CARBON-MEDIATED ECOLOGICAL AND PHYSIOLOGICAL CONTROLS ON NITROGEN CYCLING ACROSS AGRICULTURAL LANDSCAPES

By

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ABSTRACT

CARBON-MEDIATED ECOLOGICAL AND
PHYSIOLOGICAL CONTROLS ON NITROGEN CYCLING
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The sustainable intensification of agriculture relies on the efficient use of ecosystem services, particularly those provided by the microbial community. Managing for these ecosystem services can improve plant yields and reduce off-site impacts. For instance, increasing plant diversity is linked to positive effects on yield, and these beneficial effects are often mediated by the microbial community and the nutrient transformations it carries out. My dissertation has aimed to elucidate the mechanisms by which plant diversity improves agricultural production. In particular, I have focused on how changes to the amount and diversity of carbon (C) inputs affects soil microorganisms involved in the nitrogen (N) cycle. My work spans multiple scales of observation: from a global meta-analysis to mechanistic studies utilizing denitrification as a model system.

In a global meta-analysis, I found that increasing plant diversity through intercropping yields a net increase in extracellular enzyme activity. This effect varied by plant species and soil type suggesting that increases in the quality of nutrient inputs mediates these positive effects on microbial activity. Then, I performed a field study to determine how intercropping of cover crops into corn affects microbially mediated N-cycling activities and found no cover crop effects on soil nutrient pools or microbial activities. However, I observed how the availability of dissolved organic C (DOC) drives differences in microbial N-cycling processes. I then investigated how C availability drives

activity in microbial hotspots within the soil by comparing differences in denitrification potential in bulk soil versus the rhizospheres of corn and interseeded cover crops. Here, I found that denitrification rates were increased in the rhizospheres of all plant types, and this effect varied depending on the species of plant. I was able to further differentiate the impact of DOC and microbial biomass C on the rhizosphere effect and found that C availability was the primary driver of differences in denitrification rates between rhizospheres. Since plants provide many different forms of C to soil microbes, it is important to understand how the chemistry of C inputs affects microbial activity. I used a series of C-substrate additions to determine how C chemistry affects denitrifiers. I found that amino acids and organic acids stimulated the most nitrous oxide (N_2O) production and reduction. Although management and site affected overall rates of denitrification, C-utilization patterns of microbes were mostly similar between locations. To identify the mechanisms responsible for these effects, I performed a final experiment to track how denitrifiers utilized different C compounds. The C substrates that stimulated the most complete reduce of N_2O also were utilized with the lowest C-use efficiency (CUE). This suggests possible trade-offs between N_2O reduction and CUE, with important implications for how to manage microbial communities.

Overall, my work demonstrates that land management can impact microbial community activity by influencing the identity of soil C inputs. While the importance of increasing soil C inputs has been known, this dissertation supports the notion that the chemical identity of C inputs can exert significant controls on microbial activity. Moreover, by comparing microbial traits I highlight the importance of trade-offs in how microbially mediated C- and N cycling are coupled.

This dissertation is dedicated to my wife, whose sacrifices
have enabled me to embark upon this journey. Your encouragement has
been constant, your support unfailing, and your love essential.
This accomplishment is as much yours as mine.

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influence of the teachings of the Bahá'í Faith, which assert that the advancement of civilization depends upon scientific achievement proceeding hand-in-hand with social progress. Such ideas sparked an interest that has since been nurtured into a flame. Last but not least, I owe a huge debt of gratitude to my family. Since I was a child, you ensured that I had every opportunity that would enable me to pursue whatever path most interested me. And, of course, thank you to my wife, Zainab. Beyond your constant love and encouragement, you kept me organized and focused, no matter the day of the week or the time of day.

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KEY TO SYMBOLS AND ABBREVIATIONS

BEF	Biodiversity-ecosystem functioning relationship
BG	β-glucosidase
C_2H_2	Acetylene
CUE	Carbon-use efficiency
DEA	Denitrification enzyme activity
dN_2O	Relative production of N_2O
DON	Dissolved organic nitrogen
EEA	Extracellular enzyme activity
EL	East Lansing
K_2SO_4	Potassium sulfate
KBS	Kellogg Biological Station
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
MRC	Montcalm Research Center
N_2O	Nitrous oxide
NAG	N-acetylglucosaminidase (Chapter 2); N-acetylglucosamine (Chapters 4 & 5)
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
NPA	Nitrification potential activity
rN_2O	Relative N_2O reduction

RR	Response ratio
SOC	Soil organic carbon
SOM	Soil organic matter
SV	Saginaw Valley
TCA	Tricarboxylic acid cycle

CHAPTER 1:

INTERCROPPING INCREASES SOIL EXTRACELLULAR ENZYME ACTIVITY: A META-ANALYSIS

1.1. ABSTRACT

Intercropping has been shown to increase the productivity of agroecosystems, but the belowground mechanisms for this have not been well elucidated. The soil microbial community produces extracellular enzymes that are responsible for degrading plant residues and maintaining nutrient cycles in the soil. While the effects of plant diversity on enzyme activities have been studied, the results are often highly variable, both within and between studies. Therefore, to determine whether intercropping enhances nutrient cycling by increasing extracellular enzyme activity and to identify sources of variation in enzyme rates, I performed a meta-analysis drawing on 969 observations across 100 studies from around the world. I find that intercropping significantly increases enzyme activities by an average of 13% ($P < 0.001$) and that the intercropping effect varies by enzyme category, the type of plant used as the main crop or intercrop, and other experimental and environmental factors. Increased quantity and quality of plant residue inputs is a likely driver of the intercropping effect. The larger intercropping effect on increasing enzyme activity in nutrient-poor soils, together with differences between plant types, supports the view that soil nutrient status mediates patterns of microbial nutrient acquisition strategies. Future research should be aimed towards capturing regional variation within North American and Europe and towards identifying the specific drivers of the microbial response to increased plant diversity.

1.2. INTRODUCTION

Intercropping is the practice of growing two or more crops at the same time in the same area and has been used around the world, typically in small-acreage systems, to increase yield and reduce inputs (Brooker et al., 2015). Intercropping draws on the biodiversity-ecosystem functioning (BEF) relationship, wherein increased biodiversity is correlated with greater plant primary productivity (Tilman et al., 1996; Smith et al., 2008; Finney and Kaye, 2017). While this relationship has been well-studied in natural ecosystems, only limited work has thus far been performed in agroecosystems. Available studies suggest that a BEF relationship does exist within agriculture, with increased spatial diversity (e.g. through mixed cropping or intercropping) being positively correlated with increased biomass production and yield. Studies have observed a wide range in yield increases, with one review finding a mean increase of 40% compared to monoculture (Rao and Willey, 1980; Dai et al., 2013; Brooker et al., 2015; Chen et al., 2018). The mechanisms underlying the BEF relationship in agriculture and how spatial diversity affects belowground microbial communities have not been investigated.

The BEF relationship in agriculture may be related to plant-diversity effects on the soil microbial community, which is responsible for a host of ecosystem services that support agriculture (Swinton et al., 2007). These range from direct effects, such as plant-growth promotion, pathogen suppression, and the formation of symbiotic relationships involving mycorrhizal fungi and rhizobial bacteria (Van Der Heijden et al., 2008; Hayat et al., 2010). Indirect benefits provided by the soil microbial community include the maintenance of soil fertility by increasing nutrient provisioning, aggregate stability, and soil carbon (C) formation (Balestrini et al., 2015; Fierer, 2017). When properly exploited, the soil microbial

community can augment or replace synthetic pesticides and fertilizers. Indeed, legumes have been used in rotations for centuries to provide nitrogen (N) to crops by exploiting the microbial N-fixation reaction (Hayat et al., 2010). However, without proper management, the soil microbial community can also be a source of complementary ecosystem disservices, causing disease, contributing to the loss of soil organic matter, and producing greenhouse gas emissions (Robertson and Tiedje 1987; Zhang et al. 2007). For example, agriculture is responsible for 50% of global N_2O emissions, which are primarily due to nitrifying and denitrifying soil microbes (IPCC, 2014). It is therefore essential to identify the effects that various management practices have on the soil microbial community and to determine how best to enhance the ecosystem services the microbial community provides while minimizing potential negative impacts.

Plant community diversity in both managed and unmanaged systems has an impact on belowground community composition and activity. One meta-analysis of rotational diversity effects showed significant, large increases in microbial biomass compared to monoculture (McDaniel et al., 2014b), while another found significant effects of temporal diversity (i.e. rotations) on the richness and diversity of the soil microbial community compared to continuous monocultures (Venter et al., 2016). While these meta-analyses and several other studies (e.g. King and Hofmockel 2017; Tiemann et al. 2015) have indicated that management practices that increase plant diversity tend to also impact soil microbial communities, the effect this has on agroecosystem functioning remains unclear.

Nutrient cycling is a particularly important ecosystem service performed by the soil microbial community (Bardgett and Van Der Putten, 2014; Toju et al., 2018). Soil microorganisms produce extracellular enzymes to decompose organic residues and litter

inputs in order to obtain the materials required for energy production and growth (Dick, 1994; Sinsabaugh, 1994; German et al., 2011). These enzymes can become stabilized to soil particles and colloids, accruing over time (Burns, 1982). By releasing enzymes to the soil solution, microorganisms have the potential to either mineralize or immobilize essential nutrients and increase or decrease, respectively, their availability to crops. Plant diversity affects extracellular enzyme activity. For instance, enzyme activity associated with N- and phosphorous (P) acquisition was greater when cropping system diversity was increased through longer rotations compared to continuous monoculture (Tiemann et al., 2015). However, this study was focused on temporal diversity (i.e. crop rotation) rather than spatial diversity (i.e. intercropping). In studies where enzyme activities have been measured in monoculture compared to intercropping, results are highly variable, with the intercropping treatment showing greater (Kuang et al., 2010; Santos et al., 2018), similar (Wang et al., 2014), or reduced (Dai et al., 2013; Solanki et al., 2017) enzyme activity.

One of the principal difficulties associated with comparing enzyme data is the variety of methods used to assess enzyme activity. In addition, studies often range across a variety of cropping systems and a spectrum of climates and soil types, adding further to the heterogeneity between studies. A meta-analytical approach can therefore help to synthesize such wide-ranging data on enzyme activity. Meta-analysis provides a systematic and statistically rigorous way of integrating data and comparing studies, even in the face of methodological and experimental differences (Hedges and Olkin, 1985; Rosenberg et al., 2013). Meta-analysis has been widely used to synthesize soil enzyme data and assess the effects of various management practices, including fertilization, organic amendments, and

tillage (Kallenbach and Grandy, 2011; Geisseler and Scow, 2014; Zuber and Villamil, 2016; Luo et al., 2018; Zhou and Staver, 2019).

In my study, to determine the global effect of increasing spatial diversity on the functional potential of microbial communities in agroecosystems and to help me understand why results between studies are so variable I used a meta-analytical approach to combine and evaluate enzyme data. I hypothesized that (i) by increasing the plant diversity in an agriculture system, microbially-mediated nutrient cycling in the soil will be increased as indicated by elevated rates of extracellular enzyme activities, and that (ii) the effect of intercropping on enzyme activities will be affected by crop productivity, cropping system diversity, and the plant functional group of either the main crop or intercrop. I also examined other potentially moderating variables, such as climate factors, soil texture, pH, and fertilizer application rates that may help account for the variation in the intercropping effect on enzyme activities.

1.3. METHODS

1.3.1. Construction of Database

In total, my data set is comprised of 969 observations across 100 studies. Most studies were conducted in China and Asia, with a few studies located in South America and Africa. Only three field studies were identified from North America or Europe.

Studies were located using ISI Web of Knowledge using the following search terms: (intercrop* OR interseed*) AND (enzyme OR urease OR *sidase OR protease OR *terase OR *genase OR sucrase OR *olase OR *ulase OR *idase OR *terase). Abstracts were screened to find papers that met the following criteria: 1) had an intercropping treatment with a paired

monoculture control, 2) was an agricultural system, which included agroforestry, and 3) measured the activity of at least one soil extracellular enzyme.

Enzyme activities as well as associated metadata were extracted from the studies. If data were only reported on graphs, the values were extracted using WebPlotDigitizer (Rohatgi, 2019). Within studies, independent observations were considered as unique combinations of an enzyme rate and an intercropping pair. If enzyme activity for the same intercropping pair was reported multiple times within a study (i.e. multiple times across the growing season), an averaged value was used as a single observation.

Metadata associated with each study were also extracted and these included: latitude and longitude, type of experiment (greenhouse or field), mean annual precipitation, mean annual temperature (MAT), soil type, soil texture (% clay, % silt, % sand), pH, soil organic matter (SOM), soil organic carbon (SOC), total N and P, available N and P (i.e. extractable inorganic N or P), N fertilizer amount, N fertilizer type, P fertilizer amount, plant biomass or yield, length of study, sample type (rhizosphere or bulk soil), sampling depth, main crop, intercrop, and microbial biomass. Methodological information was also extracted, including general information on the assay protocol and whether fresh or air-dried soil was used.

If only mean separations were reported, standard deviations were back calculated from the smallest significant difference, using the indicated mean group size, significance level, and multiple-comparisons correction method. If no multiple-comparisons correction was specified, means were assumed to have been separated by Fisher's LSD (which would result in the most conservative back-calculated estimate of the standard deviation). If studies were missing key information, authors were contacted. If authors did not respond,

soil textural data was imputed from other studies reported from the same field site or from the Harmonized World Soil Database v1.2 (Fischer et al., 2008). If authors did not provide standard errors or standard deviations, either in the paper or upon contact, standard deviations were imputed based on the average CV for the provided values (Zuber and Villamil, 2016).

1.3.2. Meta-analysis

Statistical analyses were performed using OpenMEE (Wallace et al., 2017). Response ratios were calculated for each observation by taking the natural log of the average extracellular enzyme activity ($\bar{x}$) of the monocrop (control) and intercrop treatments, as described in Formula 1. The variance for the response ratio was calculated according to Formula 2. Formula 1 was also used to calculate an unweighted response ratio for plant biomass yield and microbial biomass C.

$$RR = \ln \frac{\bar{x}_{inter}}{\bar{x}_{mono}} \quad (1)$$

$$var(RR) = \frac{SD_{mono}^2}{n_{mono} \times \bar{x}_{mono}^2} + \frac{SD_{inter}^2}{n_{inter} \times \bar{x}_{inter}^2} \quad (2)$$

To calculate the intercropping effect overall and by sub-group, a random effects model was used, which accounts for both within- and between-study variability, using τ^2 estimated via the DerSimonian-Laird method. Accordingly, the response ratio was weighted using a variance term that accounted for between-study heterogeneity.

Analyses of various moderators were performed to identify drivers for the intercropping effect. The moderators examined included enzyme type, main crop (monocrop) type, intercrop type, sampling type (rhizosphere or bulk soil), experiment type (greenhouse or field), latitudinal zone (temperate or tropical), and fertilizer amount. I categorized enzymes based on the type of substrate they react with or the nutrient that is released with activity. Hydrolytic C enzymes are those that target relatively easy to access forms of C with a regularly repeating polymeric structure (invertase, cellulase, cellobiohydrolase, β-glucosidase, and β-xylosidase); oxidative C enzymes included peroxidase, phenoloxidase, and polyphenoloxidase; N enzymes included N-acetyl-glucosaminidase, proteases, peptidases, and urease; P enzymes included both alkaline and acid phosphatases as well as phytase (Table 1.2). A "general" category of enzymes was also included for enzymes routinely used as indicators of overall microbial activity but not associated with the acquisition of particular nutrients (Tabatabai, 1994; Alef, 1995; Prosser et al., 2015). This category included dehydrogenase and catalase as well as fluorescein diacetate (FDA) degradation activities.

To determine the role of plant type on the intercropping effect, I grouped observations according to the type of plant used as the main crop and the intercrop. In my analysis, each observation consisted of a treatment (i.e. a main crop grown with an intercrop) compared to a control (i.e. the main crop grown in monoculture). Accordingly, the main crop was defined as the crop that was grown in monoculture in the control group. Any particular species of plant could therefore be considered as the main crop or intercrop depending on how it was used in the study. For example, if the intercrop treatment included maize grown with soybean and the control group was only maize, then maize

would be considered the main crop and soybean the intercrop. On the other hand, if the intercropped treatment was compared to a control group consisting of soybean by itself, then maize would be considered the intercrop and soybean the monocrop. These treatment designations were defined according to the crops grown during the growing season(s) reported by the study. Additional elements of diversity, such as the use of rotations, were not specifically included in my analysis.

Plants were grouped according to the following categories: the grasses category included grains as well as forage grasses; legumes included pulses, oilseeds, and forages; woody species included shrubs and trees; the forbs category included non-woody plants that did not fall into the grasses or legumes categories. The same categories were used to group main crops and intercrops.

Sub-group means were determined within the categories of enzyme type, intercrop type, main crop type, sampling type, experiment type, latitudinal zone, and fertilizer amount. Differences within categories (between sub-groups) were considered to be significant if the two groups' 95% confidence intervals were non-overlapping.

I used the meta-regression tool in OpenMEE to assess relationships between the intercropping effect and the continuous experimental, edaphic, and environmental factors. For meta-regressions, τ^2 was estimated via maximum likelihood.

1.4. RESULTS

I found that, overall, intercropping increases enzyme activity by an average of 13%, a significant increase with a P-value less than 0.001 (Fig. 1.1). This effect was significant for all enzyme classes except oxidative enzymes (Fig. 1.1). The magnitude of the intercropping effect differs slightly between enzyme classes: the effect was significantly greater for N, P,

and S enzymes than oxidative enzymes. S-acquisition enzymes were also significantly higher than N- and P-acquisition enzymes as well as general enzymes.

Differences between plant types were pronounced with significant differences between the categories of plants used as intercrops and main crops (Fig. 1.1). The intercropping effect is apparent across all main crop types and all intercropping types, and these differences vary between enzyme types (Fig. 1.2). At an overall level, there is no significant difference between bulk soils and rhizosphere samples (Fig. 1.1). Likewise, there is no significant difference between greenhouse and field studies, or between studies in temperate or tropical environments.

Overall, the intercropping effect on microbial biomass C (MBC) explained a significant amount of variation in the intercropping effect on enzyme activity (Fig. 1.3). The intercropping effect on yield explained relatively less, but a still significant amount of variation in the intercropping effect on enzyme activity (Fig. 1.4). In addition, total N, total P, annual precipitation, sampling depth, and length of study are also suggested to be significant drivers of the overall intercropping effect (Table 1.1). Other environmental and experimental factors—such as, soil texture, pH, soil C, fertilization, and available nutrients—do not explain a significant amount of variation. However, some of these drivers are significant regressors when examined within different enzyme types. Interestingly, there is no clear linear relationship between amount of N fertilizer and the size of the intercropping effect but there are significant differences when fertilizer amount is combined into groups (Fig. 1.1). Moderate N-fertilizer applications have a significantly higher intercropping effect than low N-fertilizer applications, suggesting a possible unimodal response.

1.4.1. Carbon Enzymes

To determine how intercropping affected the nutrient cycling of specific elements, I analyzed the response ratio separately by enzyme class. Intercropping has a positive effect on the activity of C enzymes, increasing their rate of activity by an average of 14% (Fig. 1.1). While similar to the overall rate of increase, the increase in activity of C enzymes is subject to greater variability.

There is some variation in effect between plant types. The intercropping effect is significantly positive when grasses and woody plants are introduced as intercrops (Fig. 1.2A). The effect with grasses here is significantly higher than the effect with forbs.

Between main crop types, the effect on C enzymes is only significantly positive with legumes (Fig. 1.2B). That is, introducing an intercrop to a legume main crop significantly increases C-enzyme activities compared to when the legume is grown in monoculture. This effect with legume main crops is significantly higher than the effect with grasses or forbs.

Neither MBC nor yield were significantly related to the intercropping effect on hydrolytic C enzymes (Figs. 1.3 & 1.4). Of the other examined covariates, total N and length of study explained a significant amount of variation (Table 1.1).

1.4.2. Nitrogen Enzymes

The activity of N enzymes increases by 16% due to intercropping (Fig. 1.1). Across studies, there is relatively less variation than in C enzymes. However, there is some variation in this effect between plant types (Fig. 1.2). All plant types used as intercrop significantly increase enzyme activities (Fig. 1.2A). However, this effect does not differ significantly between intercropping plant types.

Between main crops, all plant types demonstrate a significant increase in N-acquisition enzyme activity when receiving an intercrop (Fig. 1.2B). This effect is significantly higher for woody plants as main crops than it is for legumes or grasses.

A large portion of the variation in the intercropping effect is accounted for by the response of MBC and yield to intercropping (Figs. 1.3 & 1.4). Total N and P account for a significant amount of the variation between studies, while length of study and sampling depth are also significant sources of variation (Table 1.1). Further, there is a significant inverse relationship between amount of N fertilizer and the intercropping effect within N enzymes (slope values not presented).

1.4.3. Phosphorous Enzymes

Intercropping increases P-enzyme activities by an average of 12% (Fig. 1.1). When added to a system as intercrops, all plant types except forbs result in a significant intercropping effect on P-enzyme activity (Fig. 1.2A). The effect with woody plants and legume intercrops is significantly higher than the effect with forbs. Moreover, the effect with woody plants is also significantly greater than with grasses.

Compared to growing as monoculture, intercropping into woody plants and grasses, but not into legumes or forbs, results in a significant increase in P-enzyme activity (Fig. 1.2B). The effect is greatest with woody plants, but is also significantly higher with grasses than with forbs or legumes.

Soil available N, annual precipitation, and study length were significantly related with the intercropping effect on P enzymes (Table 1.1). MBC explained the most variation within P enzymes (Fig. 1.3). Yield was not a significant covariate (Fig. 1.4).

1.4.4. Oxidative Enzymes

Intercropping elicits an overall 3% decrease in oxidative enzyme activity across all studies (Fig. 1.1), but low sample size makes it difficult to examine the sources of variation. There are no significant differences between intercrop or main crop plant types (Fig. 1.2). Intercropping with legumes and forbs shows a non-significant decrease in oxidative enzyme activity (Fig. 1.2A), while legume and grass main crops tends to have lower oxidative enzyme activities after intercropping (Fig. 1.2B).

Soil sand % and total P are significantly related with the intercropping effect on oxidative enzymes (Table 1.1).

1.4.5. General Enzymes

Similar to hydrolytic C-, N-, and P-acquisition enzymes, there is a significant 12% increase in general enzyme activity associated with intercropping (Fig. 1.1). The use of legumes and woody plants as intercrops results in a significantly positive intercropping effect, which is higher than that associated with intercropping grasses (Fig. 1.2A). Between main crops, all crop types except legumes demonstrate a significantly positive intercropping effect (Fig. 1.2B). Interestingly, intercropping into legumes is associated with a significant reduction in general microbial enzyme activity.

Significant sources of variation in the intercropping effect on general enzymes include pH, soil C, total N and P, annual precipitation, sampling depth, and length of study (Table 1.1). In addition, MBC and yield possessed significant positive relationships with the intercropping effect on general enzyme activity (Figs. 1.3 & 1.4).

1.4.6. Enzyme Assay Protocol

To ensure that differences in assay methodology or protocol did not introduce any systematic bias in the intercropping effect, in an early phase of database construction I compared effect sizes between different types of protocols. Almost every study used a bench-scale method to assess enzyme activities, with only two studies reporting the use of a microplate-scale assay. Urease and phosphatase were the only enzymes that were assayed using more than one type of method with at least ten observations, and methodology had no impact on the intercropping effect in either of these cases (Table 1.3). Moreover, within each enzyme type, there were no differences in effect size between procedures using fresh or air-dried soil (Table 1.4). Of course, studies varied further in terms of their exact assay procedures, but such variation is accounted for in my meta-analysis through the use of a random-effects model (Hedges and Olkin, 1985).

1.5. DISCUSSION

The studies in my analysis tend to represent the areas where intercropping is most commonly utilized (Brooker et al., 2015). The majority of these studies are in China, with many in Latin America and Africa. I found only a handful of reports on intercropping in North America and Europe, and only three of these included data on enzyme activity that met my criteria for inclusion in this analysis. This likely reflects the rarity of intercropping in North America and Europe; a USDA report found that as of 2012, just 2% of crop land in the United States utilized a "double cropping" system—which mostly refers to single-year rotations rather than intercropping—and only 1–2% of crop land utilized cover crops (Borchers et al., 2014). By contrast, intercropping in China occurs on about one-quarter of

arable land (Knörzer et al., 2009), and it is likewise common in Latin America and Africa (Vandermeer, 1992; Knörzer et al., 2009; Brooker et al., 2015).

Overall, I find that intercropping increases enzyme activities by an average of 13%, providing support for my hypothesis that increasing plant diversity through intercropping increases the functional potential of the belowground microbial community. This is consistent with others' findings that enzyme activities are impacted by plant species (Kourtev et al., 2002), management practice (Bandick and Dick, 1999; Tiemann et al., 2015), and land use (Waldrop et al., 2000).

