture, mineralogy and organic matter under long-term conservation systems. Soil and Tillage Research, 174, 104-112.

Enrique, A. G., Bruno, C., Christopher, A., Virgile, C., & Steven, C. (2008). Effects of enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology, Ecological Society of America 81(9),pp. 2359–2365

Gartner, T. B., & Cardon, Z. G. (2004). Decomposition dynamics in mixed-species leaf litter. Oikos 104:230–246

Geisseler, D., & Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms - A review. Soil Biology and Biochemistry, 75, 54–63.

Geisseler, D., Lazicki, P. A., & Scow, K. M. (2016). Mineral nitrogen input decreases microbial biomass in soils under grasslands but not annual crops. Applied Soil Ecology, 106, 1–10.

Grandy, A. S., Salam, D. S., Wickings, K., McDaniel, M. D., Culman, S. W., & Snapp, S. S. (2013). Soil respiration and litter decomposition responses to nitrogen fertilization rate in no-till corn systems. Agriculture, Ecosystems and Environment, 179, 35–40.

Hättenschwiler, S., Vitousek, P. M. (2000). The role of polyphenols in terrestrial Ecosystem nutrient cycling. Trends Ecol Evol 15:238–243

Hector, A., Beale, A. J., Minns, A., Otway, S. J., Lawton, J. H. (2000). Consequences of the reduction of plant diversity for litter decomposition: effects through litter quality and microenvironment. Oikos 90:357–371

Hobbie, S. E. (1996). Temperature and plant species control over litter decomposition in Alaskan tundra. – Ecol. Monogr. 66: 503–522

Hobbie, S. E. (2005). Contrasting effects of substrate and fertilizer nitrogen on the early stages of litter decomposition. Ecosystems, 8(6), 644–656.

Hobbie, S. E. (2008). Nitrogen effects on decomposition: A five-year experiment in eight temperate sites. Ecology, 89(9), 2633–2644.

Hoorens, B., Aerts, R., & Stroetenga , M. (2003). Does initial litter chemistry explain litter mixture effects on decomposition? Oecologia 137: 578–586.

Kazanski, C. E., Riggs, C. E., Reich, P. B., & Hobbie, S. E. (2019). Long-Term Nitrogen Addition Does Not Increase Soil Carbon Storage or Cycling Across Eight Temperate Forest and Grassland Sites on a Sandy Outwash Plain. Ecosystems, 1-14.

Keeler, B. L., Hobbie, S. E., & Kellogg, L. E. (2009). Effects of long-term nitrogen addition on microbial enzyme activity in eight forested and grassland sites: implications for litter and soil organic matter decomposition. Ecosystems, 12(1), 1–15.

Knorr, M., Frey, S. D., & Curtis, P. S. (2005). Nitrogen Additions and Litter Decomposition: A Meta-Analysis. Ecology, 86(12), 3252–3257.

Ladha, J. K., Reddy, C. K., Padre, A.T., & Van Kessel, C. (2011). Role of nitrogen fertilization in sustaining organic matter in cultivated soils. J. Environ. Qual. 40, 1756–1766

Liu, P., Sun, O. J., Huang, J., & Han, L. L. X. (2007). Nonadditive effects of litter mixtures on decomposition and correlation with initial litter N and P concentrations in grassland plant species of northern China. Biol Fertil Soils 44:211–216.

Makkonen, M., Berg, M. P., Van Logtestijn, R. S. P., Van Hal, J. R., & Aerts, R. (2013). Do physical plant litter traits explain non-additivity in litter mixtures? A test of the improved microenvironmental conditions theory. Oikos 122, 987e997.

McDaniel, M. D., Grandy, A. S., Tiemann, L. K., & Weintraub, M. N. (2016). Eleven years of crop diversification alters decomposition dynamics of litter mixtures incubated with soil. Ecosphere, 7(8).

McDaniel, M.D., Grandy, A.S., Tiemann, L. K., & Weintraub, M. N. (2014). Crop rotation complexity regulates the decomposition of high and low quality residues. Soil Biology & Biochemistry 78: 243e254.

Meier, C. L., & Bowman, W. D. (2008). Links between plant litter chemistry, species diversity, and below-ground ecosystem function. Proceedings of the National Academy of Sciences, 105(50), 19780–19785.

Meier, C. L., & Bowman, W. D. (2010). Chemical composition and diversity influence non-additive effects of litter mixtures on soil carbon and nitrogen cycling: Implications for plant species loss. Soil Biology and Biochemistry, 42(9), 1447–1454.

Oelmann, Y., Wilcke, W., Temperton, V. M., Buchmann, N., Roscher, C., Schumacher, J., Schulze, E. D., & Weisser, W. W. (2007). Soil and plant nitrogen pools as related to plant diversity in an experimental grassland. Soil Science Society of America Journal 71, 720e729.