1.5.1. Crop Productivity and Residue Quality

I hypothesized that the magnitude of the intercropping effect on enzyme activities would be determined by the productivity, diversity, and plant functional groups in the intercropping system. Intercropping increases C inputs through greater plant density when the space between rows of the main crop is utilized for the intercrop. Indeed, greater residue input is reported with intercropping in many of the studies included here (e.g. Chander et al., 1998; Santos et al., 2018; Zhou et al., 2011). In my analysis, of the studies that included yield or biomass data, I found a highly significant relationship between the intercropping-induced increase in plant biomass and the corresponding increase in enzyme activity; however, this relationship appears to be driven primarily by N- and general enzymes (Fig. 1.4). Increased plant productivity leads to increased C inputs to soils, which in turn stimulates the soil microbial community and promotes greater microbial biomass (Kallenbach and Grandy, 2011) and higher activity levels (Nyberg et al., 2000; Yang and Wu, 2011; Mancinelli et al., 2013). This combination of increased inputs and increased enzyme activities results in a more active nutrient cycle that often leads to greater N and P

availability for plants and a positive feedback to productivity (Randhawa et al., 2005; Piotrowska and Wilczewski, 2012).

Overall, enzyme activities in both bulk soil samples and the rhizosphere were significantly increased by intercropping with no difference in intercropping effect between the two sample types (Fig. 1.1). Bulk soil is relatively nutrient poor, and additional residue inputs are more likely to reach the bulk soil than the rhizosphere. The large intercropping effect in the bulk soil demonstrates the potential for microbes to synthesize and release hydrolytic enzymes in response to increased C and nutrient inputs from intercropping. Although average microbial activity is lower in the bulk soil, the volume of bulk soil far outweighs the volume of rhizosphere in most soils (Young et al., 2008; Kuzyakov and Blagodatskaya, 2015). Therefore, a similar increase in bulk soil enzyme activities will go a long way towards increasing overall nutrient cycling in the field. This points to intercropping as a potential avenue for improving the nutrient cycling of many annual systems, where most of the soil over a significant portion of the year is not in contact with plant roots.

The significant intercropping effect on enzyme activity in the rhizosphere suggests that intercropping elicits a systemic response that extends to the roots of individual plants. Many of the studies in this analysis focus on the rhizosphere, which is a microbial hotspot where the number of active microbes can be as much as twenty times greater than the bulk soil due to an increase in plant-derived C around the roots (Kuzyakov and Blagodatskaya, 2015; Ma et al., 2018). Belowground C inputs, such as root exudation and deposition, can increase with intercropping for the same reasons as identified for aboveground residues,

but these inputs may have a larger impact because they are already in close association with microbes and their enzymes.

Root exudates appear to play a large role in the intercropping effect. I noticed that the amount and composition of root exudates were reported to be affected by intercropping: in a field study Dai et al., (2013) described how different phenolic compounds were excreted by the same plants depending on whether they were in a monoculture or intercropping system, and B. Li et al., (2016) found that intercropping of maize and faba bean influenced the root exudation profile of maize as well as the N fixation activity of faba-bean-associated rhizobia. Further, in many of the greenhouse studies I identified, a complete or partial root-separation design was used; in both designs roots were prevented from direct physical contact but exudates could mix in the partial-separation treatments. The majority of these rhizosphere experiments report significantly less enzyme activity with complete barriers between intercrop roots, but no difference between partial- and non-separated treatments, suggesting that exudates rather than the roots themselves promoted enzyme activities (Xu et al., 2007; Hu et al., 2013; Khan et al., 2014; Q. Li et al., 2016; Li et al., 2018). Similar to how increases in the amount and diversity of aboveground C could accelerate nutrient cycling, differences in the quantity and composition of belowground root exudates could also stimulate the production of soil enzymes.

1.5.2. Plant Species and Type

In addition to increasing total C inputs, intercropping increases the diversity of C inputs to the soil, which has the potential to support greater biodiversity in soil communities (Tiemann et al., 2015; Eisenhauer, 2016; Venter et al., 2016) and increase

microbial biomass and soil C (McDaniel et al., 2014b). I was unable to test the response of enzyme activities to intercropping-system diversity *per se* due to a lack of studies at higher levels of diversity (i.e. > 2 species). However, another important impact of increasing diversity via intercropping on microbial activity is the potential for increasing overall crop residue quality, especially when transitioning from systems like monoculture maize or other crops with low-quality litters.

Residue quality, which can be roughly defined as the ease with which a residue can be decomposed, has been shown to have a strong effect on enzyme activities (Tiemann et al., 2011; McDaniel et al. 2014). Quality can be considered in terms of C:N ratio as well as the chemical composition of the residues. Residue N content has been suggested to be a stronger driver of microbial activity than soil nutrient status (Chander et al., 1998; Bini et al., 2013), and lignin content has been shown to be inversely related to mineralization rates and can be a better indicator of potential mineralization rates than N content alone (Kumar and Goh, 2003; Fanin and Bertrand, 2016). The exact C:N content of residues was rarely reported in the studies included in my meta-analysis; therefore, I used plant type as a broad-scale indicator of residue quality. Quality effects are suggested by the significant difference between systems that introduce a legume as an intercrop, which has narrow C:N residues, versus those that introduce a grass or forb that tend to have wider C:N residues (Fig. 1.1). Beyond N effects, overall nutrient balance and P content also appear to influence the degradation of crop residues. In one study included here, it was thought that greater microbial demand for P than N caused high-N litters to be decomposed more slowly than residues with a wider C:N ratio but higher P content (Santos et al., 2018).

I see further support for residue quality effects in the difference in the response of hydrolytic and oxidative C enzymes. Enzymes in these categories work on C from different types of plant tissues that differ in bioaccessibility. Oxidative enzyme activities generally increase only when systems are C limited, have high amounts of poor quality (wide C:N) residue inputs, or are experiencing high levels of stress or disturbance (Sinsabaugh, 2010; Xiao et al., 2018). On the other hand, hydrolytic C enzymes appear to be driven by different factors, and it is suggested that the ratio of hydrolytic-to-oxidative enzyme activities is positively related to C quality (Sinsabaugh and Shah, 2011). In my analysis, hydrolytic C enzyme activity significantly increases by 14%, while oxidative enzymes are not significantly different from zero and even display a trend towards decreasing activity with intercropping (Fig. 1.1). This suggests that across a variety of systems, intercropping increases the availability of higher quality litter to microbial communities and decreases nutrient stress in the soil environment.

Given the importance of residue type and quality as discussed above, it is not surprising that I also find that the magnitude of the microbial response to intercropping is sensitive to the plant type of both the intercrop and the main crop. Intercropping systems that introduce woody crops and legumes tend to elicit stronger effects on enzyme activities than other crop types (Fig. 1.1). It should be noted that some of the tree species used in the agroforestry systems examined here are leguminous and as such could provide a net input of N through fixation and the subsequent production of high-quality, narrow-C:N litter.

Insight toward the contributions of each plant species can be obtained by comparing plant-type differences within each enzyme class (Fig. 1.2). Intercropping with grasses had the most significant increase in C enzyme activities, while the effect of using a legume

intercrop on C enzyme activities was highly variable, which may be due to differences in overall residue quality with different main crop types. Unfortunately, there were not enough data points to test for differences between each main crop type paired with a legume intercrop. The molecular composition of legume and grass residues differs markedly. Grass residues have a higher composition of cellulose and structural components compared to legume residues and would require a greater investment in hydrolytic C-degradation enzymes to decompose. On the other hand, legumes generally have greater protein (N) and P content, thus an increase in N- and P-mineralization activity would be expected, as increased availability of nutrient-containing substrate can stimulate production of extracellular enzymes (Kumar and Goh, 2003; Allison and Vitousek, 2005; Sinsabaugh et al., 2008). Legumes can also increase inorganic-P mobility (Li et al., 2007), causing P from inorganic pools to be transferred into plant biomass, which can then be mineralized by microbial phosphatases. In addition, alleviation of N limitation through the introduction of an intercropped legume could stimulate the production of phosphatases in systems that are co-limited by N and P. The introduction of N would supply the nutrients required for production of phosphatases.

I also considered how the effect of intercropping varies between the plant types used as the main crop. Overall, between main crop types, woody crops had the largest overall intercropping effect and legumes the smallest, albeit still significantly positive, and forbs and grasses were in between (Fig. 1.1). I find additional significant differences between the main crop types within enzyme categories (Fig. 1.2B). For hydrolytic C enzymes, I see a significant increase after intercrops are introduced to a legume monocrop system, while no other main crop type had a significant response in C-enzyme activities. In systems with a

legume, or those with narrow-C:N residue inputs, N is assumed to be relatively more available while overall C inputs may be a more limiting factor for enzyme production and microbial activity (Tiemann and Billings, 2011; McDaniel et al., 2014a). Therefore, C- or energy-limited microbes in legume systems that receive an intercrop would respond positively to additional biomass inputs, producing more enzymes that target C substrates.

I also report that N- and P enzymes have a significantly greater response when woody monocrops receive an intercrop relative to when legume or grass monocrops receive an intercrop (Fig. 1.2B). Tree litter is typically of poor quality, and trees often translocate N and P from leaves before senescence and litterfall (Zechmeister-Boltenstern et al., 2015). This results in a C-rich but nutrient-poor residue input. Intercropping into woody species such as trees would therefore have a greater impact on the average quality of residue inputs to the soil and lead to larger increases in enzyme activities, as discussed above.

1.5.3. Effects on the Microbial Community

By far, changes in microbial biomass explained the greatest amount of variation in the overall intercropping effect on enzyme activity (Fig. 1.3; Table 1.1). While the degradation of plant and animal tissues can release enzymes to the environment, the soil microbial community is the primary producer of the enzymes responsible for nutrient cycling in the soil (Skujiņš and Burns, 1976). The strong and significant relationship between the response ratios of MBC and enzyme activity after intercropping provides compelling evidence that the increase in enzyme rates I observe after intercropping is mainly due to microbial activity. Further, the category of enzymes that indicate general microbial activity consists of enzymes thought to be mostly intracellular. The increase in

this general category therefore indicates an increase in microbial activity on a similar magnitude to the increase in most other nutrient-cycling extracellular enzymes (Fig. 1.1).

Given the role of the microbial community in the production of nutrient-cycling enzymes, it is interesting to consider how changes to the microbial community in terms of abundance and composition can affect enzyme production and nutrient cycling (Waldrop et al., 2000). For instance, saprotrophic fungi are relatively more prolific producers of cellulases than mycorrhizal fungi or bacteria (Taylor and Sinsabaugh, 2015; Wallenstein and Burns, 2015), while protease production is more widely distributed amongst members of the microbial community (Bardgett et al., 2006; Geisseler and Horwath, 2008; Geisseler et al., 2010). Therefore, intercropping effects on enzyme activities could be mediated by changes to the soil microbial community, especially over the long term.

Intercropping has been demonstrated to alter the composition of the soil microbial community (Zhou et al., 2011; Dai et al., 2013; Khan et al., 2014; Hernandez et al., 2015; Zheng et al., 2018). For example, within my analysis, microbial communities from the rhizospheres of some intercropped plants are more closely related to each other than to their monocultured counterparts (Q. Li et al., 2016; Bini et al., 2018). In some of these studies, the effect of intercropping is stronger than that of N fertilization (Dai et al., 2013; Chen et al., 2018). One study lasting multiple growing seasons shows this effect to persist over three years (Zhou et al., 2011). Bacteria tend to respond faster to intercropping treatments than fungi in some studies (Zhou et al., 2011; Khan et al., 2014); in others, intercropping increases total AMF colonization (Hernandez et al., 2015; Bini et al., 2018).

Plant-mediated effects on community composition can range from being general to specific. For example, some plants exert directional control over their associated microbial

communities (Hartmann et al., 2009), while at the same time microbial communities associated with one plant are able to inoculate the other plant in an intercropping system (Sun et al., 2009; Hernandez et al., 2015; Bini et al., 2018). However, such effects may depend on both the host plant and the presence or abundance of particular members of the microbial community. Sun et al. (2009) describe that intercropped alfalfa affected rye-associated microbial communities only when the alfalfa is first treated with a rhizobial inoculant. Other outcomes include a mixing of microbial communities, with both initial communities being represented about equally (Rachid et al., 2015; Q. Li et al., 2016), and synergistic increases during intercropping in the abundance of certain taxa that are less abundant when either crop is in monoculture (Sun et al., 2009; Rachid et al., 2015).

Functional differences may reflect changes in the composition of the microbial community in addition to, or instead of, changes to the functionality of the existing microbial community. From an observational perspective, ideally, functional shifts would occur alongside changes in the abundance of key genes involved in nutrient cycling, allowing for changes in functional potential to be detected through quantitative gene sequencing. For example, differences in N-cycling processes following intercropping with legumes have been accounted for by increases in the abundance of N-fixing microbes and ammonia-oxidizing bacteria (Sun et al., 2009; Wang et al., 2015). However, such links between function rates and the abundance of functional genes or key taxa are, more often than not, quite difficult to establish. With regard to extracellular enzyme production in particular, the relative abundance of enzyme-coding genes does not tend to have a consistent relationship to enzyme activity (Burns et al., 2013). One study included in my analysis was able to correlate cellobiohydrolase activity with greater expression of the

cellobiohydrolase gene, but within the same study the researchers did not find similar correlations between gene abundance and activity of β-glucosidase or β-xylosidase (Zheng et al., 2018). Nevertheless, since extracellular enzymes are the products of soil microbes, the membership of the microbial community and its genetic potential must exert a certain degree of control over the intercropping effect. Future work should utilize genomic tools in concert with manipulative experiments, as well as observational studies, to elucidate how environmental factors together with the abundance and expression of enzyme-coding genes interact to determine the functional potential of the soil microbial community.

1.5.4. Edaphic, Environmental, and Experimental Factors

1.5.4.1. Environmental and Edaphic Properties

Soil nutrient concentrations explained significant amounts of variation within the intercropping effect within various enzyme classes (Table 1.1). These relationships were particularly consistent within general enzymes, with significant inverse relationships with soil C, total N, and total P. Total N was also inversely related to the overall intercropping effect as well as that within C- and N-acquisition enzymes. That the intercropping effect is greater when there are fewer available nutrients suggests that intercropping tends to have the largest stimulatory effects on microbial activity in relatively more depleted soils. Such a relationship is predicted for the productivity of intercropped plants themselves by the stress gradient hypothesis, which posits that facilitative interactions between differing species become more likely as ecological conditions become more stressful (e.g. nutrient- or water limitation), resulting in greater primary productivity or nutrient complementarity (Brooker et al., 2015). The additional plant residues from intercropping would have a relatively greater effect in resource-poor soil, alleviating substrate limitation that may have

been preventing enzyme synthesis. These results could indicate that the greater effects of intercropping in stressful environments extend beyond plant productivity itself and go on to increase nutrient cycling activity in the soil and stimulate the microbial community.

Only within general enzymes was the relationship between pH and the intercropping effect statistically significant (Table 1.1). The intercropping effect was greater for general enzymes at lower pH. Differences in pH can affect cation exchange with clays, impacting the availability of enzyme substrates as well as important enzyme co-factors, such as iron, magnesium, and manganese ions, which tend to be depleted in many acidic soils. Therefore, intercropping could have a greater effect in mobilizing and distributing nutrients in lower pH soils. Enzyme activity is also directly affected by pH, with most soil enzymes exhibiting maximum activity at slightly acidic pH, although this varies by enzyme and between soils (Turner, 2010; German et al., 2011). At a lower pH, activity of enzymes with more acidic optima could therefore be more responsive to increases in substrate and nutrient availability.

Water availability, retention, and distribution can also impact the intercropping effect (Morris and Garrity, 1993; Brooker et al., 2015; Hernandez et al., 2015). Here I found that overall, mean annual precipitation was positively related to the intercropping effect, with the amount of variation explained by precipitation in the intercropping effect of P-acquisition and general enzymes being particularly high (Table 1.1). C- and nutrient mineralization are consistently linked to soil moisture (Crowther et al., 2019). Increasing plant density and diversity through intercropping can improve water infiltration by creating a more widely distributed network of soil pores formed by roots. By limiting the exposure of bare soil to the physical impact of precipitation, greater plant densities can also

reduce soil crusting and surface run-off, providing a more favorable environment for microbial activity. On the other hand, there is also the potential for intercropping to improve microbial activity in drier systems as well. Differential rooting zones between intercropped plants (especially if one is a tree or shrub) can exploit water resources from different parts of the soil profile and redistribute moisture to drier areas, stimulating microbial activity (Brooker et al., 2015; Hernandez et al., 2015). In this connection, one study demonstrates how intercropping with indigenous shrubs increases water retention during times of water stress in an arid environment; it is suggested that this promotes microbial activity (Hernandez et al. 2015; Bogie et al. 2018).

Other environmental factors not quantified here may also play a role in increasing or decreasing enzyme activity. Variables such as weather and season can be difficult to control, casting uncertainty on perceived trends within a single study. For example, studies that took multiple time points throughout the season often found widely varying data (for the purpose of my analyses, I averaged measurements taken multiple times over a season; e.g. Bini et al., 2013; Liu et al., 2014; Ma et al., 2017; Wang et al., 2014; Xiao et al., 2012). Much of this variation comes from the timing of plant development and associated differences in plant nutrient uptake, root exudation, and litter inputs (Zhou et al., 2011; Bini et al., 2013). Regardless, while the wide variation throughout the growing season makes it difficult to draw conclusions from the data presented by one study, in my meta-analysis a decided trend has emerged—across a number of sites and experimental conditions—that intercropping increases enzyme activities.

In addition, I found there were no significant differences in the intercropping effect between greenhouse and field studies, suggesting that while environmental factors could

contribute to the variation in the intercropping effect, it may not negate it. It is also worth noting in this connection that the greenhouse studies included in my analysis were, for the most part, pot experiments and were not meant to model or evaluate commercial greenhouse systems.

Interestingly, I found no significant differences in the intercropping effect between latitudinal zones (Figs. 1.1 & 1.5), even though differences may be expected given that the edaphic properties and environmental conditions between temperate and tropical soils differ widely. Tropical soils are highly weathered and are typically poor in nutrients, particularly P. That the effect of intercropping is no different between tropical and temperate environments suggests that, from a microbial perspective, the effects of increased plant diversity and nutrient inputs are similar across a wide range of locations where row-crop agriculture is practiced. Together with other studies that have found consistent microbial responses to environmental drivers at global scales (Fierer et al., 2009; Leff et al., 2015), my results support the generalizability of environmental controls on microbial function across a broad spectrum of geographic locations.

1.5.4.2. Fertilizer Application Rate

I examined the effect of N fertilizer by regressing enzyme-activity response ratio with fertilization rate and by binning studies into N-application categories. There is no significant linear relationship between the overall intercropping effect and application rate (Table 1.1), but studies that that applied a moderate amount (between 100-250 kg N ha^{-1}) have a significantly larger intercropping effect than those with low rates of fertilizer application (less than 100 kg N ha^{-1}; Fig. 1.1). The opposing influences of residue quantity and nutrient availability could account for this unimodal pattern. Compared to no

fertilization, low to moderate rates of application stimulate plant growth without exceeding plant demand. As a result, excess fertilizer nutrients are not available to the microbial community but increased residue inputs stimulate enzyme activity. On the other hand, applying fertilizer at higher rates, in excess of plant demand, could increase nutrient concentrations for the microbial community and reduce the need for microorganisms to invest in nutrient-acquisition enzymes.

While there is no relationship between fertilization and overall enzyme activity, there is a significant inverse relationship between fertilization and N-enzyme activities, as may be expected. Intercropping produces a smaller effect on stimulating N enzymes with greater fertilization (Table 1.1). In addition, total soil N also possesses a negative relationship with the intercropping effect on N-mineralization enzymes. This suggests that greater N availability moderates intercropping-induced increases in N-mineralization activity. Extracellular enzymes are expressed in response to a combination of microbial demand and substrate availability (Allison and Vitousek, 2005; Sinsabaugh et al., 2008). Economic theories of microbial metabolism suggest that certain induced enzymes, particularly those for N mineralization, are expressed only when labile sources of the target nutrient are limiting and there is a potential supply of the nutrient in complex forms (Allison and Vitousek, 2005; Chen et al., 2014). The presence of labile N provided as fertilizer could alleviate N demand and prevent the induction of enzymes for the acquisition of this already available nutrient. Nevertheless, another meta-analysis that investigated the long-term effects of fertilizers on enzyme activities found that fertilization had no effect on N-mineralization activity and significantly increased C-cycling enzymes and acid phosphatase (Geisseler and Scow, 2014). The lack of a consistent relationship of N-

fertilization on enzyme activities could be due to the manifold effects that fertilization has on the cropping system, including increases in primary production, soil pH, and overall nutrient balance.

1.5.4.3. Length of Study

One of the strongest covariates with the overall intercropping effect is length of study, with the longest studies having the largest effects (Table 1.1). Temporal dynamics of increased plant diversity have been well-studied in natural ecosystems, where the benefits of diversity—including improved biomass production and soil nutrient status—accrue over time (Cardinale et al., 2007; Reich et al., 2012). The significant length-of-study effect here demonstrates that it may also take time in agricultural systems for the effects of plant diversity to be reflected in the functioning of soil microbial communities.

In addition, previous work in agroecosystems has shown that microbial communities from more diverse rotational histories process organic residues faster than monocultured counterparts independent of plant diversity at the time of soil collection, suggesting that long-term diversity can produce persistent legacy effects in the activity of the soil microbial community (McDaniel et al., 2014a; Schipanski et al., 2014; Crème et al., 2018). This could be due in part to the fact that soil enzymes can become stabilized in the soil long after release from the cell where they were originally synthesized (Burns, 1982). Certain management practices can stimulate the production of nutrient-cycling enzymes, and as extracellular enzymes become stabilized, such increases can accumulate over the course of the practice, with potential nutrient-cycling activity increasing year-to-year.

1.6. CONCLUSION

Sustainable intensification of agricultural production will require relying more on ecosystem services and less on synthetic chemical inputs. As described by the BEF hypothesis, the provisioning of ecosystem services is enhanced through greater plant diversity. Therefore, intercropping can support agricultural production by increasing plant diversity and ecosystem functioning. Here, I argue for nutrient cycling as a possible mechanism by which intercropping improves crop productivity by increasing enzyme activity in the soil.

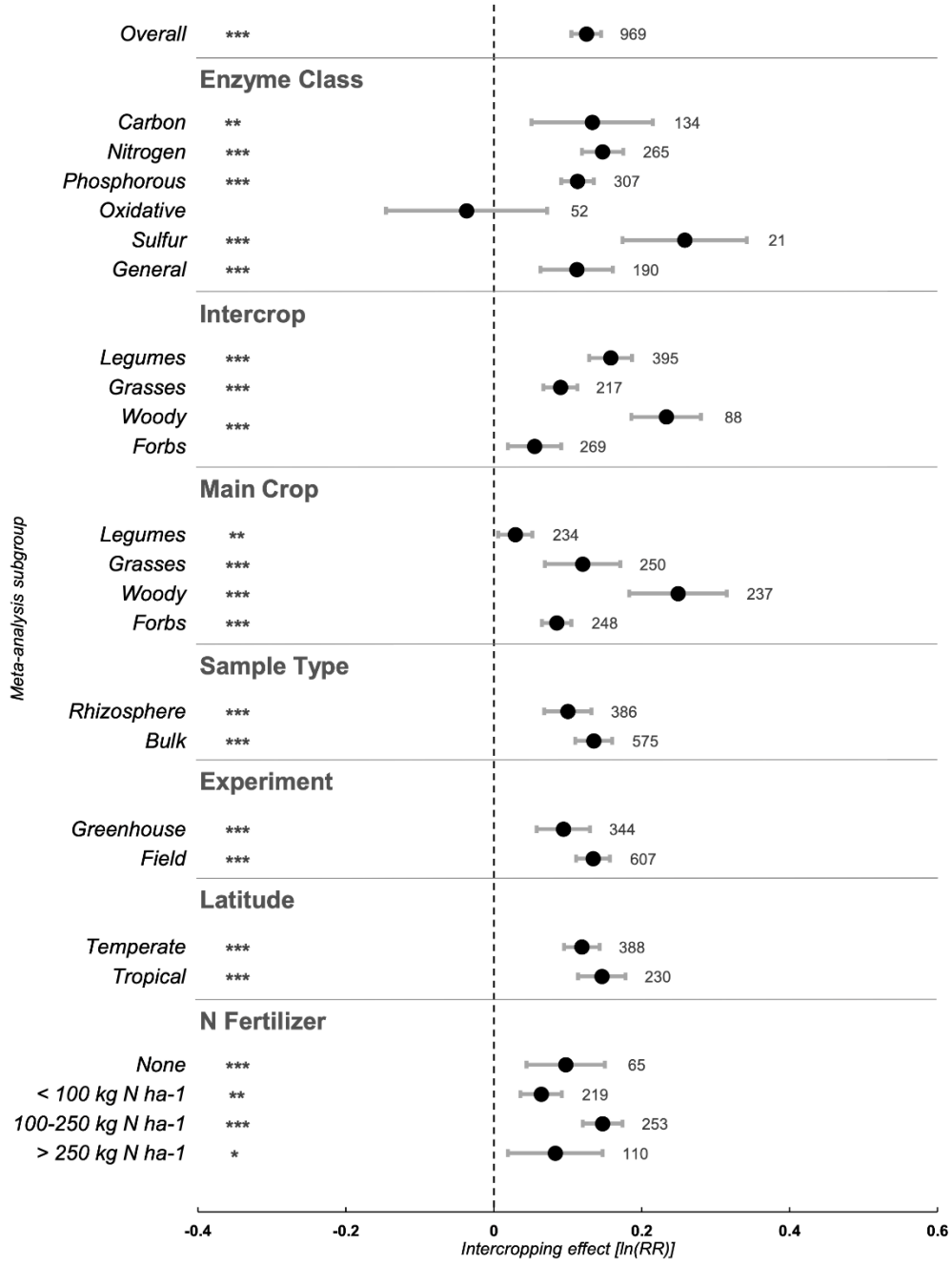
Extracellular enzymes play a key role in nutrient cycling, and I find that intercropping significantly increases enzyme activity across most enzyme classes and plant types. I suggest that this intercropping effect is mediated by responses of the soil microbial community to increases in the quantity and quality of residue inputs. I see support for this in the specific effects that intercropping has on the activity of different classes of enzymes across various plant-type combinations. In addition, much of the variation in the intercropping effect is accounted for by intercropping-induced increases in microbial biomass and general microbial activity. Given the role of the soil microbial community in producing extracellular enzymes, the effect of intercropping on microbial community structure and function remains an important question requiring further investigation.

APPENDICES

APPENDIX A:

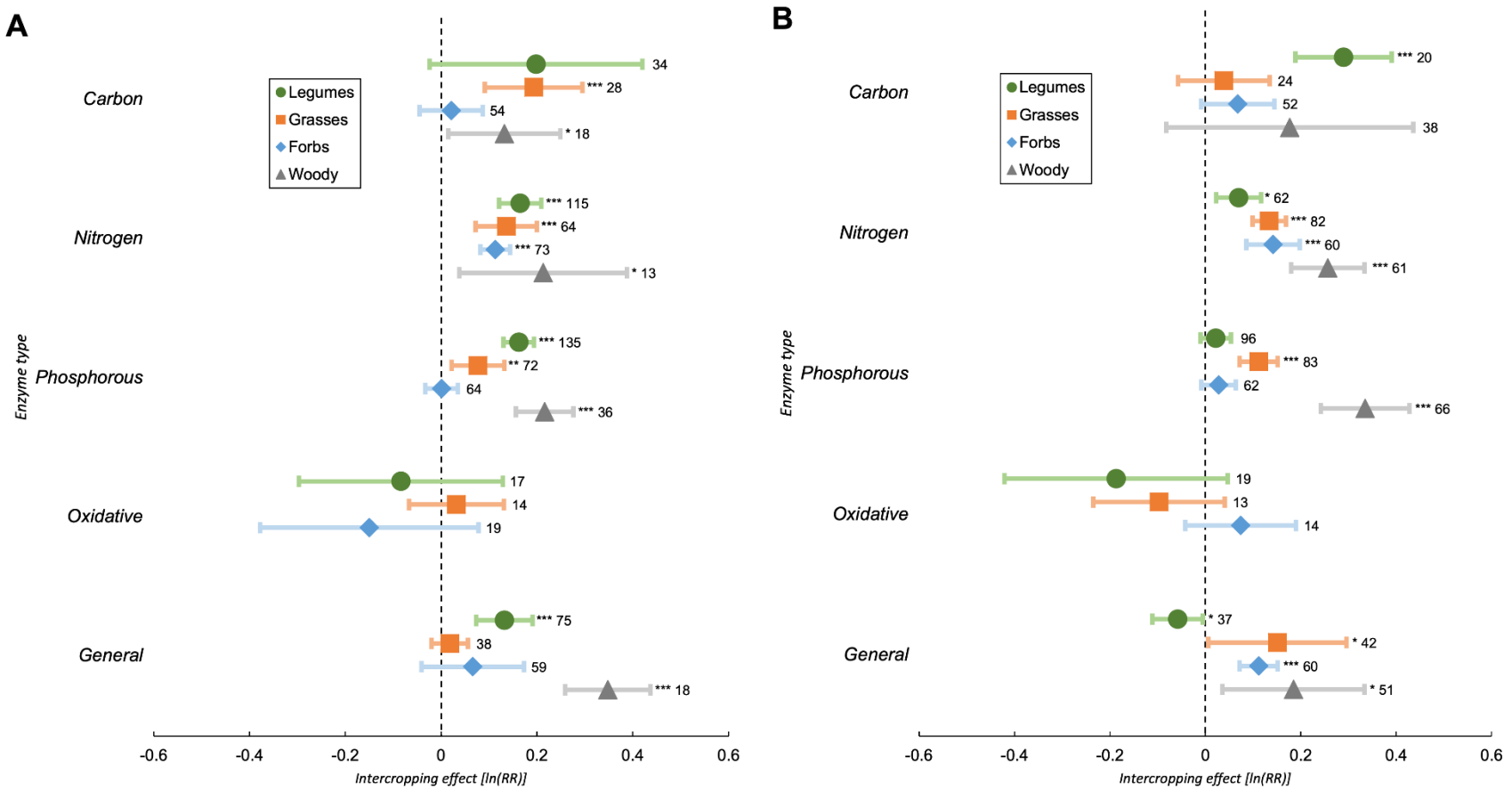
FIGURES

Figure 1.1: Overall intercropping effect and intercropping effect within sub-group categories



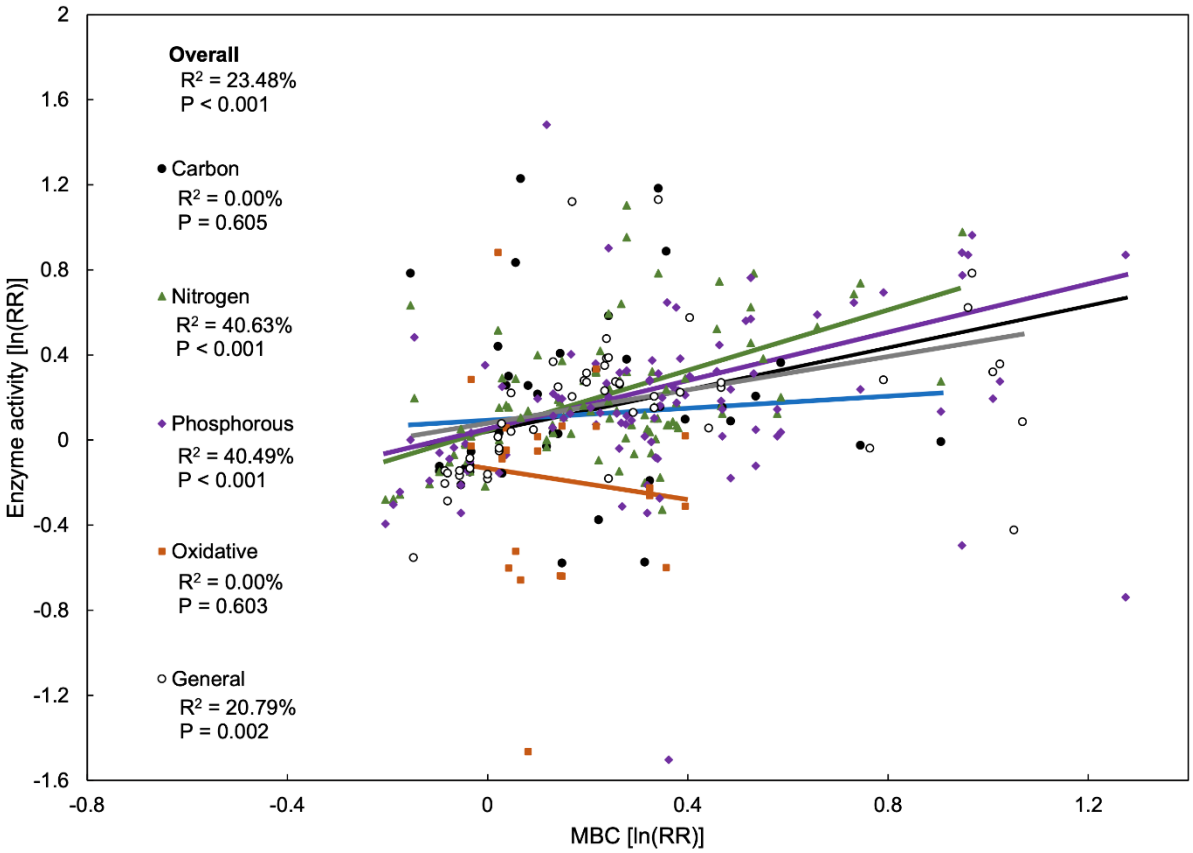
Values are shown as the mean log response ratio of the intercropping effect with 95% confidence intervals. Means significantly different from zero are indicated by asterisks next to sub-group labels (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The number of observations within each sub-group is indicated to the right of the confidence interval.

Figure 1.2: Intercropping effect by enzyme type and between plant type of the intercrops (A) and main crops (B)



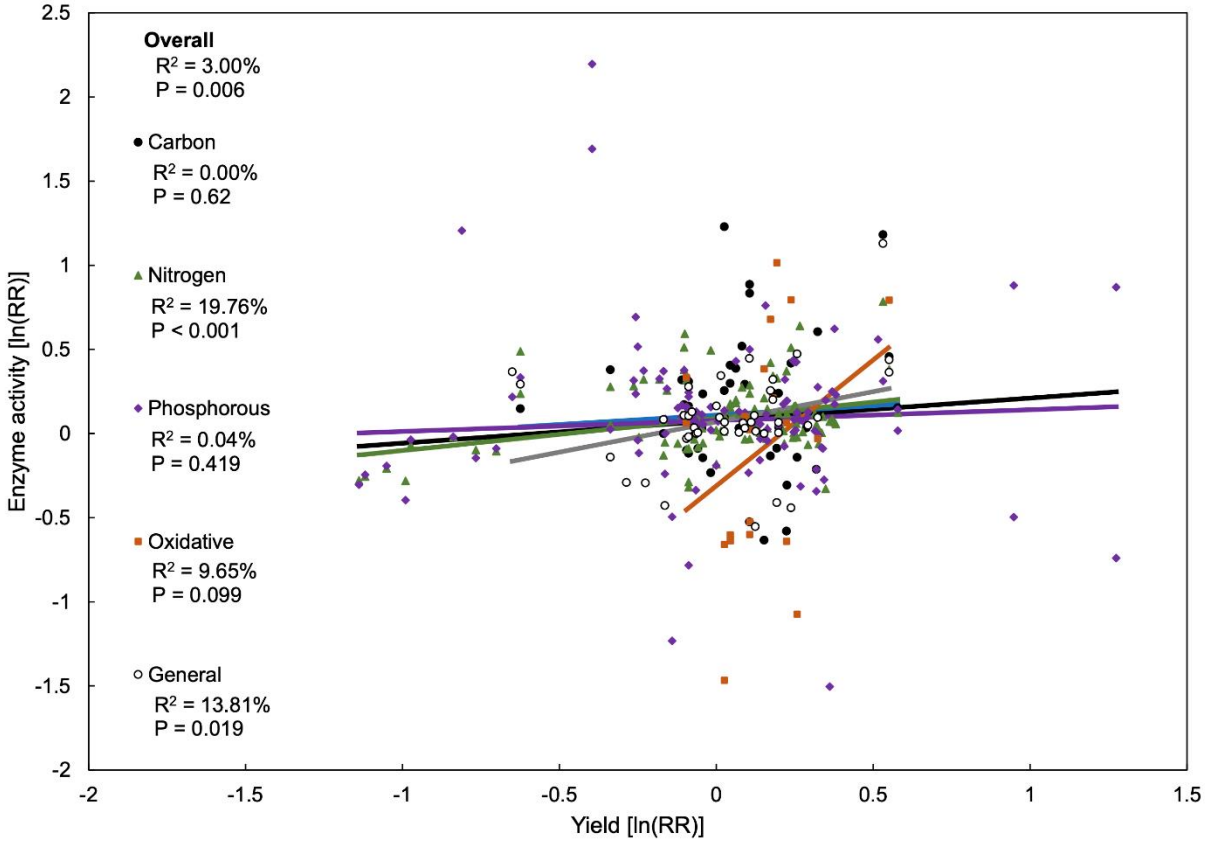
Values are shown as the mean log response ratio of the intercropping effect with 95% confidence intervals. Means significantly different from zero are indicated by asterisks to the right of the confidence interval (*, P < 0.05; **, P < 0.01; ***, P < 0.001), followed by the number of observations within each sub-group.

Figure 1.3: Relationship between the intercropping effect on enzyme activities and the intercropping effect on microbial biomass carbon



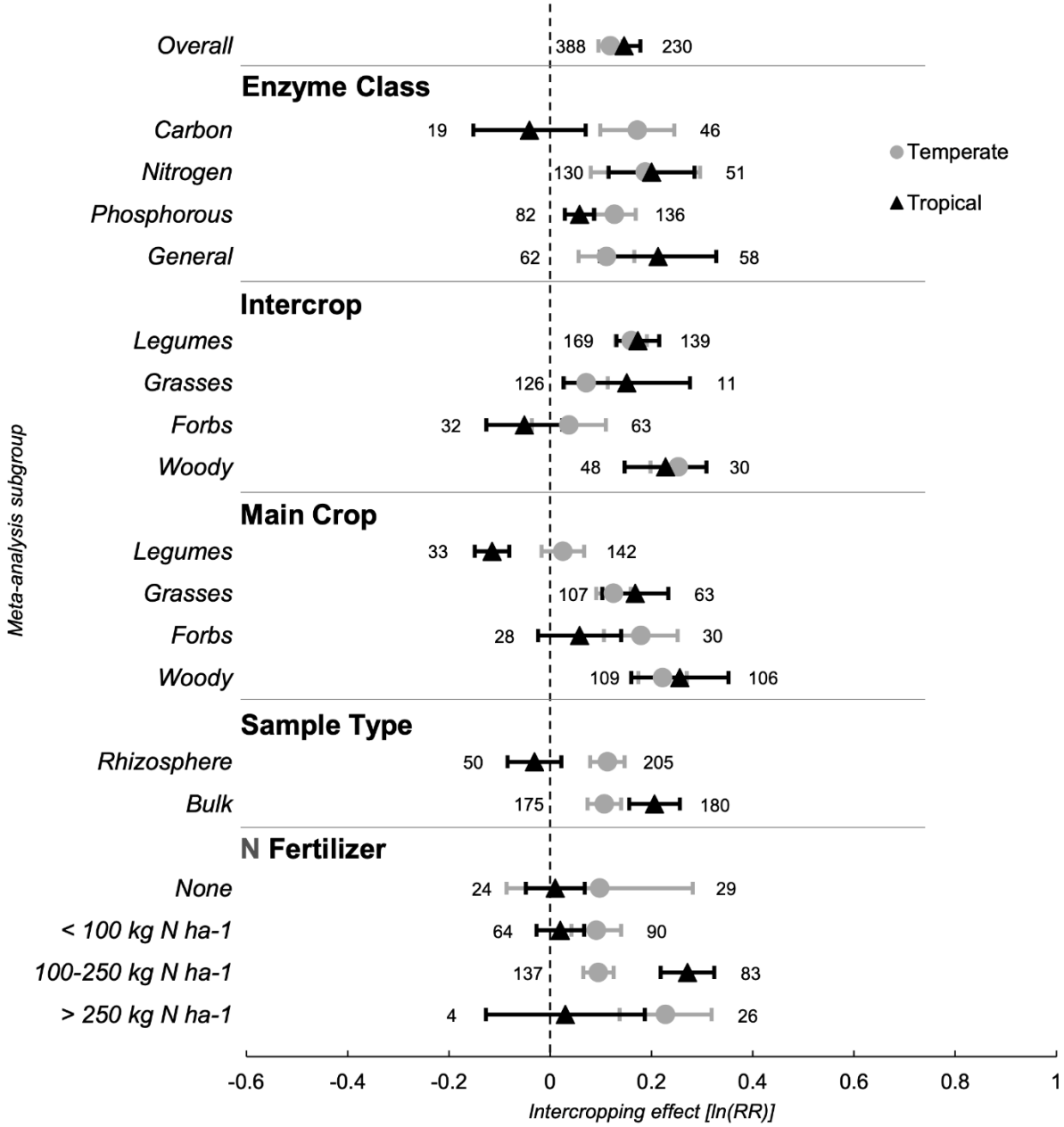
Effects are expressed as log response ratios. Regression lines show the relationship between microbial biomass carbon (MBC) and the enzyme intercropping effect for the overall dataset (black) and within the sub-categories of hydrolytic carbon-acquisition enzymes (blue), nitrogen-acquisition enzymes (green), phosphorous-acquisition enzymes (purple), oxidative carbon-acquisition enzymes (orange), and enzymes indicative of general microbial activity (gray).

Figure 1.4: Relationship between the intercropping effect on enzyme activities and the intercropping effect on plant biomass yield



Effects are expressed as log response ratios. Regression lines show the relationship between plant yield and the enzyme intercropping effect for the overall dataset (black) and within the sub-categories of hydrolytic carbon-acquisition enzymes (blue), nitrogen-acquisition enzymes (green), phosphorous-acquisition enzymes (purple), oxidative carbon-acquisition enzymes (orange), and enzymes indicative of general microbial activity (gray).

Figure 1.5: Differences in intercropping effect between studies in temperate and tropical regions



Differences are presented for the overall intercropping effect and the intercropping effect within sub-group categories. Values are shown as the mean log response ratio of the intercropping effect with 95% confidence intervals. The number of observations within each sub-group is indicated next to the confidence intervals.

APPENDIX B:

TABLES

Table 1.1: Strength of correlations between continuous moderators and the intercropping effect

	Overall			Carbon Enzymes			Nitrogen Enzymes		
	R^2	P	*n*	R^2	P	*n*	R^2	P	*n*
Environmental and Edaphic Factors									
% Clay	0.15%	0.152	592	0.17%	0.268	79	0.61%	0.206	163
% Sand	0.23%	0.171	557	0.00%	0.552	78	0.00%	0.389	162
pH	0.22%	0.177	882	2.01%	0.132	115	1.97%	0.091	226
Soil C	0.00%	0.516	775	0.00%	0.852	104	0.00%	0.407	221
Total N	**1.67%**	**0.003**	**555**	**10.38%**	**0.004**	**78**	**5.40%**	**0.004**	**160**
Available N	0.25%	0.173	604	0.00%	0.932	93	0.00%	0.315	165
Total P	**2.35%**	**0.002**	**400**	0.00%	0.398	67	**5.33%**	**0.007**	**121**
Available P	0.00%	0.447	814	2.22%	0.054	104	0.00%	0.823	217
Annual Precipitation	**2.95%**	**<0.001**	**478**	3.33%	0.298	48	0.00%	0.787	145
Experimental Factors									
N Fertilizer	0.00%	0.692	563	0.00%	0.913	70	**2.92%**	**0.024**	**153**
Length of Study	**8.36%**	**<0.001**	**885**	**3.03%**	**0.045**	**125**	**6.41%**	**<0.001**	**226**
Sampling Depth	**0.23%**	**0.029**	**432**	3.70%	0.089	64	**1.71%**	**0.016**	**117**

Correlations are given as R^2 and associated P-value, with *n* number of observations. Values are reported for the overall intercropping effect and for the intercropping effect within each enzyme type.

Table 1.1 (cont'd)

	Phosphorous Enzymes			Oxidative Enzymes			General Enzymes		
	R^2	P	*n*	R^2	P	*n*	R^2	P	*n*
Environmental and Edaphic Factors									
% Clay	1.22%	0.024	215	0.00%	0.902	19	0.00%	0.877	96
% Sand	0.00%	0.996	182	**15.87%**	**0.042**	**19**	1.61%	0.103	96
pH	0.01%	0.367	298	0.00%	0.116	52	**3.84%**	**0.012**	**170**
Soil C	0.00%	0.884	243	0.00%	0.587	40	**2.44%**	**0.041**	**147**
Total N	1.29%	0.080	172	3.33%	0.093	38	**6.75%**	**0.006**	**101**
Available N	**10.79%**	**<0.001**	**175**	1.69%	0.147	43	0.00%	0.897	126
Total P	0.65%	0.193	100	**34.82%**	**<0.001**	**38**	**7.49%**	**0.019**	**72**
Available P	0.00%	0.610	282	2.22%	0.078	49	0.00%	0.762	144
Annual Precipitation	**12.72%**	**<0.001**	**168**	1.15%	0.279	12	**9.44%**	**0.004**	**84**
Experimental Factors									
N Fertilizer	0.86%	0.176	215	0.00%	0.683	29	0.62%	0.214	93
Length of Study	**8.02%**	**<0.001**	**294**	8.31%	0.063	48	**9.18%**	**<0.001**	**171**
Sampling Depth	1.13%	0.218	133	0.00%	0.842	6	**4.59%**	**0.040**	**91**

Table 1.2: Listing of enzyme categories used in the meta-analysis

Enzyme	*EC number*	*Methods*	
		Description	*n*
Hydrolytic C enzymes			
Invertase	3.2.1.26	3,5-dinitrosalicylic acid (e.g. Frankenberger and Johanson, 1983)	77
		n.s.	24
Cellulase	3.2.1.4	Carboxymethyl-cellulose (e.g. Guan, 1986)	7
Cellobiohydrolase	3.2.1.91	4-methylumbelliferyl-cellobioside (e.g. Qi et al., 2016)	3
β-glucosidase	3.2.1.21	4-methylumbelliferyl-glucopyranoside (e.g. Qi et al., 2016)	3
		p-nitrophenyl-β-D-glucoside (e.g. Hayano, 1973)	19
β-xylosidase	3.2.1.37	4-methylumbelliferyl-xylopyranoside (e.g. Qi et al., 2016)	1
Oxidative C enzymes			
Peroxidase	1.11.1.7	Pyrogallic acid (e.g. Chen et al., 2004)	2
		n.s.	16
Phenoloxidase (laccase)	1.10.3.2	ABTS (e.g. Floch et al., 2007)	2
Polyphenoloxidase (catechol oxidase)	1.10.3.1	Catechol (e.g. Perucci et al., 2000)	13
		Pyrogallol (e.g. Guan, 1986)	5
		n.s.	14
General			
Catalase	1.11.1.6	$KMnO_4$ (e.g. Johnson and Temple, 1964)	50
		n.s.	31
Dehydrogenase	1.1.1.-	2,3,5-triphenyltetrazolium chloride (e.g. Casida, 1977)	51
		2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (e.g. Prosser et al., 2015)	15
		n.s.	16
Fluorescein diacetate hydrolase	*n.a.*	Pure-culture method (e.g. Schnürer and Rosswall, 1982)	16
		Soil-optimized method (e.g. Prosser et al., 2015)	13

The references for each enzyme assay method are those specified by at least one of the studies utilizing the respective method. EC number refers to the Enzyme Commission classification for that enzyme or class of enzymes. n refers to the number of observations that were obtained using the specified method. n.s. indicates the method was not specified in sufficient detail to describe.

Table 1.2 (cont'd)

Enzyme	*EC number*	*Methods*	
		Description	*n*
N enzymes			
N-acetyl-glucosaminidase	3.2.1.50	4-nitrophenyl N-acetyl-β-D-glucosaminide (e.g. Parham and Deng, 2000)	2
		4-methylumbelliferyl-N-acetyl-β-D-glucosaminidine (e.g. Qi et al., 2016)	4
Proteases (peptidases)	3.4.-	Folin-Ciocalteu reagent (e.g. Alef and Nannipieri, 1995)	7
		Ninhydrin (e.g. Zhou et al., 1987)	7
		n.s.	23
Urease	3.5.1.5	Phenate-hypochlorite (e.g. Guan, 1986)	115
		Steam distillation of ammonium (e.g. Tabatabai and Bremner, 1972)	48
		Indophenol blue (e.g. Kandeler and Gerber, 1988)	16
		n.s.	47
P enzymes			
Alkaline phosphatase	3.1.3.1	Benzene/phenol phosphate (e.g. Guan, 1986)	17
		p-nitrophenyl phosphate (e.g. Tabatabai and Bremner, 1969)	51
		4-methylumbelliferyl-phosphate (e.g. Qi et al., 2016)	1
		n.s.	3
Acid phosphatase	3.1.3.2	Benzene/phenol phosphate (e.g. Guan, 1986)	12
		p-nitrophenyl phosphate (e.g. Tabatabai and Bremner, 1969)	169
		4-methylumbelliferyl-phosphate (e.g. Qi et al., 2016)	4
		n.s.	7
Phosphatase (non-buffered or not specified)	3.1.3.-	Benzene/phenol phosphate (e.g. Guan, 1986)	2
		p-nitrophenyl phosphate (e.g. Tabatabai and Bremner, 1969)	26
		n.s.	22

Table 1.3: Estimates of the intercropping effect by assay method type

	n§	LCI	Estimate	UCI
Urease†				
Overall	154	0.102	0.139	0.176
Sodium hypochlorite	88	0.082	0.128	0.173
Steam distillation	33	0.065	0.148	0.231
Phosphatase‡				
Overall	156	0.101	0.133	0.165
PNP	130	0.090	0.121	0.151
Na_2-R-P	18	0.042	0.087	0.132

Methods were compared within enzyme groups only if there were multiple method types that had at least ten observations each. Individual methods were not significantly different from each other or the subgroup average for the respective enzyme, as determined by non-overlapping confidence intervals. LCI and UCI indicate the lower- and upper 95% confidence intervals. n indicates the number of observations associated with each method type.

† Sodium hypochlorite methods measure the amount of ammonium released through urease activity, as determined by a color change measured at 578 nm (e.g. Guan, 1986). Steam distillation methods measure the amount of ammonium released using steam distillation and a boric acid indicator solution (e.g. Tabatabai and Bremner, 1972).

‡ Acid phosphatases and alkaline phosphatases were combined. PNP denotes methods that utilize *p*-nitrophenyl phosphate as a substrate, which when acted upon by phosphatase, produces a color change readable at 400-420 nm (e.g. Tabatabai and Bremner, 1969). Na_2-R-P denotes methods that utilize a disodium benzyl/phenyl phosphate solution that reacts to produce a color change at 660 nm (e.g. Guan, 1986).