Paul, E. A. (2016). The nature and dynamics of soil organic matter: plant inputs, microbial transformations, and organic matter stabilization. Soil Biology and Biochemistry, 98, 109-126.

Ramirez, K. S., Craine, J. M., & Fierer, N. (2012). Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. Global Change Biology, 18(6), 1918–1927.

Sainju, U. M., Singh, B. P., & Whitehead, W. F. (2000). Cover crops and nitrogen fertilization effects on soil carbon and nitrogen and tomato yield. Canadian Journal of Soil Science, 80(3), 523–532

Saiya-Cork, K., Sinsabaugh, R., & Zak, D. (2002). The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biology and Biochemistry, 34(9), 1309–1315

Sinsabaugh, R.L., Reynolds, H., Long, T.M., 2000. Rapid assay for amidohydrolase (urease) activity in environmental samples. Soil Biology & Biochemistry 32, 2095e2097.

Sinsabaugh, R. L., Carreiro, M. M., & Repert, D. A. (2002). Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. Biogeochemistry, 60(1), 1–24.

Sinsabaugh, R. L., Belnap, J., Findlay, S. G., Shah, J. J. F., Hill, B.H., & Kuehn, K. A. (2014). Extracellular enzyme kinetics scale with resource availability. Biogeochemistry, 121, 287–304.

Stark, C., Condron, L. M., Stewart, A., Di, H. J., & O'Callaghan, M. (2007). Influence of organic and mineral amendments on microbial soil properties and processes. Applied Soil Ecology, 35(1), 79–93.

Tiemann, L. K., & Billings, S. A. (2011). Changes in variability of soil moisture alter microbial community C and N resource use. Soil Biology and Biochemistry, 43(9), 1837–1847.

Tiemann, L. K., Grandy, A. S., Atkinson, E. E., Marin-Spiotta, E., & McDaniel, M. D. (2015). Crop rotational diversity enhances belowground communities and functions in an agroecosystem. Ecology Letters, 18: 761–771.

Treseder, K. (2008). Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. Ecology Letters, 11, 1111–1120

Tu, L. H., Hu, H. L., Chen, G., Peng, Y., Xiao, Y. L., Hu, T. X., Tang, Y. (2014). Nitrogen addition significantly affects forest litter decomposition under high levels of ambient nitrogen deposition. PLoS ONE, 9(2)

Waldrop, M. P., Zak, D. R., & Sinsabaugh, R. L. (2004). Microbial community response to nitrogen deposition in northern forest ecosystems. Soil Biology and Biochemistry, 36(9), 1443–1451

Wang, J., Bu, W., Zhao, B., Zhao, X., Zhang, C., Fan, J., & Gadow, K. V. (2015). Effects of nitrogen addition on leaf decomposition of single-species and litter mixture in Pinus tabulaeformis forests. Forests, 6(12), 4462–4476.

Wardle, D. A., Bonner, K. I., & Nicholson, K. S. (1997). Biodiversity and plant litter: experimental evidence which does not support the view that enhanced species richness improves ecosystem function. Oikos 79:247–258

Wickings, K., Stuart G. A., Sasha, C. R., & Cory, C. C. (2012). The origin of litter chemical complexity during decomposition. Ecology Letters,15: 1180–1188.

Yevdokimov, I., Gattinger, A., Buegger, F., Munch, J. C., & Schloter, M. (2008). Changes in microbial community structure in soil as a result of different amounts of nitrogen fertilization. Biology and Fertility of Soils, 44(8), 1103–1106.

Zak, D. R., Holmes, W. E., White, D. C., Peacock, A. D., Ecology, S., & Aug, N. (2014). Plant Diversity , Soil Microbial Communities , and Ecosystem Function : Are There Any Links ? Plant Diversity , Soil Microbial Communities , And Ecosystem Function : Are There Any Links ? Ecology, 84(8), 2042–2050.

Zeglin, L. H., Stursova, M., Sinsabaugh, R. L., & Collins, S. L. (2007). Microbial responses to nitrogen addition in three contrasting grassland ecosystems. Oecologia, 154(2), 349–359.

Zhang, N., Shiqiang, W., Linghao, L., Jie, B., Mingming, Z., & Keping, M. (2008). Impacts of urea N addition on soil microbial community in a semi-arid temperate steppe in northern China. Plant Soil 311:19–28.

Zhang, W., Chao, L., Yang, Q., Wang, Q., Fang, Y., & Wang, S. (2016). Litter quality mediated nitrogen effect on plant litter decomposition regardless of soil fauna presence. Ecology, 97(10), 2834–2843.

Zhong, W., Gu, T., Wang, W., Zhang, B., Lin, X., Huang, Q., & Shen, W. (2010). The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant and Soil, 326(1), 511–522.

Zhou, X., Chen, C., Wu, H., & Xu, Z. (2012). Dynamics of soil extractable carbon and nitrogen under different cover crop residues. J Soils Sediments 12:844–853