§ Comparisons were performed following the first round of literature review, which only included studies published up to 2018.

Table 1.4: Estimates of the intercropping effect for studies reporting the use of fresh or air-dried soil

	n†	LCI	Estimate	UCI
Carbon enzymes				
Fresh	25	-0.105	-0.018	0.069
Air dried	43	0.038	0.113	0.188
Nitrogen enzymes				
Fresh	66	0.123	0.174	0.226
Air dried	41	0.086	0.132	0.178
Phosphorous enzymes				
Fresh	79	0.115	0.151	0.188
Air dried	37	0.078	0.112	0.146
Oxidative enzymes				
Fresh	24	-0.376	-0.161	0.054
Air dried	6	-0.144	0.349	0.842

Within each enzyme type, studies utilizing fresh soil did not have a significantly different intercropping effect than those using air-dried soil, as determined by non-overlapping confidence intervals. LCI and UCI indicate the lower- and upper 95% confidence intervals. *n* indicates the number of observations associated with each method type.

† Comparisons were performed following the first round of literature review, which only included studies published up to 2018.

APPENDIX C:

STUDIES INCLUDED IN META-ANALYSIS

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CHAPTER 2:

AVAILABILITY OF DISSOLVED ORGANIC CARBON DRIVES DIFFERENCES IN MICROBIAL NITROGEN-CYCLING PROCESSES BETWEEN TWO SITES WITH COVER CROPS INTERSEEDED INTO CORN

2.1. ABSTRACT

Winter cover crops are often used to build soil health. However, narrow windows for planting cover crops between harvest and the first frost can restrict their use in northern regions. Interseeding cover crops into corn has been proposed as a technique to extend the cover crop growing window, but questions remain as to the effect of interseeded cover crops on nitrogen (N) availability and whether they will compete with corn for available nutrients. To assess N-cycling dynamics in soils where cover crops have been interseeded into corn, plots were established at two sites with differing edaphic properties. Two sets of plots were established at both locations, each for two years. Annual ryegrass (*Lolium multiflorum* Lam), crimson clover (*Trifolium incarnatum* L.), oilseed radish (*Raphanus sativus* L.), and a mixture of ryegrass and clover were interseeded into corn at the V3 or V6 stages of corn growth, for a total of eight cover-cropping treatments. These were compared to control plots that did not receive cover crops. I measured how cover crops affected the availability of carbon (C) and N during the growing season and after harvest. In addition, I determined how potential activities of microbial nutrient cycling processes—extracellular enzyme activities, nitrification, and denitrification—were impacted by cover cropping. I found that after two years of cover cropping, cover-cropping treatments had no effect on available C and N or on microbial nutrient-cycling activities in most site years. I observed major differences between sites, including in associations with soil nutrient pools and

microbial activities. Differences in the availability of dissolved organic C (DOC) appeared to primarily influence N cycling at the two locations. Despite a more rapid flux of N through the N cycle, one location with finer-textured soils and qualitatively better soil health exhibited lower potential for N loss.

2.2. INTRODUCTION

Cover cropping has been promoted as a practice that promotes soil health (Snapp et al., 2005; Finney et al., 2017; Wegner et al., 2018). Studies have shown how integrating cover crops into a rotation increases many ecosystem services, such as protection against erosion, the formation of soil carbon (C), improved water retention, and reductions in nutrient loss (Schipanski et al., 2014; Daryanto et al., 2018; Blanco-Canqui and Ruis, 2020). In turn, cover cropping is also frequently associated with increased yields of the main crop (Fageria et al., 2005; Smith et al., 2008; Marcillo and Miguez, 2017; Daryanto et al., 2018). Nevertheless, the benefits of cover cropping can take time to manifest, and often depend on how cover crops are managed as well as on climate and soil properties (Snapp et al., 2005; Abdalla et al., 2019; Kallenbach et al., 2019; Xu et al., 2020). Understanding the mechanisms of how cover crops improve soil health can inform the selection of appropriate cover cropping strategies for growers.

Plant productivity is dependent on the timely supply of nitrogen (N) to meet crop demands. Often N is supplied through an input of chemical or organic fertilizer once or twice a year. Maximizing the efficient use of N inputs requires timing N additions with the critical period of plant growth, which often depends on environmental factors that are difficult to control or predict, such as precipitation and temperature (Cassman et al., 2002; Robertson and Vitousek, 2009). When anthropogenic N additions do not match demand, N

accumulates in various forms and is subject to transformations and subsequent loss from the ecosystem (Schlesinger, 2009; Robertson and Groffman, 2015). The soil N cycle also provides plant-available N through biological N fixation and by releasing N contained within plant residues and soil organic matter. In most natural systems, the N provided by the microbial community is completely utilized by the native vegetation (Galloway et al., 2004). Therefore, internal N cycling in the soil is a double-edged sword. It can provide nutrients to plants during their peak demand, but it can also drive nutrient losses from ecosystems when N is in excess. This presents many opportunities to understand and optimize N cycling to maximize ecosystem services (Van Groenigen et al., 2015).

Cover crops are often specifically chosen to improve N cycling in the soil by both retaining and releasing N. Some cover crops, particularly grasses, are often used as "catch crops" to capture excess N, helping to keep this vital nutrient in the field. Such uses of cover crops are particularly effective in the winter, when excess moisture in bare soils can result in substantial amounts of N leaching (Komatsuzaki and Wagger, 2015; Hirsh et al., 2021). Leguminous cover crops can also be utilized to provide "N credits" through their symbiotic association with N-fixing bacteria (Ebelhar et al., 1984; Snapp et al., 2005; Tonitto et al., 2006). As N-containing cover crop residues decompose, their N is released for utilization by the main crop. These benefits are particularly evident in wheat-clover systems (Thorsted et al., 2006; Gaudin et al., 2014). In addition to cover crop selection, the timing of cover crop planting can determine the potential of cover crops to scavenge nutrients and produce biomass (Hashemi et al., 2013; Lawson et al., 2015; Komainda et al., 2016).

Typically, cover crops are planted after harvest, but in some locations, particularly northern latitudes, this can leave little time for cover crops to grow and establish before

winter (Komainda et al., 2016; CTIC et al., 2020). Interseeding can help overcome these narrow cover cropping windows. Interseeding is a practice where cover crops are planted during the growing season between rows of a main crop, such as corn, allowing for a longer growing season for the cover crop. On the other hand, there are some concerns that if planted too early, interseeded cover crops may compete with the main crop for needed nutrients, such as N (Fageria and Baligar, 2005; Snapp et al., 2005; Wachendorf et al., 2006). Therefore, it is important to understand how interseeded cover crops impact the N cycle both during the growing season and after harvest, when cover crop residues are decomposed in the soil.

The soil microbial community is responsible for carrying out the N transformations that make up the soil N cycle (e.g., Booth et al., 2005; Robertson and Groffman, 2015). The balance between N supply and microbial demand determines the amount of N that is mineralized from organic matter by the microbial community and made available for plant uptake or immobilized in microbial biomass. Soil microbes also carry out a host of mineral N transformations, such as nitrification, the conversion of ammonium to nitrite and nitrate, and denitrification, which closes the N cycle by converting nitrate to nitrous oxide (N_2O) and/or dinitrogen gas. Cover crops have the potential to influence the soil microbial community by altering the types and diversity of organic inputs to the soil as well as by increasing the amount of residues (e.g., Aulakh et al., 1991; Tiemann et al., 2015). For example, C-rich grass residues could increase microbial N demand and lead to N immobilization, while the degradation of N-rich clover residues could either help provide plant available N or lead to greater N losses if degradation does not coincide with plant demand (McKenney et al., 1993, 1995; Steenwerth and Belina, 2008; O'Connell et al., 2015).

Ultimately, the impact of cover crops on soil N availability depends on the N transformations carried out by the microbial community. Understanding the dynamics of the microbially mediated N cycle is therefore essential to maximizing the benefits of cover crops for increasing plant yields and building soil health.

I sought to determine how interseeding cover crops into corn impacts measures of soil health and soil N cycling and provisioning. Since these factors are heavily influenced by environmental and edaphic factors, the experiment was initiated at two locations with varying soil and climate. I sampled soils during the growing season and after harvest over three years. To distinguish legacy effects of cover cropping from year-to-year variation, the experiment was repeated at each site in two consecutive years and maintained for two years each, for a total of eight site-years. I hypothesized that (1) interseeded cover crops would improve soil health by increasing "active" soil C pools and microbial activity; (2) a mixture of grass and legume interseeded cover crops would provide greater soil health benefits than either grass or legume interseeded alone; (3) soil N provisioning would be greater and potential N losses reduced with interseeded cover crops, with these benefits most pronounced under the grass-legume mixture, as indicated by reductions in concentrations of inorganic forms of N, especially nitrate, and reduced rates of nitrification and denitrification; and (4) soils sampled after consecutive years of interseeding cover crops compared to an initial year would have greater "active" soil C pools, greater microbial activity, and more efficient N provisioning.

2.3. METHODS

2.3.1. Site Description and Experimental Design

Experimental plots were established at two research locations in central Michigan, USA, in 2017. The first site was at the East Lansing Agronomy Farm (42.7100°N, 84.4663°W). The soils are an Aubbeenaubbee–Capac sandy loam (fine-loamy, mixed, active, mesic Aeric Epiaqualf; fine-loamy, mixed, active, mesic Aquic Glossudalf). Soil organic matter (SOM) was between 2.8 and 2.9%, with pH between 5.8 and 7.6. The second site was located at the Saginaw Valley Research and Extension Center (43.3952°N, 83.6831°W). Soils at this location are a Tappan-Londo loam (fine-loamy, mixed, active, calcareous, mesic Typic Epiaquolls; fine-loamy, mixed, semiactive, mesic Aeric Glossaqualfs), with 3.0% SOM and pH of 7.5. The following year, 2018, a second, identical set of plots was established at each location in a different field. Each set of plots was maintained for two years (Fig. 2.1; Brooker et al., 2020).

Cover crop treatments were established within four blocks at each location for both establishment years. Cover crop treatments included two effects: cover crop species and cover crop seeding time. The four cover crop species were annual ryegrass (*Lolium multiflorum* Lam.), crimson clover (*Trifolium incarnatum* L.), oilseed radish (*Raphanus sativus* L.), and a mixture of ryegrass and clover. Within these cover crop species, a seeding time treatment was applied, where covers were seeding during either the V3 or V6 stage of corn growth. These cover crop treatments were compared to plots that did not receive any cover crop treatment. Since the seeding time treatment could not be applied across all levels of cover crop species (i.e., the no-cover treatment did not have a seeding-time treatment), this resulted in an incomplete-randomized-block design. Cover crop biomass was determined at

the time of corn harvest by sampling aboveground biomass of cover crops twice within 0.25 m^2 quadrats randomly placed within the plot. Cover crop biomass was dried prior to weighing.

2.3.2. Soil Chemical Properties

Soil samples were taken throughout the year using a 1.9 cm diameter soil probe to 10 cm depth (Table 2.1). Samples were sieved through a 4 mm mesh. Soil water content was determined gravimetrically. I extracted dissolved soil organic C and total N by combining 8 g of field moist soil with 40 mL 0.5 M K_2SO_4 and shaking for 1 hour followed by filtration with Whatman #1 filters. Soil ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were determined colorimetrically in clear 96-well plates. Ammonium determination was done using salicylate and cyanurate color change chemistry (Sinsabaugh et al., 2000), and NO_3^- concentrations were determined by first reducing NO_3^- to NO_2^- using nitrate reductase from *Arabidopsis thaliana* (EC 1.7.1.1; NECi, USA) followed by standard procedures for nitrite determination (NECi, 2014). Total dissolved organic C (DOC) and N were determined using a vario TOC select elemental analyzer (Elementar Americas, USA). Dissolved organic N (DON) levels were calculated by subtracting concentrations of NO_3^- and NH_4^+ from total dissolved N concentrations. I estimated microbial biomass using a modified chloroform-fumigation method (Jenkinson et al., 2004). In brief, 1 mL of chloroform was added to 8 g of soil and incubated in sealed tubes for 24 hrs. After venting chloroform for one hour under a fume hood, I performed K_2SO_4 extractions and quantified total DOC and DON as described above. The difference in DOC and DON levels between fumigated and non-fumigated samples is considered microbial biomass, following the application of a correction factor of 0.45.

2.3.3. Soil Biological Properties

I used fluorescently labelled substrates to estimate the activity of eight extracellular enzymes: β-glucosidase (BG), β-1,4-N-acetyl-glucosaminidase (NAG), phosphatase, alanine aminopeptidase, arginine aminopeptidase, leucine aminopeptidase, glutamate aminopeptidase, and tyrosine aminopeptidase. BG, NAG, and phosphatase substrates were labelled with 4-methylumbelliferone, and aminopeptidase substrates were labelled with 7-amino-4-methylcoumarin, according to the high-throughput microplate method (German et al., 2011). Urease activity was determined via the microplate method (Sinsabaugh et al., 2000) by quantifying ammonium release colorimetrically, as described above, following the addition of urea.

Potential rates of denitrification were determined using a modified denitrification enzyme activity (DEA) assay (Groffman et al., 1999). Five grams of soil were placed in airtight jars and combined with 15 mL of a solution containing 4.75 mM KNO_3 and 20 mM glucose. Jars were evacuated and flushed with N_2. To inhibit N_2O reduction, acetylene was added to the headspace of each jar to a final concentration of 10% (v/v). Jars were incubated at 22°C on an orbital shaker. Headspace gas samples were taken after 30 and 90 minutes and placed in pre-evacuated 12 mL vials. Gas samples were analyzed for N_2O and CO_2 concentrations using a TRACE 1310 gas chromatograph equipped with electron capture and thermal conductivity detectors (Thermo Fisher Scientific, USA).

Nitrification potential activity (NPA) was determined using the shaken soil-slurry method (Hart et al., 1994). Briefly, soil slurries were prepared by combining 15 g of field moist soil with 100 mL of a solution containing 1 mM phosphate, 1.5 mM NH_4^+, pH 7.2. Jars were flushed with O_2 to prevent denitrification and incubated at 22°C on an orbital shaker.

Over the course of 24 hours, soil slurry samples were taken four times at evenly spaced intervals. Nitrate concentrations in the soil slurry were determined using the microplate method described above, and changes through time were used to calculate the rate of nitrification.

2.3.4. Statistical Analyses

Because the experimental design, with seeding time and cover crop species in an incomplete factorial, would not allow me to simultaneously analyze these two cover crop effects, I first analyzed the main effect of cover crop species alone and its interactions with season and environment. When the cover-crop-species treatment was found to be significant, I omitted the no-cover-crop treatment and performed a second analysis that included the effects of both cover crop species and seeding time as well as their interaction.

To model how season impacted cover crop treatment effects, sampling times were grouped by seasons: samples taken between corn emergence and corn harvest were categorized as "growing season", and samples taken after harvest were "post-harvest." Treatment differences in single response variables were determined using linear models and analysis of variance (ANOVA). Linear models were constructed using the *lme4* package in R (Bates et al., 2015). Fixed effects were season, site, and cover crop species, and when cover crop species effects were significant, additional analyses included seeding time as a fixed effect. Random effects were specified as block (nested within site and establishment year) and its interactions with the various fixed effects. In addition, with respect to season, plot was considered a random effect to account for repeated measures over time. I utilized the *lmerTest* package (Kuznetsova et al., 2017) to determine the significance of main effects and interactions by performing Type III tests with numerator degrees of freedom calculated

using the Kenward-Roger method. When main effects or interactions were found to be significant, pairwise comparisons were conducted with the *emmeans* package (Lenth et al., 2019) and Fisher's LSD at $\alpha = 0.05$ (e.g., I looked for significant differences between sites by season when the site-by-season interaction was significant). Since the five peptidase activities were similar, these were averaged to produce a single peptidase activity.

I analyzed correlations between variables separately within each site. Correlation matrices between variables were generated using the *cor* function in R to calculate Pearson's correlation coefficients. Significance of Pearson correlation coefficients were based on $\alpha = 0.05$ using the *cor.test* function, as implemented within the *corrplot* package.

I performed a multivariate analysis to determine how soil chemical properties explained variation among samples in biological process rates. I used redundancy analysis (RDA) using the *vegan* package (Oksanen et al., 2007) with all enzyme data, DEA, and NPA as response variables, and all chemical data as explanatory variables.

2.4. RESULTS

2.4.1. Cover Crop Biomass

Cover crop biomass varied between cover crop types and seeding times as well as by location and year (Table 2.2). For example, in the first year of the study at East Lansing (EL), tillage radish seeded at V3 had nearly twice as much biomass as the next highest cover crop (13.99 versus 7.28 g 0.25 m^{-2}). However, at the same location, this cover crop failed to emerge when seeded at V6 during the first year of the 2018 plots and did not emerge at all in 2019. In general, 2018 had less cover crop biomass compared to other years. Only V3-seeded clover and mixture at Saginaw Valley (SV) had more than 0.5 g 0.25 m^{-2} biomass. In contrast, cover crop biomass varied from 0.95 to 4.88 g 0.25 m^{-2} at SV in 2017. The lack of

cover crop biomass in 2018 was likely due to a shortfall of precipitation during the period when cover crops were interseeded (Fig. 2.1).

2.4.2. Soil C and N Pools

I measured dissolved organic C (DOC) and microbial biomass C (MBC) to determine how cover crops influenced fast turnover pools of soil C and found no significant main or interactive effects of cover cropping (Table 2.3). Instead, DOC and MBC varied significantly by site and season, with site differences depending on sampling season. DOC was significantly higher at SV in four out of six seasons (Fig. 2.2A) and MBC was significantly higher at SV in two out of six seasons, but one season was not observed at EL due to loss of samples (Fig. 2.2B). At EL, DOC concentrations were greater during the growing season than after harvest. There was no consistent temporal pattern to DOC concentrations at SV.

Soil N pools were largely unaffected by cover cropping, regardless of sampling site or season (Table 2.3). I found greater NH_4^+ at EL compared to SV at all seasons except one, but the magnitude of the site difference varied by season (Fig. 2.3A). Soil nitrate (NO_3^-) concentrations did not differ by cover crop and were not consistently different between the two locations (Fig. 2.3B). Within each location, NO_3^- tended to be greater during growing seasons. Dissolved organic N (DON) was impacted by site and season, although the seasonal effects varied by site (Fig. 2.3C). At EL, DON was significantly higher during the growing season compared to post harvest, while at SV, post-harvest levels of DON were significantly greater in two out of three years. Microbial biomass N (MBN) was significantly affected by cover crop (Table 2.3), with significantly greater MBN in the mixture (34.3 ± 1.4 mg MBN-N kg^{-1} soil), radish (33.6 ± 1.3 mg MBN-N kg^{-1} soil), and no-cover (33.5 ± 1.8 mg MBN-N kg^{-1} soil) treatments compared to clover (29.4 ± 1.2 mg MBN-N kg^{-1} soil). MBN was

consistently higher at SV than EL and, with no significant site-by-season interaction, was not driven by seasonality effects (Fig. 2.3D).

2.4.3. Soil Biological Activity

As with soil chemical parameters, cover cropping did not account for a significant amount of variation in extracellular enzyme activity (EEA), but I did see significant site, season, and site-by-season effects (Table 2.4). EEA potential rates significantly differed between locations for most enzymes (Fig. 2.4). BG and NAG activities tended to be significantly greater at EL than SV in most seasons (Fig. 2.4A, B). Conversely, peptidase rates were significantly greater at SV than EL in all seasons (Fig. 2.4C). Phosphatase activities were significantly greater at EL in four out of six seasons but were significantly higher at SV in the last season observed (Fig. 2.4D). Although activities varied significantly over time at both locations, there were no consistent seasonal trends.

Nitrification potential activity (NPA) and denitrification potential (DEA) both differed by site and season, with significant site-by-season effects (Table 2.5). NPA rates were significantly greater at SV than EL across all seasons except the 2018 growing season (Fig. 2.5A). Conversely, DEA was significantly greater at EL than SV in most seasons (Fig. 2.5B).

2.4.4. Correlations between Soil Biological and Chemical Properties

DOC was correlated with enzyme activities at both locations, but these relationships were stronger at SV (Fig. 2.6). At EL, DOC was inversely correlated to BG and NPA, while at SV, DOC was positively correlated with peptidase, phosphatase, and NPA. DOC was inversely related to BG and NAG activity at SV. There were fewer significant relationships between soil N and enzyme activities at EL than SV. NPA and NH_4^+ as well as DON and

peptidase were strongly correlated at SV but not at EL. Among enzyme activities, I found that BG and peptidase were strongly positively correlated at EL but possessed a significant inverse relationship at SV. At both locations, NPA and peptidase activities were positively correlated.

2.4.5. RDA Analysis

I used RDA to determine which soil characteristics were most important in driving differences in microbially mediated nutrient-cycling processes (Fig. 2.7). A total of 42% of the variation in biological process rates can be explained by the environmental variables included in my analysis, with 92% of this variation being expressed on the first two axes of the RDA. The two sites differentiate from one another along the first RDA axis. This axis is most strongly positively correlated with DOC and soil moisture, while it is negatively correlated with NH_4^+. Total N and MBN account for much of the variation explained by RDA2, but these environmental factors are not highly correlated with any of the response factors.

2.5. DISCUSSION

The benefits of cover crops to soil health and soil C are well known (e.g., Fageria et al., 2005; Snapp et al., 2005; Daryanto et al., 2018). Cover crops can help to build soil C stocks, thereby improving soil health, and this is often a stated goal of utilizing cover crops (CTIC et al., 2020). Nevertheless, across the four plot locations in this study, I did not observe consistent effects of cover crops on any of the soil chemical or biological indicators of soil health. The lack of significant effects is likely due to a combination of low amounts of C inputs supplied by cover crops (Table 2.2) and the relatively short duration of the study. Other studies have also failed to detect significant differences in various soil C metrics after

only a few years of cover cropping. In one study, there were no differences in SOM after 5 years of cover cropping, but detectable effects did emerge by 7 years, with this effect dependent on main cropping system type and management strategy (Wegner et al., 2015, 2018). Another study in Michigan also found that 5 years was insufficient time to produce significant differences in SOC (Ladoni et al., 2016), but in a similar experiment, differences in SOC were apparent after 12 years between a management system with cover crops and one without (Syswerda et al., 2011). The increase in soil C over time is likely linked to accumulation of cover crop biomass. Long-term studies show that soil C concentrations are positively related to increases in C inputs (Mazzoncini et al., 2011; Barbera et al., 2012). Across all years of the study, cover crop biomass inputs were relatively modest, especially when compared to the contributions of biomass C from the corn main crop (Table 2.2). Cover crop biomass was typically between 1 and 10 g 0.25 m^{-2}, equivalent to roughly 50 to 500 g ha^{-1}. Compared to corn residue additions that can be measured in megagrams per hectare, the cover crop biomass made a modest contribution to soil C inputs. A primary reason for the low biomass was likely a lack of precipitation during the key growing phase for the cover crops after interseeding, especially in 2018 (Fig. 2.1; Brooker et al., 2020). Precipitation occurring later in the season after the corn canopy closes would not likely benefit cover crops.

The strongest effects I observed on all measured parameters were between seasons and sites (Table 2.3). In general, seasonal trends were more pronounced at EL. For instance, all soil N pools were usually higher during the growing season. Greater amounts of N during the growing season are typical of agricultural systems, where anthropogenic N additions make up the overwhelming majority of N input, with less than half being taken

up by the crop (Fageria and Baligar, 2005; Robertson and Vitousek, 2009). In contrast, seasonal patterns in inorganic N were not as pronounced at SV. Although NO_3^- concentrations tended to be higher during the growing season at SV, levels of soil NH_4^+ did not follow a consistent seasonal pattern. In addition, soil NH_4^+ was consistently lower at SV compared to EL at every sampling date. Greater microbial N demand at SV may have helped to maintain lower levels of inorganic N throughout the year compared to EL. Microbial immobilization of inorganic N can vary greatly between soils and is driven largely by soil organic C content (Barrett and Burke, 2000). When C is available, net N immobilization by microbes occurs, reducing the concentration of dissolved inorganic N (Aulakh et al., 1991; McKenney et al., 1995; Hume et al., 2002). I found higher DOC concentrations at SV compared to EL in most seasons (Fig. 2.2A), and MBN was also greater at SV (Fig. 2.3D), indicating more N in the microbial pool. While higher DOC levels may have driven microbial immobilization at SV, C limitation at EL could have hampered the ability of microbes to utilize N when it was available, allowing mobile forms of inorganic N to build up in the soil and become susceptible to loss.

DOC was higher and subject to less temporal variation at SV, suggesting a steady supply of DOC. Ultimately, this is due to more DOC being released through the degradation of crop residues and/or SOM (Kalbitz et al., 2000; Neff and Asner, 2001), but it is not immediately evident whether these site differences are caused by variable efficiencies in degradation dynamics or by differences in stocks of SOM. Enzyme activities were significantly different between sites. Further, at SV, enzyme activities were both positively and negatively correlated with DOC, indicating a complex relationship between DOC availability and the regulation of extracellular enzyme production. It is also possible that

DOC levels were maintained through rapid turnover of MBC. MBC concentrations were not always higher at SV, but they did tend to exhibit greater variability, with season-to-season changes larger than the total pool of DOC. While some of MBC turnover becomes stabilized as necromass (Miltner et al., 2012; Buckeridge et al., 2022), a substantial portion can feed back into the pool of DOC (Blazewicz et al., 2014; Shahbaz et al., 2017). Greater DOC availability can in turn lead to increased microbial activity, stimulating a virtuous cycle where C is actively cycled back and forth between DOC and MBC.

Regardless of its source, the availability of DOC appeared to drive differences in nutrient-acquisition strategies between the microbial communities at the two locations. For instance, I found that BG and peptidase activities were strongly positively correlated at EL but strongly negatively correlated at SV (Fig. 2.6B). Although extracellular enzyme activities tend to increase together (Sinsabaugh et al., 2008, 2009), others have also described a negative pattern between peptidase and glucosidase activities at the field scale (Weedon et al., 2014). These distinct relationships between enzyme activities indicate differences in nutrient limitation (Chen et al., 2014; Mooshammer et al., 2014). Stoichiometric decomposition theory describes how microorganisms shift their strategies for C- and nutrient acquisition to overcome differences in the stoichiometry of available resources and the relatively narrow C:N requirements of biomass (Sinsabaugh and Shah, 2012). Increased C availability at SV stimulated microbial N demand, resulting in a shift in allocation of microbial resources towards acquisition of N by producing peptidases (Allison and Vitousek, 2005; Geisseler et al., 2009). Indeed, I found that DOC and peptidase activities were tightly correlated at SV but not at EL (Fig. 2.6). This agrees with other studies that

have also shown a relative increase in peptidase activity associated with DOC and have attributed this to increased microbial N demand (Bowles et al., 2014).

Greater peptidase activity can result in more available DON at SV (Schimel and Bennett, 2004). As may be expected, I found that peptidase activity and DON were positively related at SV but not at EL (Fig. 2.6). In addition, I found that DON tended to be higher post-harvest at SV (Fig. 2.3C). Compared to corn stover, cover crop residues typically have much lower C:N ratios, especially clover residue, and the degradation of these N-rich substrate can increase soluble N levels during the fall and winter (McKenney et al., 1995; Abdalla et al., 2019). Nevertheless, as with other pools of N, I saw no cover crop effects on the availability of DON at SV. This leaves SOM as the most likely source of DON at SV. SOM is a rich source of N in some soils, and microorganisms often mineralize SOM to obtain needed N (Moorhead and Sinsabaugh, 2006; Craine et al., 2007). Inputs of corn stover could provide an influx of C that stimulates the microbial community to increase N mining from SOM (Shahbaz et al., 2017).

NPA was higher at SV, potentially explaining the lower concentrations of NH_4^+, but interestingly, the correlation between NH_4^+ and NPA was strongly positive (Fig. 2.6). If NPA was the primary process responsible for the consumption of NH_4^+, there should be an inverse relationship. Rather, it appears that nitrifiers are responding to an increase in NH_4^+ concentrations. The high potential activity of peptidases at SV could be driving a mineralization process that creates a niche for nitrifiers. Other studies have found nitrification to be well correlated with N mineralization (Booth et al., 2005; Steenwerth and Belina, 2008; Liang et al., 2014; Ouyang et al., 2016), and it has been suggested accordingly that N mineralization is a better determinant of nitrification substrate supply than

concentrations of NH_4^+ (Stark and Hart, 1997; Norton and Ouyang, 2019). In support of this possibility, both locations had significant positive correlations between NPA and peptidase activity. While potential peptidase activity does not measure N mineralization *per se*, the depolymerization of extracellular proteins and polypeptides is the rate-limiting step in the process of N mineralization (Schimel and Bennett, 2004). Depolymerization is all the more influential in tightly coupled systems where the demand for N is high and where rapid immobilization maintains a small pool of inorganic N, such as at SV. Only a few studies have explicitly looked at the relationship between potential rates of peptidase activity and nitrification (Zaman et al., 1999; Schloter et al., 2003). Often, no correlation or a negative correlation is found, but these studies are usually performed during the growing season and are confounded by treatments and field-management practices that provide fertilizer or DON, underscoring the importance of understanding microbial activity and nutrient-cycling dynamics throughout the entire year within agricultural soils.

A more active N cycle at SV may or may not lead to increased N losses. For example, NPA is linked to N loss via the production of NO_3^-, which is the dominant form of N lost in agricultural ecosystems (Robertson and Vitousek, 2009). Nitrate is highly mobile and can be easily leached through the soil profile; in addition, NO_3^- is susceptible to loss via denitrification. Although I did not measure *in situ* fluxes of N into and out of the system, I found that DEA and inorganic N levels, including NO_3^-, tended to be lower at SV than EL. This suggests minimal N loss pathways at SV, where despite the more active fluxes between soil N pools, the greater DOC content drove microbial demand for N. On the other hand, N availability at EL was driven by exogenous inputs, with higher DEA and concentrations of inorganic N suggesting greater potential for nutrient loss.

2.6. CONCLUSION

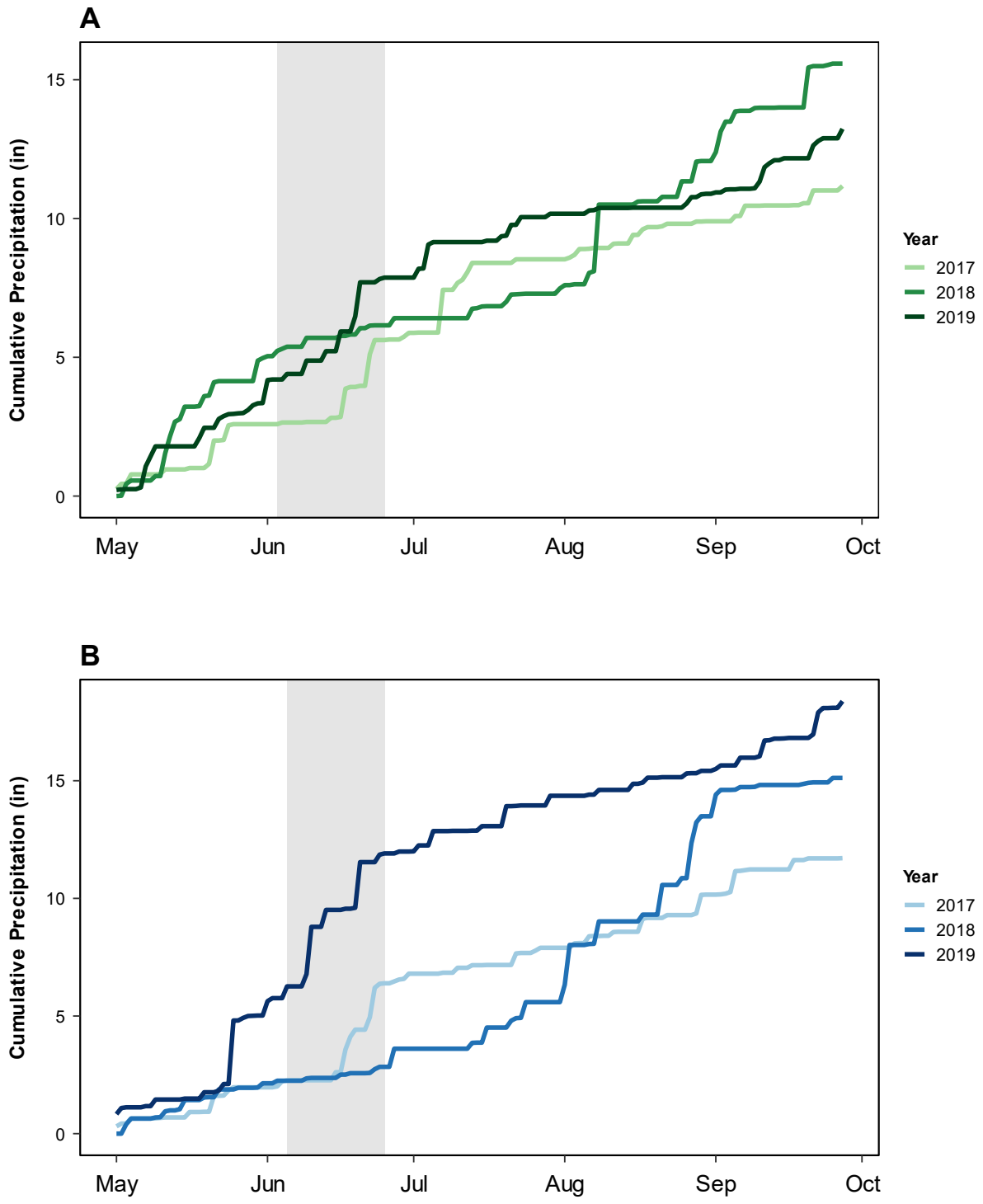
Interseeded cover crops did not produce consistent changes to soil nutrient pools or microbial activities in the two-year cover cropping treatments utilized in this study. The lack of a strong cover crop effect may have been due in part to the relatively modest cover crop biomass additions that were a result of low precipitation during key moments for cover crop growth. Instead, differences between site and season were far more pronounced. The microbial communities had distinct nutrient cycling strategies between the two locations observed in the study, including patterns of extracellular enzyme activity. I suggest that resource availability and relative nutrient demand drove these differences in nutrient transformations between the two sites, with C availability being associated with a more active N cycle and lower concentrations of inorganic N. These large and consistent differences in N cycling between locations indicates the importance of having site-specific management recommendations to improve N provisioning. For example, the contrasting seasonal patterns of organic N availability suggest that the N contained within cover crop residues will be mineralized differently depending on microbial demand and the strength of various N-cycling processes. Additional research is needed to further describe the mechanisms underlying the relationship between available C and microbial N-cycling processes and how practices such as cover cropping impact these interactions.

APPENDICES

APPENDIX A:

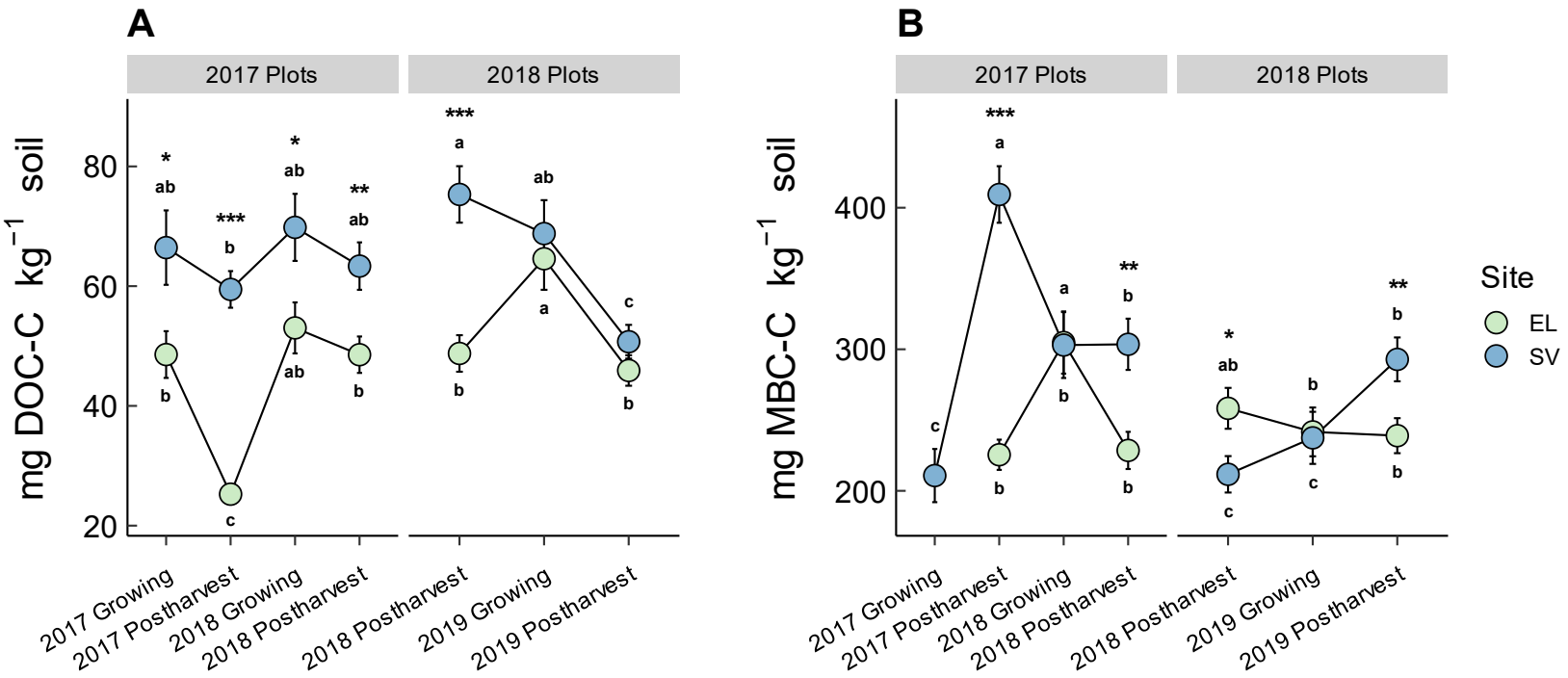
FIGURES

Figure 2.1: Cumulative precipitation during growing season



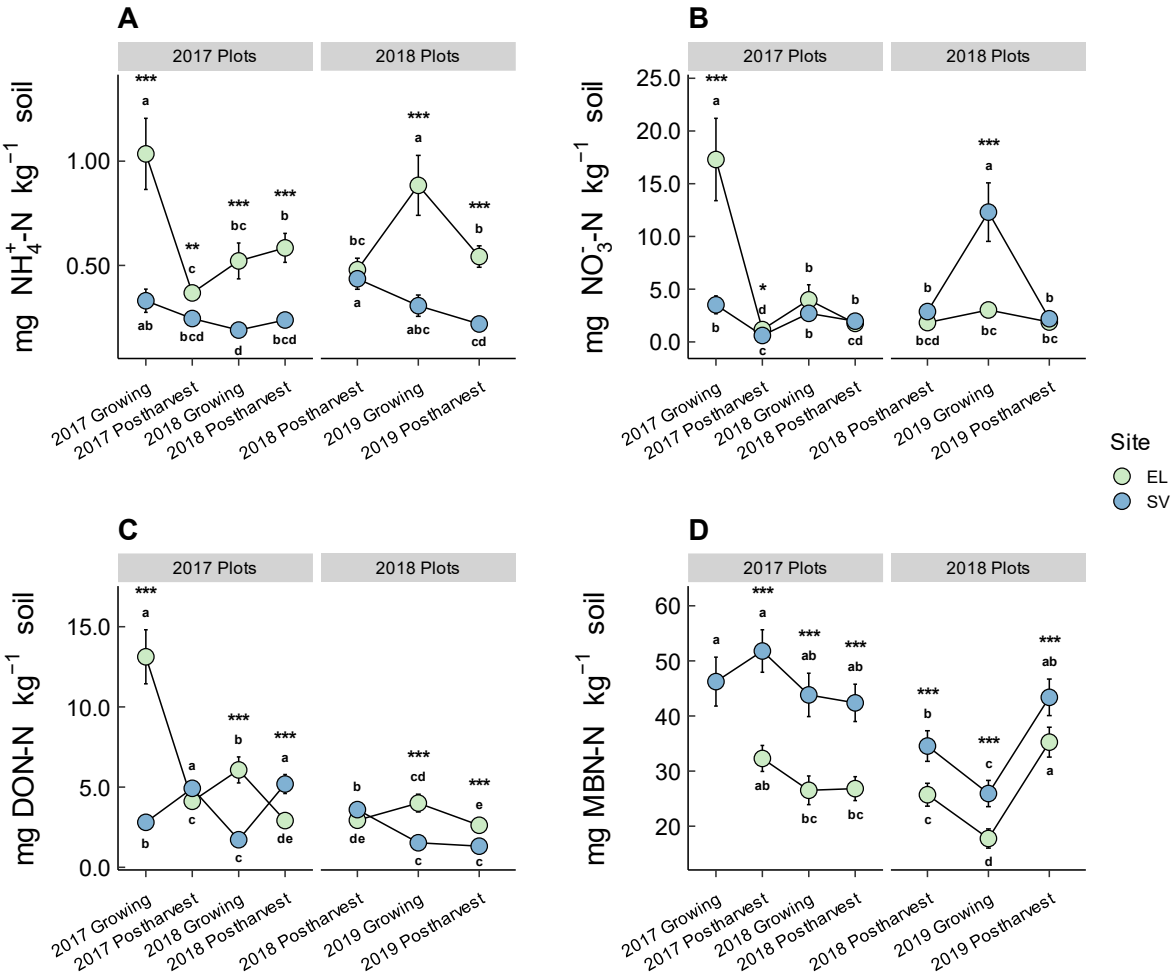
Cumulative precipitation at East Lansing (A) and Saginaw Valley (B) during the maize growing season. Shaded region indicates the period when cover crops were interseeded.

Figure 2.2: Concentrations of soil carbon pools



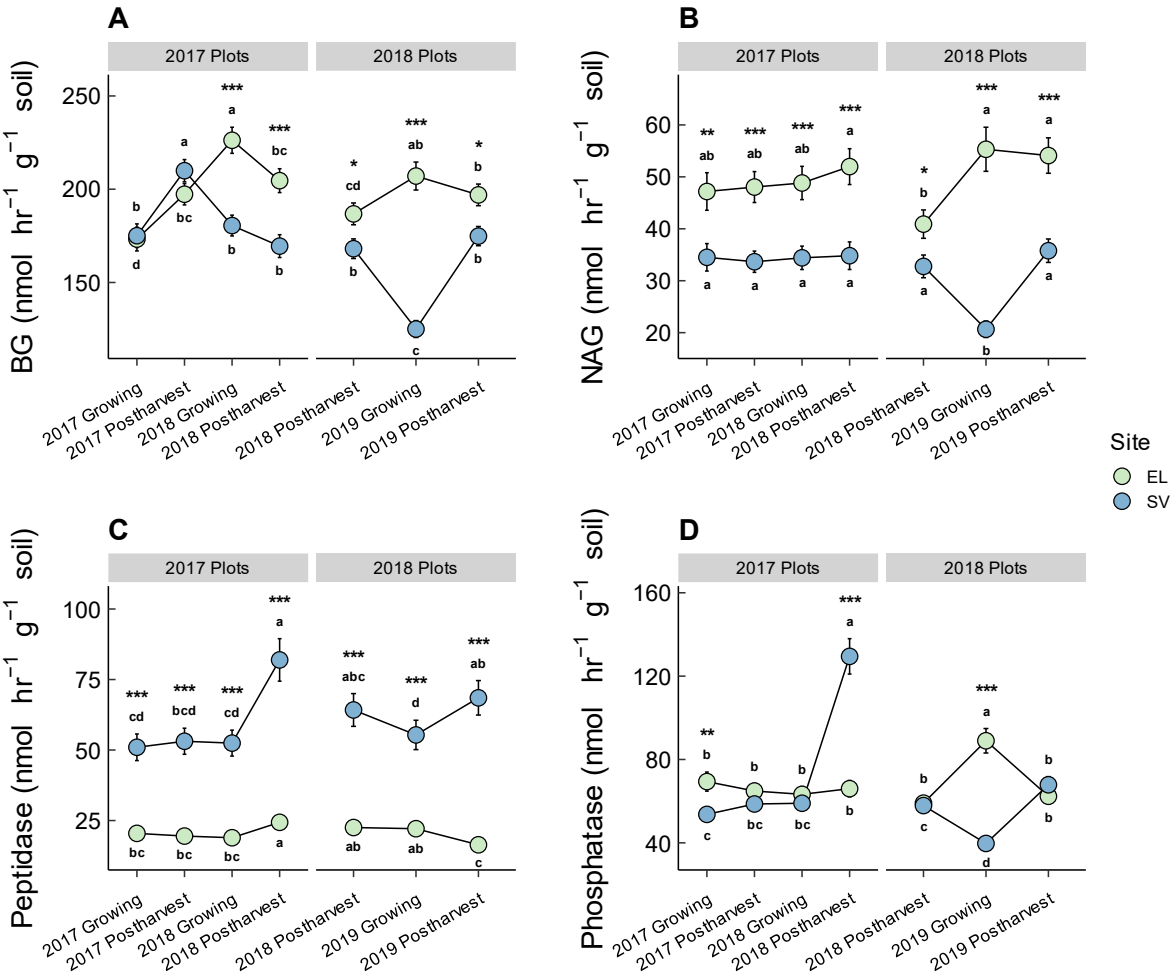
Mean values of dissolved organic carbon (A) and microbial biomass carbon (B), with error bars representing one standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Within each location, significantly different means between seasons are indicated by lowercase letters (α = 0.05).

Figure 2.3: Concentrations of soil nitrogen



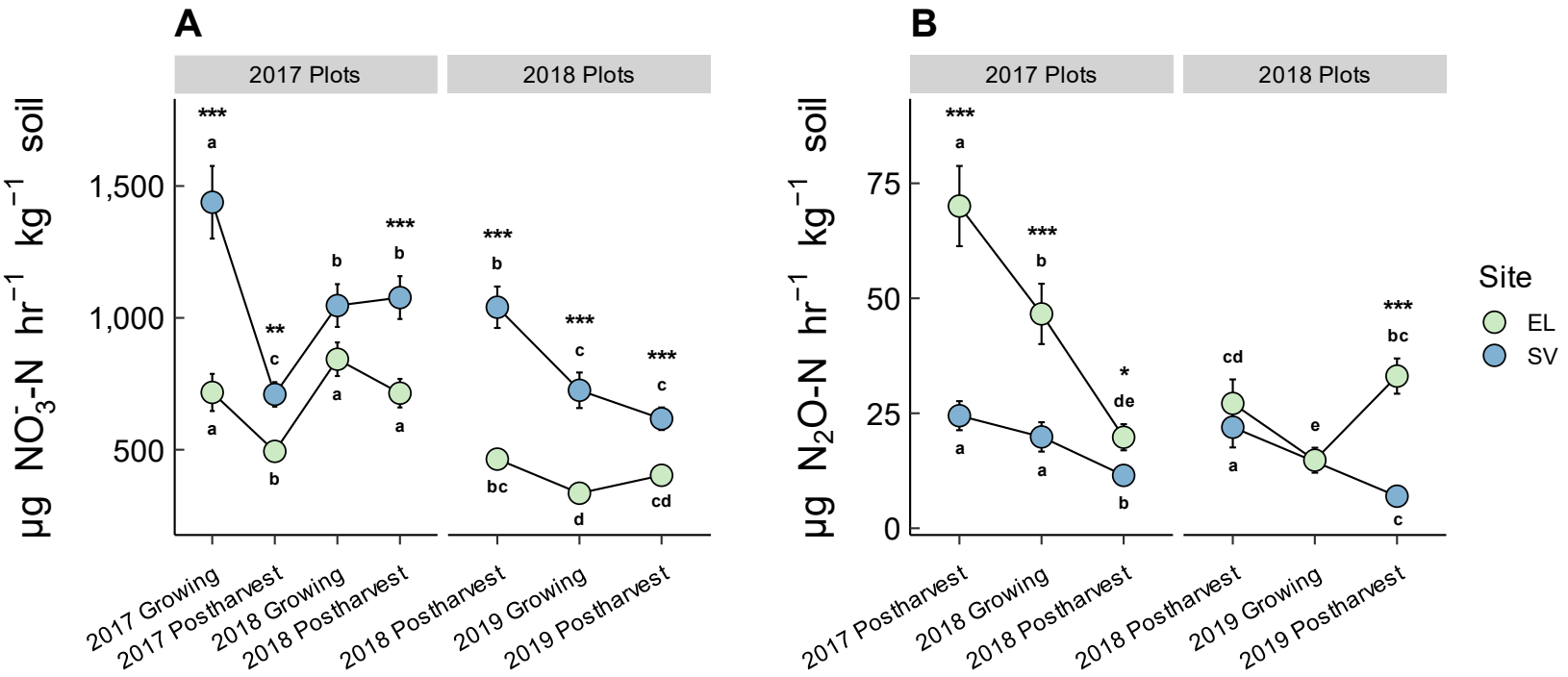
Means values of ammonium (A), nitrate (B), dissolved organic nitrogen (C), and microbial biomass N (D); error bars represent one standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Within each location, significantly different means between seasons are indicated by lowercase letters ($\alpha = 0.05$).

Figure 2.4: Microbial extracellular enzyme activities



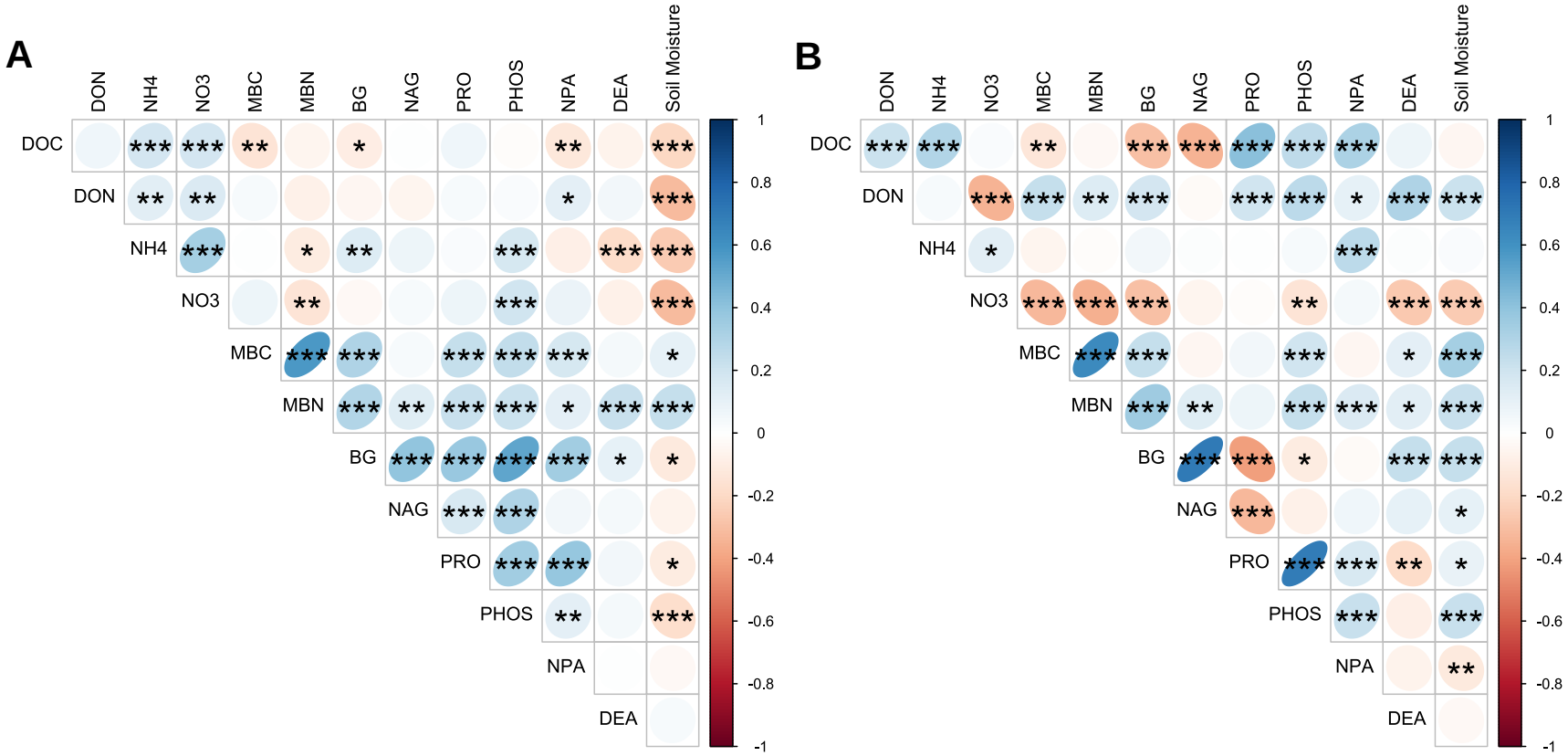
Values indicate average potential activities of β-glucosidase (A), N-acetyl-glucosaminidase (B), peptidase (C), and phosphatase (D). Error bars are one standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Within each location, significantly different means between seasons are indicated by lowercase letters (α = 0.05).

Figure 2.5: Potential nitrification and denitrification



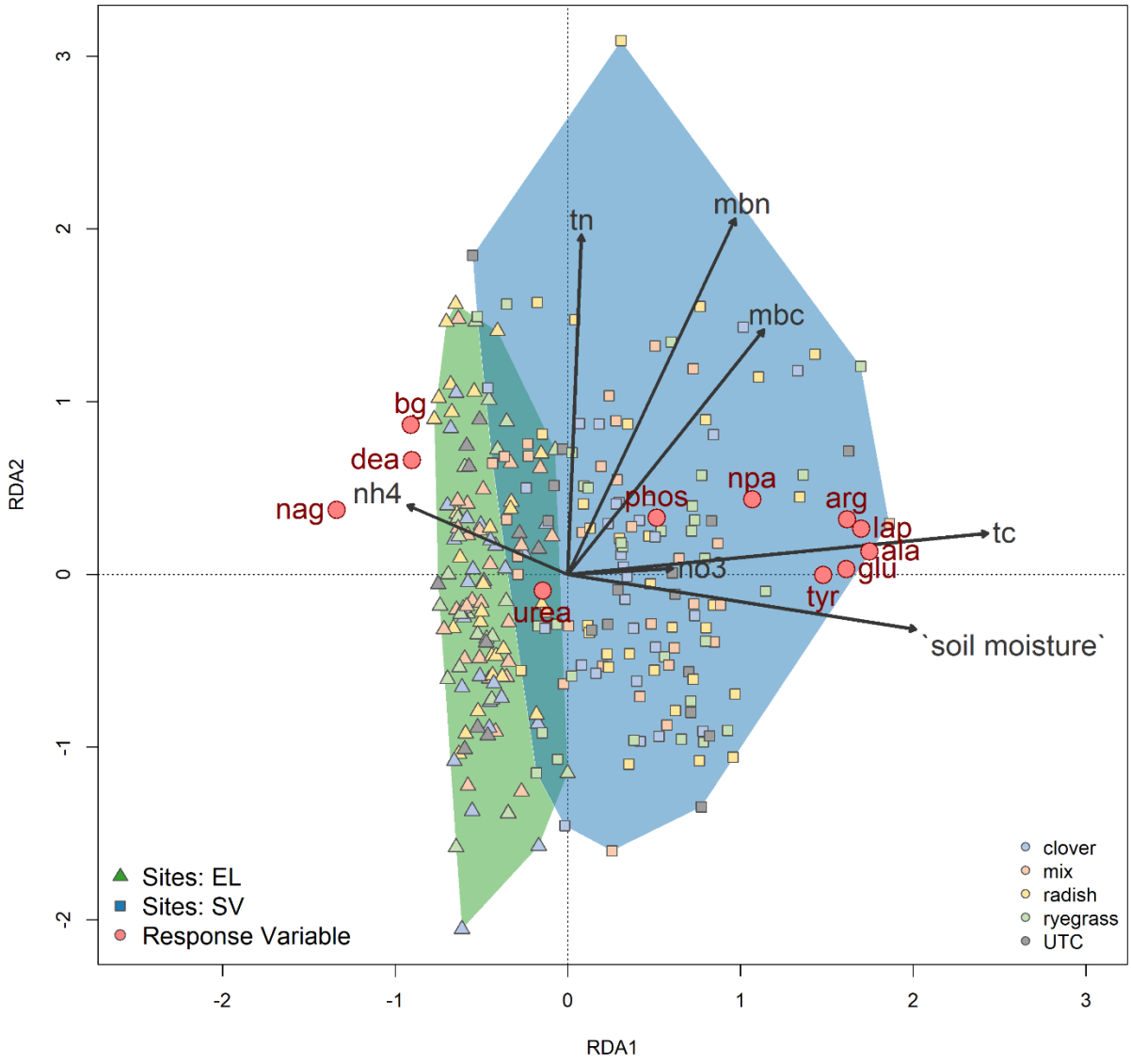
Values indicate average potential rates of nitrification (A) and denitrification (B). Error bars are standard error (n = 4). Means that significantly differ between locations for each season are indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Within each location, significantly different means between seasons are indicated by lowercase letters (α = 0.05).

Figure 2.6: Correlation plots of soil chemical and biological factors at East Lansing (A) and Saginaw Valley (B)



Color scale represents the Pearson correlation coefficient, the significance of which is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 2.7: Redundancy analysis (RDA) ordination of soil biological properties constrained to the variation exhibited within soil chemical properties



Black vectors correspond to soil chemical properties, and red points indicate microbial activities. Blue polygons encompass the samples taken from Saginaw Valley, and green polygons encompass those from East Lansing.

APPENDIX B:

TABLES

Table 2.1: Sampling dates

		2017 Plots		2018 Plots	
Year	Season	East Lansing	Saginaw Valley	East Lansing	Saginaw Valley
2017	Growing	July 10	July 17	–	–
	Postharvest	Nov. 7; Dec. 8; Feb. 26; May 1	Nov. 9; Dec. 19; Feb. 27; Apr. 27	–	–
2018	Growing	July 26; Sept. 12	July 27; Sept. 17	–	–
	Postharvest	Nov. 19; Mar. 27	Nov. 2; Mar. 28	Dec. 3; Apr. 16	Nov. 2; Apr. 22
2019	Growing	–	–	Aug. 14	July 30
	Postharvest	–	–	Nov. 5; Mar. 9; Apr. 22	Oct. 4; Mar. 4; Apr. 28

Soils were sampled at multiple times throughout the year. For analysis sampling dates were combined by season as indicated in the table. Two distinct sets of plots were established at each location in 2017 and 2018. For each set of plots, soil samples were taken in the first year of establishment and during the second year of cover cropping.

Table 2.2: Cover crop biomass

		East Lansing				Saginaw Valley			
		2017 Plots		2018 Plots		2017 Plots		2018 Plots	
Seeding	Crop	2017	2018	2018	2019	2017	2018	2018	2019
V3	Ryegrass	3.05 (1.76)	0.82 (0.28)	1.46 (0.29)	3.47 (1.71)	4.88 (0.5)	0.46 (0.32)	0.41 (0.38)	12.55 (3.8)
	Clover	7.28 (1.7)	0.03 (0.03)	2.44 (0.91)	1.21 (0.73)	1.65 (0.36)	2.49 (0.94)	0.03 (0.02)	2.49 (1.12)
	Mixture	6.39 (1.08)	0.7 (0.35)	0.5 (0.36)	4.07 (1.96)	3.67 (0.73)	2.26 (1.34)	0.23 (0.23)	9.88 (1.47)
	Radish	13.99 (4.14)	1.38 (1.23)	4.5 (3.47)	0 (0)	0.95 (0.61)	0.42 (0.42)	0.26 (0.26)	0 (0)
V6	Ryegrass	2.83 (1.44)	1.94 (0.84)	0.79 (0.47)	3.44 (1.31)	4.58 (0.83)	0.11 (0.04)	0.18 (0.13)	0 (0)
	Clover	3.5 (0.71)	0.64 (0.64)	0.18 (0.11)	2.12 (0.84)	2.81 (0.4)	0.11 (0.06)	0.36 (0.32)	0.19 (0.19)
	Mixture	2.04 (0.45)	0.45 (0.15)	0.21 (0.12)	1.76 (0.65)	3.8 (0.85)	0.3 (0.24)	0.11 (0.04)	0.24 (0.15)
	Radish	5.39 (0.92)	0.67 (0.67)	0 (0)	0 (0)	2.01 (0.5)	0 (0)	0.43 (0.38)	0 (0)

Dry weight of aboveground biomass (g 0.25 m^{-2}) of cover crops at the time of maize harvest. Values presented are means with standard error in parentheses (n = 4).

Table 2.3: Type III ANOVA table of fixed effects for soil chemical properties

	DOC				MBC				NH_4^+			
	F	df	P-value		F	df	P-value		F	df	P-value	
Site (St)	45.86	1	<0.001	***	11.87	1	0.003	**	119.12	1	<0.001	***
Season (Sn)	26.61	6	<0.001	***	7.89	5	<0.001	***	7.28	6	<0.001	***
Crop (C)	0.37	4	0.826		0.81	4	0.520		1.75	4	0.147	
St × Sn	14.77	6	<0.001	***	17.73	5	<0.001	***	5.00	6	<0.001	**
St × C	0.46	4	0.766		0.16	4	0.959		0.19	4	0.945	
Sn × C	0.49	24	0.981		0.59	20	0.922		0.76	24	0.792	
St × Sn × C	0.38	24	0.997		0.69	20	0.840		1.28	24	0.171	

Asterisks next to P-values indicate thresholds of significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Table 2.3 (cont'd)

	NO_3^-				DON				MBN			
	F	df	P-value		F	df	P-value		F	df	P-value	
Site (St)	0.43	1	0.520		62.02	1	<0.001	***	59.63	1	<0.001	***
Season (Sn)	37.63	6	<0.001	***	30.46	6	<0.001	***	24.70	5	<0.001	***
Crop (C)	1.86	4	0.126		0.60	4	0.666		2.81	4	0.031	*
St × Sn	10.10	6	<0.001	***	24.95	6	<0.001	***	1.34	5	0.254	
St × C	1.92	4	0.115		0.51	4	0.728		1.35	4	0.259	
Sn × C	0.55	24	0.958		0.95	24	0.537		0.85	20	0.657	
St × Sn × C	0.70	24	0.851		0.70	24	0.857		0.77	20	0.747	

Table 2.4: Type III ANOVA table of fixed effects for extracellular enzyme activities

	BG				NAG				Peptidase				Phosphatase			
	F	df	P-value		F	df	P-value		F	df	P-value		F	df	P-value	
Site (St)	31.02	1	<0.001	***	71.15	1	<0.001	***	155.56	1	<0.001	***	1.87	1	0.194	
Season (Sn)	21.44	6	<0.001	***	8.23	6	<0.001	***	21.29	6	<0.001	***	36.97	6	<0.001	***
Crop (C)	0.78	4	0.541		1.00	4	0.412		0.21	4	0.932		0.75	4	0.564	
St × Sn	30.93	6	<0.001	***	11.23	6	<0.001	***	16.38	6	<0.001	***	63.12	6	<0.001	***
St × C	0.48	4	0.753		0.27	4	0.898		0.47	4	0.760		0.70	4	0.595	
Sn × C	1.08	24	0.368		0.96	24	0.513		0.87	24	0.650		0.65	24	0.901	
St × Sn × C	1.37	24	0.118		1.28	24	0.170		0.85	24	0.669		0.64	24	0.907	

Asterisks next to P-values indicate thresholds of significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Table 2.5: Type III ANOVA table of fixed effects for potential nitrification and denitrification

	NPA				DEA			
	F	df	P-value		F	df	P-value	
Site (St)	76.09	1	<0.001	***	42.64	1	<0.001	***
Season (Sn)	37.45	6	<0.001	***	23.86	4	<0.001	***
Crop (C)	0.38	4	0.822		0.36	4	0.833	
St × Sn	5.66	6	<0.001	***	5.94	4	<0.001	***
St × C	2.16	4	0.083		1.66	4	0.173	
Sn × C	0.86	24	0.655		1.04	16	0.411	
St × Sn × C	0.67	24	0.878		0.23	16	0.999	

Asterisks next to P-values indicate thresholds of significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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CHAPTER 3:

CARBON AVAILABILITY MEDIATES DIFFERENCES IN RHIZOSPHERE DENITRIFICATION POTENTIAL BETWEEN PLANT SPECIES

3.1. ABSTRACT

Denitrification is an important loss pathway for nitrogen (N) in agricultural systems; however, it is at times unpredictable. Some of the uncertainty surrounding denitrification rates may be due to soil heterogeneity and spatial variability, especially with regard to carbon (C) availability, as it is the energy source fueling denitrification. Nowhere in soils are differences in C concentration and type more evident than in the rhizosphere compared to bulk soils. The rhizosphere is a hotspot of microbial activity, where the chemical composition and quantity of C is quite variable depending on both plant species and plant community diversity. However, we lack an understanding of how rhizosphere-driven differences in C will impact denitrification. Here, I assessed denitrification in laboratory assays of bulk soils and rhizosphere soils of monoculture maize (*Zea mays* L.) and maize interseeded with annual ryegrass (*Lolium multiflorum* Lam.) and crimson clover (*Trifolium incarnatum* L.). In addition, I measured potential denitrification in rhizosphere soils of ryegrass and clover. I found that denitrification potential was enhanced in all rhizosphere soils compared to bulk soil and this effect varied depending on the species of plant, with significantly increased rates of denitrification in maize rhizosphere compared to clover rhizosphere. The availability of dissolved organic C, but not microbial biomass C, accounted for differences in denitrification rates among rhizosphere soils from different plants. Although not consistently enhanced in the rhizosphere, the reduction of N_2O in bulk

soils was significantly lower when maize was intercropped with ryegrass compared to maize grown with clover or in monoculture, demonstrating the effects of increased plant diversity on denitrifiers. This study indicates how denitrification rates can differ significantly within the rhizospheres of different plants and highlights the importance of C availability as a driver of differences in denitrification rates in the rhizospheres of maize and cover crops.

3.2. INTRODUCTION

Denitrification is responsible for the production of nitrous oxide (N_2O) from agriculture, but micro-scale variation and episodic fluxes make N_2O emissions difficult to predict (Groffman et al., 2009). The rhizosphere is one such hot spot where overall microbial activity is elevated, due in large part to greater availability of plant-derived carbon (C) (Philippot et al., 2009). Denitrification is an anaerobic respiratory process, wherein microbes from all three domains of life utilize various nitrogen-oxide species as terminal electron acceptors (Shapleigh, 2013). Denitrification proceeds in a stepwise function and produces N_2O, which some denitrifiers will reduce further to N_2 (Butterbach-Bahl et al., 2013). The primary drivers of denitrification include C and nitrogen (N) availability as well as anaerobicity (Robertson and Groffman, 2015). These factors can vary greatly throughout the soil profile and over time, with for instance a greater portion of anaerobic regions following a large rainfall event. Understanding how these drivers of denitrification vary can improve predictions of N_2O emissions and facilitate the management of ecosystems to produce less N_2O.

The rhizosphere is the area of the soil that is in direct contact with plant roots. This area of the soil receives large inputs of C from plants and is often anaerobic, due to high

levels of respiration from roots and microbes utilizing root-derived C. It is therefore not surprising that past studies have demonstrated an increase in denitrification in the rhizosphere (e.g. Smith and Tiedje, 1979; Højberg et al., 1996; Philippot et al., 2006; Hamonts et al., 2013). Nevertheless, it is not clear which factors in particular drive an increase in denitrification within the rhizosphere. For instance, either changes in C and N availability, anaerobicity, or microbial community composition could all impact denitrification potential. With regard to community composition, others have investigated how the abundance of denitrification genes differs among the rhizospheres of different plant species, possibly indicating unique denitrifier community compositions among plant types (Sharma et al., 2005; Pivato et al., 2017). Such changes to community composition could be caused by the availability of different types of C in the rhizospheres of different plants. The chemical identity of plant-derived C in the rhizosphere differs among plant types and over the life cycle of a single plant (Badri and Vivanco, 2009; Jones et al., 2009). The effect of these differences in root-derived C on denitrification potential in the rhizosphere is unknown.

Intercropping is a practice where multiple plant species are grown in the same plot of land at the same time (Brooker et al., 2015). Interseeding cover crops into maize is emerging as a strategy for promoting cover crop establishment, particularly in regions where the window for seeding cover crops following maize harvest is short (Brooker et al., 2020; CTIC et al., 2020). By increasing plant diversity, interseeding can affect the functioning of the soil microbial community (Tiemann et al., 2015; Finney and Kaye, 2017; Chapter 1). Intercropping-mediated increases in microbial nutrient-cycling activity are not confined to the bulk soil and have been observed within the rhizosphere (Chapter 1). Therefore, intercropping has the potential to impact denitrification rates in both

rhizosphere and bulk soils by altering the type and amount of C inputs to the soil (McGill et al., 2010; Leloup et al., 2018). The effect of interseeding cover crops in maize on denitrification potential in bulk soil and the rhizosphere has not yet been described.

I hypothesized that denitrification rates would be elevated in the rhizosphere, and this increase would vary between plant species. Due to increased demand for N in the rhizosphere, I hypothesized that the potential for N_2O reduction would be greater, since denitrifiers would need to be more efficient in their use of N. Finally, I hypothesized that intercropping would increase potential rates of denitrification by increasing the availability of C and N and stimulating overall microbial community activity.

3.3. METHODS

Bulk and rhizosphere soil samples were taken from a maize field in East Lansing, Michigan, USA, in August 2019. The maize field had been interseeded in June 2019 with two cover crop treatments: annual ryegrass (*Lolium multiflorum* Lam.) and crimson clover (*Trifolium incarnatum* L.). A no-cover-crop control was not seeded with cover crops. Replicate plots were established in four blocks. Further details of the intercropping experiment can be found in Chapter 2 and Brooker et al. (2020). I took maize rhizosphere samples from all three interseeded cover-crop treatments including the no-cover-crop control. In addition, rhizosphere soils from the ryegrass and clover covers interseeded into maize were also sampled. Rhizosphere soil samples were obtained by uprooting plants, vigorously shaking to remove loosely adhering soil, and taking the tightly adhering soil as "rhizosphere soil". Bulk soil samples (1.9 cm diameter core, 10 cm depth) were also taken from all plots. All soil samples were kept on ice until being returned to the lab for

processing, where soils were sieved through a 4 mm mesh and large roots and plant material that did not pass through the sieve were removed.

Fresh soils were then stored at -80^{o}C for later use in denitrification assays. Prior to assays, soils were thawed at 4^{o}C over three days before weighing 5 g of soil into serum bottles, which were then covered and pre-incubated at room temperature for four days before the assay. Potential rates of denitrification and N_2O reduction were obtained by adding 15 mL of nitrate solution (100 mg N g^{-1} soil) to serum bottles, following established protocols for determining denitrification enzyme activities (Groffman et al., 1999). Bottles were stoppered with rubber septa, evacuated, and flushed with N_2. Either C_2H_2 (10% v/v final concentration) or N_2 was added to the headspace of jars to estimate denitrification and net N_2O production, respectively. Headspace samples were taken at 24, 26, and 28 hours and analyzed on a GC equipped with TCD and ECD (TRACE 1310, Thermo Fisher, USA) to determine concentrations of CO_2 and N_2O.

Soil C and N measurements were made by performing soil extractions in 0.5 M K_2SO_4 (5 mL g^{-1} soil). Nitrate and ammonium were measured colorimetrically. Dissolved organic C (DOC) and total dissolved N were determined on an elemental analyzer (vario TOC select, Elementar, Germany). Microbial biomass C (MBC) was determined by adding 1 mL of chloroform to 8 g of soil and incubating for 24 hours. Soil extractions were then performed as described above, and the difference in DOC content between fumigated and non-fumigated samples was used to estimate microbial biomass by applying a correction factor of 0.45 to account for fumigation efficiency (Jenkinson et al., 2004).

Total N_2O production (gross N_2O) was determined by the amount of N_2O in jars that received C_2H_2. N_2 production was calculated by subtracting net N_2O production from gross

N_2O production. The portion of total N_2O production that was not reduced to N_2—referred to as dN_2O—was calculated by dividing gross N_2O production by net N_2O production. The rhizosphere effect, or the relative increase in denitrification activity compared to the surrounding bulk soil, was calculated from total N_2O and N_2 production. For estimating the rhizosphere effect, total N_2O and N_2 production in each rhizosphere sample was divided by total N_2O and N_2 production in the bulk soil sample taken from the same plot.

Statistical analyses were performed in R. Mixed models were constructed using the lmer function of the *lme4* package. Cover crop and soil type (i.e., bulk soil, maize rhizosphere, clover rhizosphere, ryegrass rhizosphere) were fixed effects, and field block and its interaction with fixed effects were coded as random effects. Given the nature of the fixed effects, cover crop and soil type could not be fully crossed (i.e., while maize rhizosphere or bulk soil could be obtained from plots with or without cover crops, no treatments included ryegrass- and clover rhizosphere soil with other cover crops). Therefore, a two-step process was used for modelling soil type and cover crop effects. In the first step, I used a subset of the data that only included data from the maize rhizosphere and bulk soil (i.e., cover crop rhizosphere-soil data were excluded). I used these data to test for main and interactive effects of soil type (bulk versus maize rhizosphere) and cover crop (no cover, ryegrass, or clover) using a full-factorial two-way ANOVA. If no interaction was found between soil type and cover crop—meaning that the soil-type effect was not influenced by cover crop type—then cover crop was omitted from the model and only the effect of soil type was tested on the full dataset. If the interaction was significant, then all cover crop and soil-type treatment combinations were coded as a single factor for a one-way ANOVA. Significance of fixed effects were determined using the *lmerTest* package to

perform F tests with denominator degrees of freedom estimated using the Kenward-Roger method. When fixed effects were significant at a Type I error rate of 0.05, means were separated using Fisher's LSD.

3.4. RESULTS AND DISCUSSION

Rhizosphere soils had up to twice as much denitrification as bulk soil (Fig. 3.1); denitrification rates in maize rhizosphere soil and bulk soil were 12.73 and 6.88 μg N_2O-N g^{-1} soil d^{-1}, respectively. This is in agreement with previous findings that have also found elevated rates of denitrification potential in plant rhizospheres (Smith and Tiedje, 1979; Højberg et al., 1996; Philippot et al., 2006; Hamonts et al., 2013), though this is not always the case (Graf et al., 2016; Ai et al., 2017).

Importantly, I show in this study that rates of denitrification differ among plant rhizospheres: maize rhizosphere soil had significantly greater activity than clover (12.73 versus 9.13 μg N_2O-N g^{-1} soil d^{-1}). These plant-based differences could be due to unique root-exudation profiles between plants. Previous work has shown that denitrifiers possess distinct rates of activity depending on the type of available C (Henry et al., 2008). At the same time, the composition of carbohydrates, organic acids, amino acids, and secondary metabolites within root exudates often differs between, and even within, plant species (e.g., Badri and Vivanco, 2009; Jones et al., 2009). Differences in denitrifier communities and *in situ* denitrification rates within rhizospheres have been correlated to root-exudate compositions (Wu et al., 2017; Maurer et al., 2021). Moreover, these differences in root-exudate composition can shape the structure of rhizosphere microbial communities (Zhalnina et al., 2018), and rates of denitrification could be determined in part by the unique composition of the denitrifier community within each rhizosphere. A recent meta-

analysis found that the genetic potential for denitrification based on quantification of denitrification genes in the rhizosphere differed between plant types, though only few differences among plant types were demonstrated within agricultural systems (Ling et al., 2022). This meta-analysis did not determine whether differences in genetic potential were related to increases in functional potential of denitrifiers, and so whether these community-level changes affect denitrification rates remains unknown.

Higher potential rates of denitrification in the rhizosphere could be due to an overall increase of microbial activity in the rhizosphere, or denitrification potential may be specifically enhanced because of an increased availability of dissolved C. I found that DOC was consistently higher in the rhizospheres of all plants (ryegrass, 105.1 μg DOC-C g^{-1} soil; maize, 89.1 μg DOC-C g^{-1} soil; clover, 82.4 μg DOC-C g^{-1} soil) compared to bulk soil (61.8 μg DOC-C g^{-1} soil), but microbial biomass was only significantly greater in the rhizosphere of maize compared to bulk soil (334 versus 197 μg MBC-C g^{-1} soil; Fig. 3.2). Both DOC and MBC were strongly and significantly correlated with denitrification ($r = 0.695$ and 0.656, respectively; Table 3.1). However, the correlation of MBC with denitrification appeared to be driven by low biomass levels in bulk soil compared to rhizospheres. When rhizosphere denitrification was expressed as a percentage of activity in the surrounding bulk soil, biomass itself was not a strong driver ($r = 0.073$) and only DOC explained a significant amount of variation ($r = 0.615$). Since the increased denitrification potential in the rhizosphere is independent of the size of the microbial community, it appears that denitrification is disproportionately stimulated in the rhizosphere compared to overall microbial growth and activity. In these assays, CO_2 production in rhizosphere soils was only significantly greater than bulk soil in maize rhizosphere (51.5 versus 27.8 μg CO_2-C g^{-1}

soil d^{-1}; Fig. 3.3). CO_2 production in ryegrass rhizosphere soil was only 33.5 μg CO_2-C g^{-1} soil d^{-1} and was not significantly different from bulk soil, which is in contrast to the significantly greater denitrification rates in ryegrass rhizosphere compared to bulk soil (Fig. 3.1).

I also investigated dN_2O, which is the amount of N_2O that is produced and not ultimately reduced to N_2. I found that dN_2O ratios were extremely high in all soil samples, with the vast majority of N_2O production remaining unreduced (Fig. 3.4). High dN_2O values have been shown before, often under conditions with high O_2 or low pH (Cavigelli and Robertson, 2000; Senbayram et al., 2012; Qu et al., 2016). Contrary to my hypothesis, I did not observe significantly lower dN_2O values in rhizosphere soils compared to bulk soils; dN_2O varied between 75 and 100% in both bulk soil and rhizosphere soil. The response of N_2O reducers to rhizosphere conditions can be complicated. While studies have found that genes for N_2O reduction are often enriched in the rhizosphere (Ai et al., 2020; Saghaï et al., 2022), the potential for N_2O reduction is not always increased (Graf et al., 2016).

Competition for N is particularly strong in the rhizosphere (Kuzyakov and Xu, 2013), and I hypothesized that this would drive an increase in N_2O reduction as a way of conserving scarce N. However, I found that inorganic N availability was not consistently lower in rhizosphere soil (Fig. 3.2B). In fact, concentrations of NH_4^+ and NO_3^- were highest in ryegrass rhizospheres. Maize rhizospheres did tend to have the lowest concentrations of inorganic N, but this was not significantly different from bulk soil concentrations. This may have been due in part to dry soil conditions at the time of sampling, which could have reduced plant uptake of inorganic N (Comerford, 2005; Kuzyakov and Xu, 2013). Across all

soil types, inorganic N concentrations did not explain a significant amount of variation in dN_2O (Table 3.1). This is in contrast to previous studies that have shown that dN_2O is sensitive to soil NO_3^- concentrations (Firestone et al., 1979; Qin et al., 2017a; Senbayram et al., 2018). Although N availability may influence the potential for N_2O reduction, a strong concentration gradient did not appear to exist between the different soils I used in the study. Of course, these assays were designed to measure denitrification potential, and non-limiting amounts of NO_3^- were added. Therefore, differences in dN_2O in this study would only reflect the potential for N_2O reduction; *in situ* efficiencies may be quite different under field concentrations of NO_3^-.

Within maize rhizosphere and bulk soil, cover cropping had a significant effect on dN_2O (Table 3.2, Fig. 3.4). Although I found that interseeding cover crops tended to result in a lower proportion of N_2O being reduced, lower rates of denitrification means that cover cropping will not likely increase the potential for net N_2O emissions. Intercropping effects on denitrifiers can be mediated through changes to C and nutrient inputs (Finney et al., 2016) or by broader impacts on the microbial community, such as increased extracellular enzyme activity (Chapter 1). However, C and nutrient levels were not significantly affected by intercropping in this study (Table 3.2), making it difficult to identify the mechanisms behind the intercropping effect on dN_2O.

Overall, I found that the potential for N_2O production is higher in the rhizosphere and this effect differed among plant species. This provides further evidence for the importance of plant-derived compounds for influencing the rhizosphere microbial community. Here, I show that denitrification potential is significantly different among plant species intercropped together within the same plot. The availability of DOC was the

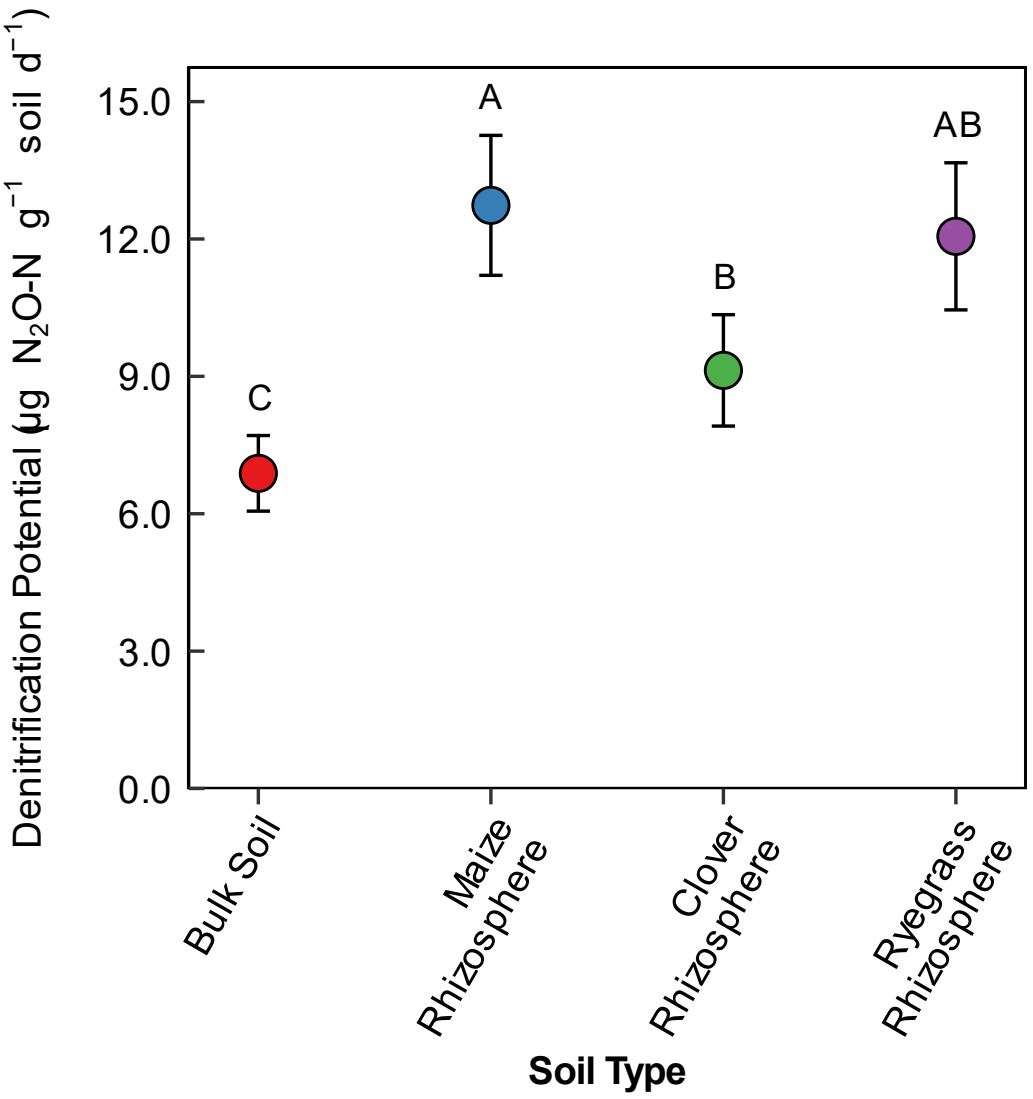
strongest driver of this effect. dN_2O did not differ significantly between bulk and rhizosphere soils, but this value was affected by intercropping, where intercropping led to slightly less efficient denitrification pathways. In conclusion, this study demonstrates the importance of root-derived C inputs on increasing the potential for denitrification in a field system.

APPENDICES

APPENDIX A:

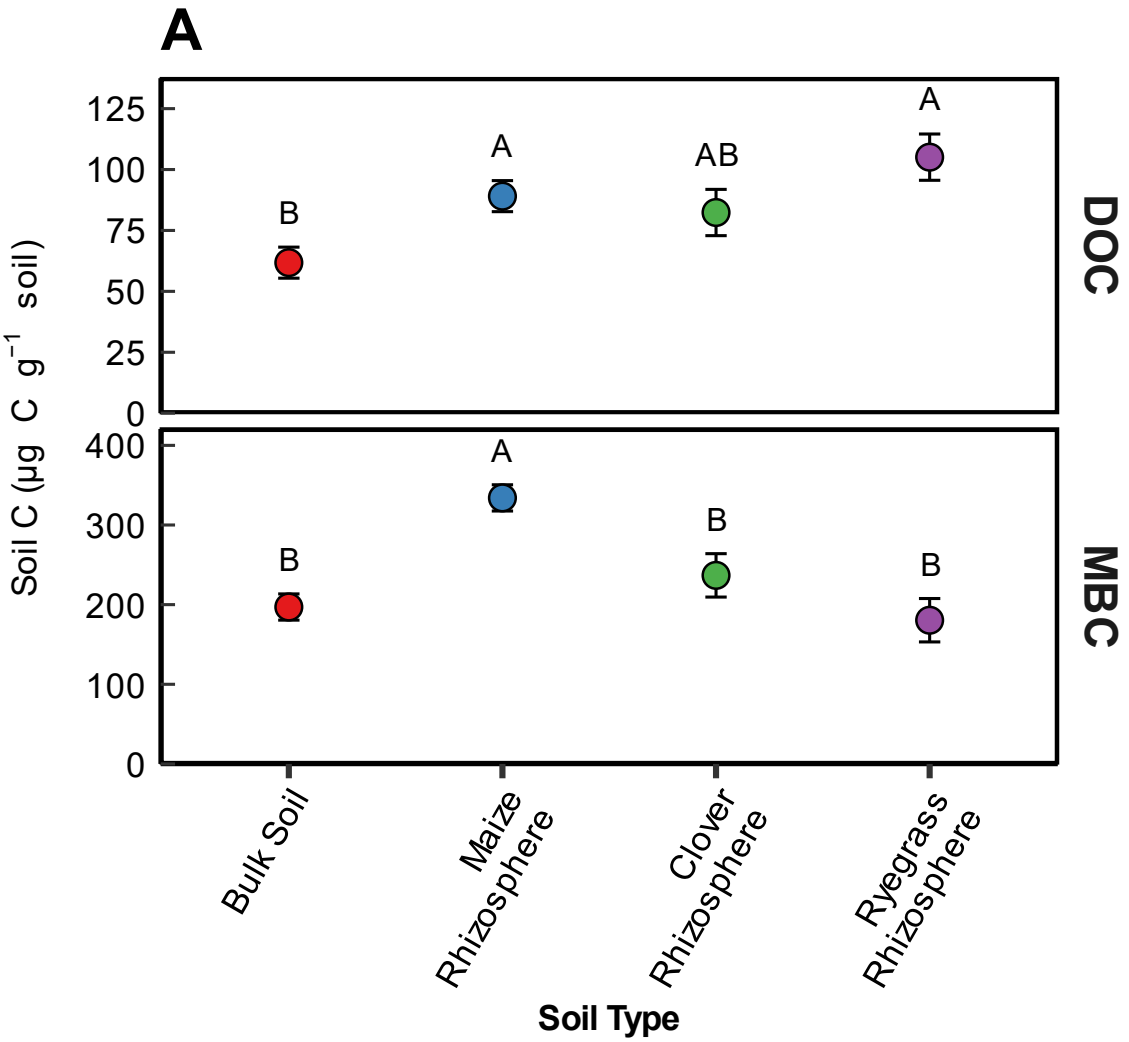
FIGURES

Figure 3.1: Differences in denitrification potential among soil types



Maize, clover, and ryegrass refer to rhizosphere soil from respective plant types. Error bars are standard error (n = 4). Letters indicate significant differences between means at α = 0.05.

Figure 3.2: Differences in soil DOC and MBC (A) and soil NH_4^+, NO_3^-, and DON (B) between soil types



Maize, clover, and ryegrass refer to rhizosphere soil from respective plant types. Error bars are standard error (n = 4). Letters indicate significant differences between means at α = 0.05.

Figure 3.2 (cont'd)

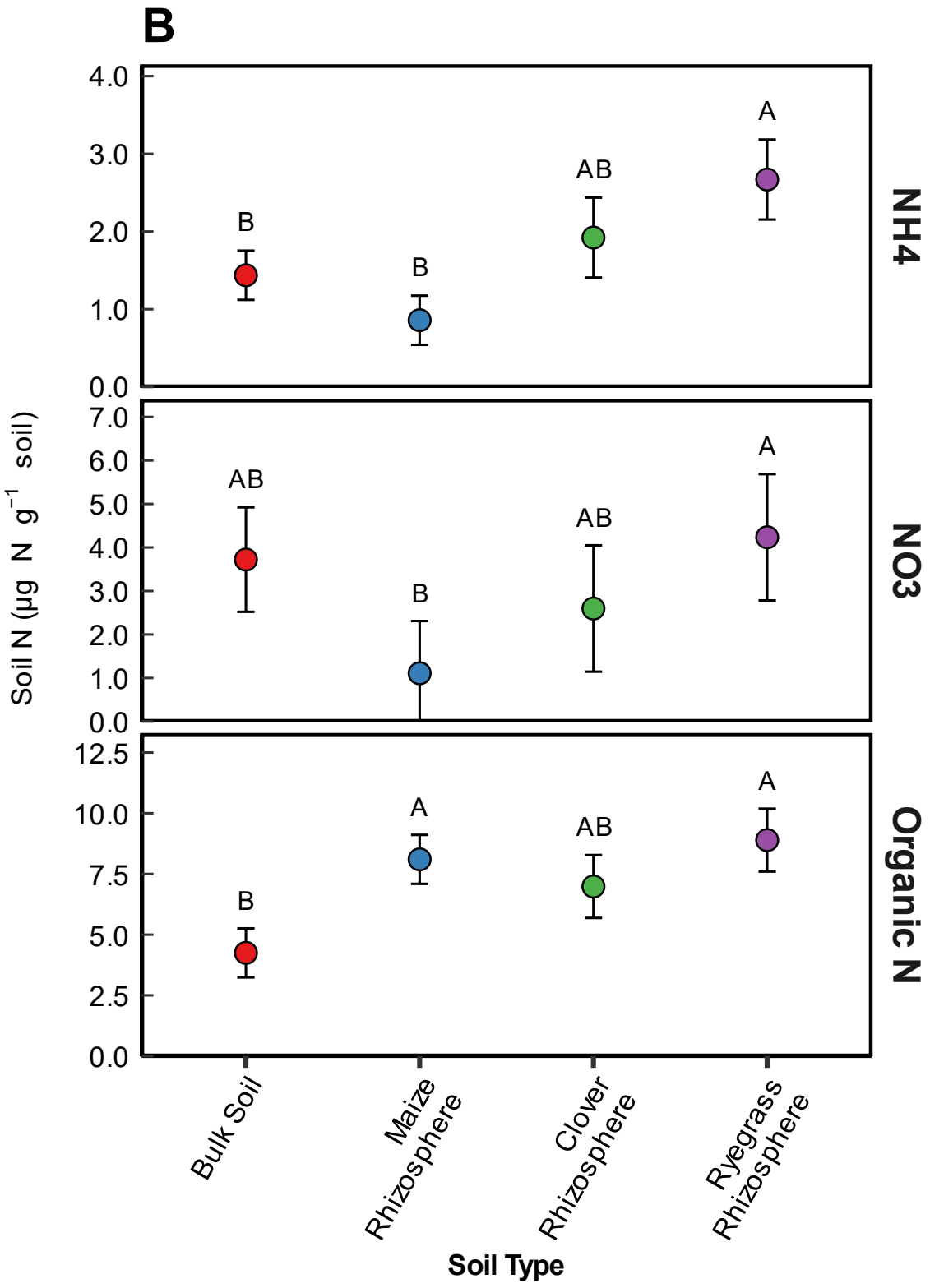
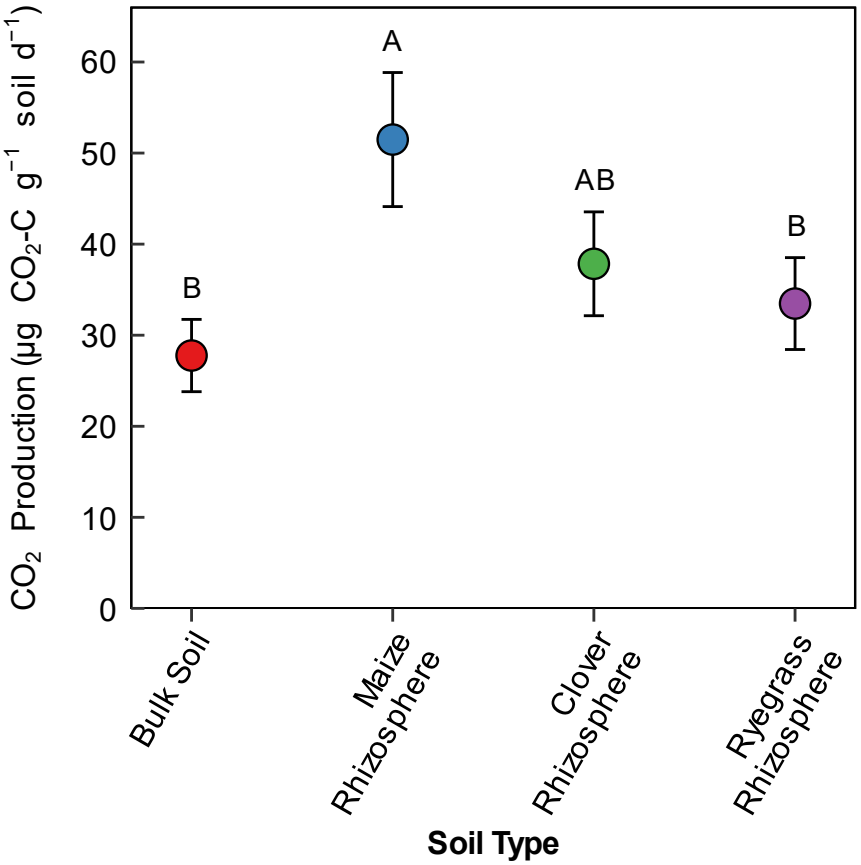
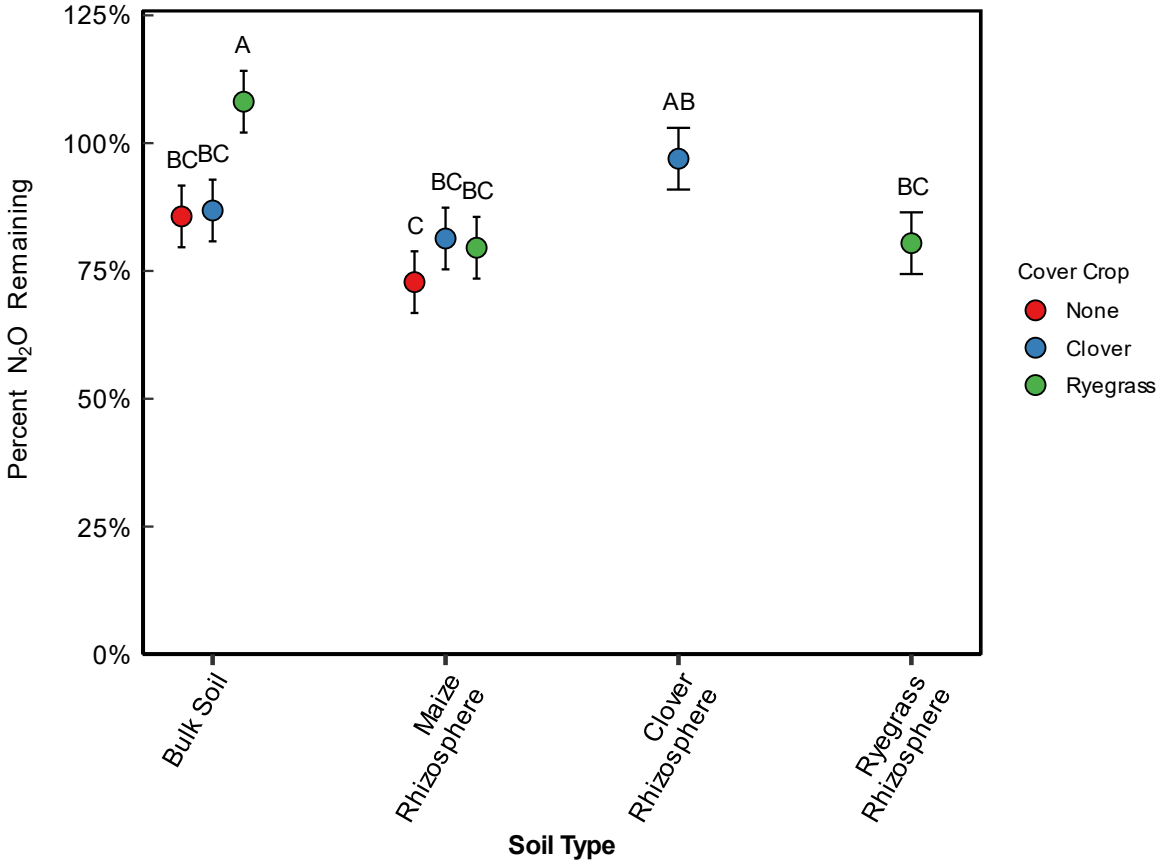


Figure 3.3: Differences in CO_2 production among soil types



Mean values of CO_2 production from bulk soil and maize, clover, and ryegrass rhizospheres. Error bars are standard error (n = 4). Letters indicate significant differences between means at $\alpha = 0.05$.

Figure 3.4: Differences in the percent N_2O remaining after N_2O reduction to N_2 occurs



Maize, clover, and ryegrass refer to rhizosphere soil from respective plant types. Letters indicate significant differences between means at $\alpha = 0.05$.

APPENDIX B:

TABLES

Table 3.1: Correlation coefficients between potential denitrification activities and soil chemical parameters

	DOC	MBC	NO_3	NH_4	DON
Total N_2O Production	0.695***	0.656***	-0.065	0.013	0.620***
dN_2O	-0.334	-0.402*	0.055	0.234	-0.231
Rhizosphere Effect[a]	0.615**	0.073	0.252	0.211	0.526*

dN_2O is the proportion of N_2O remaining following N_2O reduction to N_2. The rhizosphere effect was the relative increase in total denitrification activity (N_2O production and N_2O reduction) in the rhizosphere compared to the surrounding bulk soil. Significance of the correlations is indicated by asterisks (*, P-value < 0.05; **, P-value < 0.01; ***, P-value < 0.001). [a]The rhizosphere effect was correlated against concentrations of C and N in the rhizosphere.

Table 3.2: Type III ANOVA table of fixed effects

	Gross N_2O		dN_2O		DOC		MBC	
	F	P	F	P	F	P	F	P
Model 1: Subset								
Soil Type	36.0	0.009	13.7	0.034	15.5	0.029	65.3	0.004
Cover Crop	3.2	0.114	9.9	0.013	0.6	0.557	4.5	0.065
Soil Type × *Cover Crop*	0.0	0.984	6.2	0.034	0.8	0.505	0.1	0.913
Model 2: All Data								
Soil Type	13.3	0.001	–	–	8.2	0.005	15.9	< 0.001
Soil Type : Cover Crop	–	–	3.4	0.013	–	–	–	–

Model 1 was performed on a subset of data that included only maize rhizosphere and bulk soil, allowing for the significance of the interaction between soil type and cover crop to be assessed in a two-way ANOVA. If the interaction was found to be significant, then a second model constructed from the full dataset incorporated each treatment combination of the two factors in a one-way ANOVA. If the interaction was not significant, then cover crop was omitted from the model and only the effect of soil type was tested.

Table 3.2 (cont'd)

	NO_3		NH_4		Organic N	
	F	P	F	P	F	P
Model 1: Subset						
Soil Type	3.9	0.144	2.9	0.185	5.3	0.105
Cover Crop	0.4	0.681	1.3	0.332	2.1	0.205
Soil Type × *Cover Crop*	2.2	0.192	0.6	0.566	0.0	0.960
Model 2: All Data						
Soil Type	2.5	0.128	3.6	0.054	4.2	0.038
Soil Type : Cover Crop	–	–	–	–	–	–

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CHAPTER 4:

CHEMICAL IDENTITY OF CARBON SUBSTRATES DRIVES DIFFERENCES IN DENITRIFICATION AND N_2O REDUCTION WITHIN AGRICULTURAL SOILS

4.1. ABSTRACT

Rates of nitrous oxide (N_2O) production from agricultural soils are highly variable across space and time. Improving predictions of N_2O emissions will require improving our understanding of the drivers of denitrification and the sources of variability. While the availability of carbon (C) is a known control on denitrification and N_2O reduction, relatively little attention has been paid to the effect of the chemical identity of C substrates on rates of denitrification and N_2O reduction. I investigated the effects of twelve different C-substrate additions on the production and reduction of N_2O in five soils taken from two distinct agricultural locations in Michigan under multiple land uses. I provided additions of glucose, cellulose, N-acetyl-glucosamine, chitin, amino acids, protein, vanillyl alcohol, lignin, citrate, succinate, methanol, and water in laboratory denitrification potential assays to determine the effects of denitrifier C preference on denitrification rates. I found that amino acids, protein, and organic acids stimulated the greatest amount of denitrification potential across all land uses. Similarly, I found these same substrates resulted in the most N_2O reduction and lowest net concentrations of N_2O. Agricultural rotations without cover crops had overall lower rates of enzyme activity, leading to less net N_2O production. In general, C-utilization patterns were similar among all soils, and C-substrate identity had a much stronger effect than site. These results suggest that denitrifier C preference gives rise to variability in denitrification rates depending on the chemical identity of available C. This

highlights the importance of considering the heterogeneity of environmental factors, such as different types of C, when attempting to predict the functioning of the soil microbial community.

4.2. INTRODUCTION

Nitrous oxide (N_2O) is a greenhouse gas with a global warming potential nearly 300 times greater than that of CO_2 (IPCC, 2014). Nearly half of global N_2O emissions are anthropogenic, with agricultural accounting for the largest share by far (Tian et al., 2020). Net emissions of N_2O are the result of multiple microbially mediated processes but denitrification is thought to be the predominant N_2O generation pathway in agricultural systems (Opdyke et al., 2009; Liang and Robertson, 2021). Denitrification is an anaerobic, respiratory metabolism where inorganic N species are reduced in a stepwise manner within the electron transport chain to generate ATP through oxidative phosphorylation. The end products of denitrification are N_2O and N_2. There are multiple controls on the process of denitrification in general and on the end-product ratio in particular (Firestone and Davidson, 1989).

Even though the main drivers of denitrification are known to include carbon (C), nitrogen (N) and O_2 availability, denitrification rates and net N_2O emissions remain difficult to predict and are subject to large spatial and temporal variation. Much of this variation is due to the spatial distribution of the main drivers of denitrification throughout the soil profile, giving rise to micro-scale variation and episodic fluxes in denitrification rates (Groffman et al., 2009; Kuzyakov and Blagodatskaya, 2015). For example, anaerobic microsites can exist even within well-aerated soils, promoting denitrification (Hojberg and Sorensen, 1993; Kravchenko et al., 2017; Schlüter et al., 2018). In addition, the distribution of

particulate C substrates throughout the soil creates zones of high N_2O production (Parkin, 1987; K. Kim et al., 2020), but the effect of the chemical identity of these heterogeneously distributed forms of C on rates of N_2O production and reduction has not been well described.

One of the most important yet least explored factors determining denitrification rates and N_2O production or consumption is the biochemistry of the C compounds that supply the electrons used by denitrifiers to reduce N oxides. The importance of the quantity of C available seems obvious, with more available C yielding more electrons, but there are complex interactions that control how C quantity affects denitrification and net N_2O production. For instance, when C is limiting, net N_2O production tends to be higher, due to a lower demand for terminal electron acceptors (Pidello et al., 1996). Likewise, N_2O reduction has been shown to be inversely related to the availability of alternative electron acceptors, such as NO_3^- (Firestone et al., 1980; Miller et al., 2008; Senbayram et al., 2012). Therefore, higher rates of C availability should stimulate more N_2O reduction and a lower N_2O:N_2 ratio.

In addition to C quantity, the quality or type of C substrate and its accessibility is important. Although the idea that the chemical identity of available C is a driver of denitrification has been recognized for decades (e.g., de Catanzaro and Beauchamp, 1985), we still lack a clear understanding of how substrate identity is tied to rates of denitrification. Multiple studies have come to widely different conclusions on the effects of particular C substrates on denitrification. Some studies have described glucose and other simple carbohydrates stimulating more denitrification than organic acids and amino acids (Smith and Tiedje, 1979; Dendooven et al., 1996; Miller et al., 2008; Morley and Baggs, 2010),

while others have found the opposite (Morley and Baggs, 2010; Morley et al., 2014). Likewise, great variability exists in how denitrifiers respond to whole plant residues compared to low-molecular-weight C additions (de Catanzaro and Beauchamp, 1985; Senbayram et al., 2012; Giles et al., 2017). Much of this inconsistency comes from comparing studies that were performed under a variety of conditions, making it difficult to differentiate the effects of C chemistry from soil characteristics and environmental factors, such as N availability and anaerobicity. Further work is needed to identify the particular characteristics of C substrates that affect denitrification and whether C-based effects are consistent across different soils and microbial communities.

Setting aside other factors, the C compounds available in the soil possess various inherent characteristics that may increase or decrease denitrification. For instance, the accessibility of C differs between monomeric and polymeric compounds, with the degradation of polymeric residues by extracellular enzymes often thought to be the rate-limiting step in the mineralization of complex C residues (Sinsabaugh, 1994). Monomeric forms of C should then result in lower net N_2O emissions than polymeric forms because of the extra step involved in making the C accessible. Moreover, the C:N ratio of C substrates plays an important role in influencing denitrification and is a good predictor of N_2O production (Huang et al., 2004; Millar and Baggs, 2004; Toma and Hatano, 2007). As the C:N ratio narrows, N_2O production tends to increase. This is likely the result of low-C:N-ratio residues both supplying N to fuel denitrification and being a good source of C since these residues also tend to be easier to decompose. Finally, the redox state of compounds can influence denitrification, with more electron-rich, highly reduced substrates able to reduce more units of nitrate and therefore drive greater rates of denitrification. On the other

hand, more highly reduced substrates will often be available to organisms with other anaerobic metabolisms, such as fermentation (Reddy et al., 1982; Pidello et al., 1996), leading to competition, which could reduce denitrifier access to such C sources and provide an ecological opportunity for denitrifiers to specialize on more highly oxidized compounds such as organic acids. Indeed, succinate has been used as the C source in denitrifier isolation media (Heylen et al., 2006). If competition with other microbes prevents denitrifiers from obtaining C, then compounds available more exclusively to denitrifiers, such as succinate, should increase denitrification rates.

Denitrifier community composition and how different denitrifier species respond to different C substrates is also likely to be a critical factor. Previous research has demonstrated that individual denitrifier isolates possess their own C preferences and that synthetic communities composed of denitrifiers with complementary C preferences can produce denitrification rates greater than other synthetic communities with overlapping resource niches (Salles et al., 2009, 2012). In addition, denitrifiers have differing capacities to carry out denitrification. Denitrifier communities vary across land uses within the same landscape (Cavigelli and Robertson, 2001; Juhanson et al., 2017; Maul et al., 2019) and possess unique rates of denitrification and distinct sensitivities to environmental factors (Cavigelli and Robertson, 2000; Krause et al., 2017; Maul et al., 2019). These variations could extend to their use of C. In general, soil microbial communities from separate land uses often differ in terms of C utilization. This is the basis for community-level physiological profiling and popular techniques such as the Biolog plate assay (Garland and Mills, 1991). Underlying these patterns, differing legacies of C inputs between land-use histories can dictate the C preference of soil microbial communities, giving rise to a home-field

advantage where microbes more quickly mineralize the C types they have historically been exposed to (Ayres et al., 2009). In addition, extracellular enzyme activity has been linked to the quality and diversity of C-input legacies, with rotational diversity and intercropping both increasing extracellular enzyme activity (McDaniel et al., 2014; Chapter 1). Such effects on enzyme activity could potentially lead to differences in the amount of denitrification stimulated by polymeric forms of C. Therefore, the C utilization profiles of denitrifiers may differ between land uses, with higher overall rates of denitrification in soils with greater aboveground diversity. Such land use effects could potentially account for discrepancies in C-utilization studies between denitrifiers from different soils. However, how land use influences the C preference of denitrifiers has not yet been examined.

Using lab incubations of soils from two agricultural field experiments with varying management practices, I explore the interacting effects of land-use history, denitrifier community structure, and C-compound quality and accessibility on N_2O production and consumption due to denitrification. I hypothesized that in the same soils, the chemical identity of C inputs would result in different levels of denitrification and N_2O reduction. Moreover, I hypothesized that different land uses would lead to denitrifier communities with distinct C-utilization profiles.

4.3. METHODS

4.3.1. Land Uses and Sampling

I sampled soils from two locations in Michigan. The first location was the Kellogg Biological Station (KBS) (Hickory Corners, MI, 42° 24′ N, 85° 24′ W), where I utilized field treatments from the KBS Main Cropping System Experiment, a Long-Term Ecological Research site established in 1989. Soils at this location are Typic Hapludalfs (fine-loamy,

mixed, mesic; Table 4.1). I utilized three field treatments from this site: conventional agriculture, reduced-input agricultural, and perennial switchgrass. The conventional and reduced-input agriculture treatments are in a corn (*Zea mays*)-soybean (*Glycine max*)-wheat (*Triticum aestivum*) rotation with conventional tillage. The conventional treatment receives synthetic fertilizer inputs, and the reduced-input treatment receives a portion of its N inputs through winter leguminous cover crops. Both conventional and reduced input receive herbicide treatments to manage weeds. The perennial treatment previously contained continuous alfalfa but was switched to continuous switchgrass (*Panicum virgatum* L.) in 2019. Additional details can be found in Robertson and Hamilton (2015). Treatments are organized in a randomized-block design, and I utilized soils from four blocks. Soil cores (1.9 cm diameter) were taken to a depth of 10 cm in April 2021, following a corn rotation and prior to soybean planting.

The second set of soils was sampled from the Montcalm Research Center (MRC) (Montcalm, MI, 43° 3′ N, 85° 1′ W). These soils are Oxyaquic Glossudalfs (fine, mixed, frigid; Table 4.1). I sampled soils from two treatments of a field experiment established in 2015. The two treatments differed only in the use of cover crops; one treatment is seeded with a mixture of annual rye (*Lolium multiflorum* Lam.) and hairy vetch (*Vicia villosa*), while the other treatment has no cover crops. Both treatments are in a potato (*Solanum tuberosum*)-corn (*Zea mays*) rotation and receive conventional fertilizer and herbicide applications. Field treatments are organized in a randomized-block design. I took soil cores to a depth of 10 cm in October 2020, one week following potato harvest.

For each set of soil samples, soil cores were kept on ice in the field and brought back to the lab for processing. Soils were sieved through a 2 mm mesh, and soil moisture content

was assessed gravimetrically. Soils were kept at 4°C until utilized in denitrification assays, within two weeks of sampling.

4.3.2. Denitrification Assays

To assess denitrifier response to various C substrate additions, I modified a standard denitrification enzyme activity assay protocol (Groffman et al., 1999). Specifically, 5 g of soil were measured into 60 mL serum bottles. To each bottle, 5 mL of H_2O was added. After one hour, 10 mL of KNO_3 solution (0.1 mg N mL^{-1}) was added together with one of 12 different C-substrate treatments to a final concentration of 4.4 mg C g^{-1} soil. The different C substrates used were glucose, cellulose, N-acetyl-glucosamine (NAG), chitin, amino acids, soy protein isolate, vanillyl alcohol, lignin, citrate, succinate, and methanol. In addition, a twelfth treatment contained no C addition. Soluble forms of C were provided dissolved in the KNO_3 solution. Insoluble forms of C were added as dry additions together with an equivalent amount of KNO_3 solution. Jars were crimp capped with butyl-rubber septa.

I then evacuated and flushed each jar with N_2 three times to atmospheric pressure to create an anaerobic atmosphere. Jars were divided into two sets; one set received acetylene (C_2H_2) at 10% v/v and the other set received an equivalent amount of N_2. C_2H_2 inhibits the enzyme responsible for the reduction of N_2O to N_2, allowing for an estimation of gross versus net N_2O production (Yoshinari and Knowles, 1976).

Jars were kept on an orbital shaker at 150 rpm between gas sampling times, which were determined based on earlier optimization studies that demonstrated nitrous oxide reductase was fully induced after ~24 hours and that soil microbes had not yet reached an exponential growth phase. I took two sets of headspace gas samples (3 mL) injected into pre-evacuated 12 mL GC vials: the first at 4, 6, and 8 hours and the second at 24, 26, and 28

hours. The remaining volume of the GC vials was filled with N_2. Following each gas sampling, the headspace removed from each jar was replaced using either N_2 or a 90:10 mixture of N_2:C_2H_2. Gas samples were analyzed for N_2O and CO_2 concentrations on a TRACE 1310 (Thermo Fisher, USA) gas chromatograph equipped with an ECD and TCD.

4.3.3. Amino-Acid Assays

I performed a follow-up experiment to explore the effects of the amino acid mixture on N_2O production and reduction. For these assays, I used soils from the reduced-input field treatment from KBS. Assays were performed as described above with single amino-acid additions that represent different amino-acid side-chain functional groups. Substrate additions included L-alanine, L-aspartate, L-glutamate, L-tryptophan, L-serine, and L-histidine. In addition, I compared these individual amino acid additions to an equimolar mixture of all six amino acids, as well as to the addition of casamino acids and glucose.

4.3.4. Statistics

Concentrations of N_2O in the jars that contained C_2H_2 were used for estimates of gross N_2O production. Nitrous oxide concentrations in jars that did not receive C_2H_2 were used to estimate net N_2O productions. The proportional difference between net and gross N_2O production is often used to estimate the efficiency of the complete denitrification pathway. I calculated this value as dN_2O according to the following equation.

$$dN_2O = \frac{N_2O_{net}}{N_2O_{gross}}$$

Statistical analyses were performed in R. Data from each study site, KBS and MRC, were analyzed separately. For analysis, all concentration data were log transformed to achieve normality. The *lme4* package (Bates et al., 2015) was utilized to create mixed-effects

models with land-use, substrate addition, and their interaction as fixed effects and field-treatment block as a random effect. Marginal means were calculated using the *emmeans* package (Lenth et al., 2019). Using the *lmerTest* package (Kuznetsova et al., 2017), type III tests for fixed effects were performed using the Kenward-Roger method for calculating the denominator degrees of freedom. When fixed effects were found to be significant, mean comparisons between substrate treatments within fields and between fields within substrate were performed using Fisher's LSD at a Type I error rate of 0.05.

4.4. RESULTS

4.4.1. C-Substrate Effects on Potential Gross N_2O Production

C substrate treatments had large effects on gross N_2O production in soils from all land uses across both locations (Table 4.2). I found that amino acids and protein stimulated the most gross N_2O production across all soils, regardless of land management, with up to 42 μg N_2O-N g^{-1} soil d^{-1} being produced from the amino-acid-amended soils from the KBS reduced input treatment (Fig. 4.1). Organic acids also consistently stimulated some of the largest amounts of gross N_2O (citrate produced 20 μg N_2O-N g^{-1} soil d^{-1} in reduced-input soils), followed by glucose (13.4 μg N_2O-N g^{-1} soil d^{-1}) and NAG (8.7 μg N_2O-N g^{-1} soil d^{-1}). Vanillyl alcohol, lignin, and methanol had the lowest rates of gross N_2O production. However, these substrates still stimulated significantly more gross N_2O production than the no-C additions, which resulted in between 2.3 and 4.8 μg N_2O-N g^{-1} soil d^{-1} among the land uses at KBS and 2.4 and 2.9 μg N_2O-N g^{-1} soil d^{-1} in the MRC soils.

The monomeric compounds inconsistently stimulated more gross N_2O production than their polymeric counterparts. At KBS, glucose and amino acids stimulated about twice as much gross N_2O production than cellulose and protein, respectively. However, the

denitrifier response to amino acids was stronger than that of protein only in the KBS soils (Fig. 4.1A); in the MRC soil with no cover crops, protein stimulated approximately 75% more gross N_2O production than amino acids (Fig. 4.1B). Vanillyl alcohol never stimulated significantly more gross N_2O production than lignin.

4.4.2. C-Substrate Effects on Potential Net N_2O Production

Across all KBS soils, amino acids and protein additions resulted in the lowest rates of net potential N_2O production (between 0.31 and 1.0 μg N_2O-N g^{-1} soil d^{-1}), indicating the greatest N_2O reduction (Fig. 4.2A). Organic acids also tended to have lower net N_2O production, but only the succinate addition in the reduced-input treatment (0.59 μg N_2O-N g^{-1} soil d^{-1}) was significantly different from the no-C additions (between 1.9 and 3.4 μg N_2O-N g^{-1} soil d^{-1}). Meanwhile, no C substrate resulted in net N_2O production significantly greater than that of water alone, indicating that N_2O reduction kept pace with the N_2O production stimulated by each C substrate.

dN_2O describes the portion of total N_2O production that remains as N_2O following N_2O reduction. Proteins and amino acids had the lowest dN_2O (3.4–14%; Fig. 4.3A). Organic acids (7–24%) also had significantly lower dN_2O than other substrates. Glucose and NAG had intermediate dN_2O. Within the conventional treatment, lignin had a low dN_2O comparable to that of glucose and NAG. In addition, methanol-induced N_2O reduction was significantly greater than that of water, but only in the conventional treatment. All other substrates had dN_2O values not significantly different from that of water (~7%).

Within MRC soils, proteins and citrate had the lowest net N_2O production with only 0.05 and 0.08 μg N_2O-N g^{-1} soil d^{-1} being produced by soils amended with protein in the cover cropped and no-cover treatments, respectively (Fig. 4.2B). In contrast to the KBS soils,

amino acids (~2.6 μg N_2O-N g^{-1} soil d^{-1}) did not result in lower net N_2O production than other substrates. In fact, amino acids had significantly higher net N_2O production than water in the no-cover treatment (2.8 versus 1.4 μg N_2O-N g^{-1} soil d^{-1}**)**. Within these soils, glucose had the highest amount of net N_2O production (4.7 μg N_2O-N g^{-1} soil d^{-1}), while cellulose, NAG, chitin, and lignin also had significantly higher net N_2O production compared to the water-only addition. In the cover crop treatment, only glucose, cellulose, and lignin had significantly greater net N_2O production than the water-only treatment (1.9 μg N_2O-N g^{-1} soil d^{-1}).

Protein and citrate stimulated denitrification with the lowest dN_2O values in both treatments at MRC (Fig. 4.3B). Amino acids and succinate also had low dN_2O values. In the cover crop treatment, NAG and chitin had dN_2O values significantly lower than that of water. Within the conventional treatment, glucose, vanillyl alcohol, and lignin had dN_2O values significantly higher than water.

4.4.3. Land-Use Effects on Gross N_2O Production

Within the KBS treatments, I found that the conventional treatment had significantly less potential N_2O production across most C substrate additions, with almost half as much N_2O production in some additions (Fig. 4.1A). Interestingly, the reduced-input treatment tended to be more similar to the perennial system than the conventional system, despite sharing a corn-soy-wheat rotation. Land-use differences were similar across most substrates. Amino acids were the only substrate that did not have a significant land-use effect.

To better distinguish the stimulatory effects of C substrate from differences in basal denitrifier activity, I scaled the N_2O production stimulated by each substrate to the amount

of N_2O produced in response to water alone. From this perspective, amino acids, proteins, succinate, and lignin showed the largest differences between land uses, with the conventional treatment showing the greatest stimulation in response to each of these substrates (Fig. 4.4A).

At MRC, land-use effects were not significant either on gross N_2O production or when scaling substrate-induced N_2O production to water additions (Table 4.2).

4.4.4. Land-Use Effects on Net N_2O Production

At KBS, land-use effects were significant, but this depended on the substrate addition treatment (Table 4.2). The conventional-agriculture soils had significantly lower net N_2O production in response to glucose, vanillyl alcohol, lignin, succinate, and methanol (Fig. 4.2A). Protein resulted in higher net N_2O levels in the conventional-agriculture soils compared to the other two treatments. The reduced-input and perennial systems tended to have similar levels of net N_2O production across most substrates, but reduced input had significantly lower net N_2O levels in response to succinate.

When comparing dN_2O, a significantly greater portion of N_2O remained when soils from the perennial management were amended with glucose compared to soils under conventional management (Fig. 4.3A). Lignin stimulated significantly lower amounts of dN_2O in the conventional treatment compared to the other two land managements. dN_2O was higher in conventional following protein addition and in perennial following succinate, but these differences were not significant.

Among the MRC treatments, the cover cropped soils had higher net N_2O levels than the no-cover-crop treatment and this effect did not depend on substrate additions (Table

4.2). Comparing net N_2O as a percentage of total N_2O production, there were no significant land-use effects.

4.4.5. Amino-Acid Assays

Among the individual amino-acid additions, the two negatively charged, polar acidic amino acids stimulated the greatest amount of gross N_2O production, with glutamate stimulating significantly more N_2O production than aspartate (Fig. 4.5A). Tryptophan stimulated the lowest amount of N_2O production of all additions. The mixture of six amino acids stimulated slightly more N_2O than the glutamate addition, but the cas-amino-acid addition resulted in more than twice as much N_2O production than the next highest treatment. Glucose additions stimulated about as much N_2O production as the aspartate addition.

The lowest net production of N_2O resulted from the addition of the six-amino-acid mixture (Fig. 4.5B). Aspartate and glutamate had similar levels of net N_2O production, which were significantly lower than the remaining treatments. Glucose additions resulted in the greatest amount of net N_2O production, but this did not differ significantly from the alanine, tryptophan, serine, and histidine additions.

4.5. DISCUSSION

My results support the hypothesis that the chemical identity of available C affects rates of N_2O production and reduction. Specifically, I saw that simple and less-reduced forms of C stimulated denitrifiers the most and that monomers didn't always results in greater denitrification rates. I found denitrification rates varied nearly ten-fold within the same soil, depending on the types of added C. This suggests that denitrifier C preference can account for variability in denitrification rates even within the same soil. My results also

supported the hypothesis that different microbial communities from different land use histories have distinct use preferences and profiles, although these differences were small compared to substrate effects.

The handful of previous studies that examined denitrification rates following the addition of different forms of C have yielded varying conclusions. Some found no significant effect of organic acid or amino acid content in C cocktails (Henry et al., 2008), while others found amino acids and organic acids to elicit widely different rates of N_2O production and reduction (Morley et al., 2014; Giles et al., 2017). As may be expected, differences between C substrates tend to be smallest when denitrification rates are lowest—such as when oxygen is available or nitrate is limiting (Miller et al., 2008; Morley and Baggs, 2010; Langarica-Fuentes et al., 2018). To focus in on the effects of C substrate, *per se*, I included non-limiting amounts of nitrate under anaerobic conditions. In addition, I chose the twelve C substrate treatments in order to systematically identify the key aspects of C compounds that stimulate N_2O production and reduction.

4.5.1. C-Substrate Characteristics

Amino acids and proteins tended to stimulate by far the most N_2O production, with nearly ten times more N_2O production than the no-C addition and a five-fold increase compared to glucose (Fig. 4.1). One possible reason is the supply of N. Amino acids contain varying levels of N, and this could have further stimulated denitrifiers. For instance, among different residue additions, those with greater amounts of N tend to stimulate more denitrification (de Catanzaro and Beauchamp, 1985; Aulakh et al., 1991; Huang et al., 2004). However, in this experiment, all C substrate additions also received non-limiting quantities of nitrate, ensuring adequate amounts of N to serve as electron acceptors. On the other

hand, the N available in amino acids is more reduce than NO_3^- and may be preferentially incorporated into biomass (Geisseler et al., 2010). Nevertheless, the reduced N that amino acids contain is likely not the primary driver of the increased denitrification rates. Among the C substrates I utilized were glucose and NAG. Following the release of an amine group and acetyl group, NAG is metabolized in a manner similar to glucose. Despite this additional reduced N, I never saw NAG stimulate more denitrification than glucose. On the other hand, the cellular transporters for amino acids may be more prevalent than those for NAG. Another line of evidence against reduced forms of N being the most important characteristic for C substrates is that organic acids often stimulated nearly as much denitrification as amino acids and proteins. Organic acids do not have the reduced N groups possessed by amino acids, indicating that another attribute of these molecules facilitates their ready utilization by denitrifiers.

C substrates stimulating the most denitrification may be more easily incorporated into biomass. Amino acids are the building blocks of proteins, and environmental amino acids can be recycled into new biomass (Geisseler et al., 2009). This could result in faster production of denitrification enzymes. The organic acids I utilized in this experiment are key intermediates in the TCA cycle. In addition to generating the electrons for respiratory catabolism, the TCA cycle provides precursor molecules for biomass synthesis. The organic acids can therefore be relatively easily incorporated into new biomass. In contrast, glucose needs to proceed through glycolysis which may constitute a rate-limiting step in the utilization of this molecule by denitrifiers. However, when directly compared, organic acids and amino acids have tended to show lower C-use efficiencies than glucose (Brant et al., 2006; Frey et al., 2013), suggesting they are not preferentially used to build biomass. Since I

did not track the C-use efficiency of denitrifiers in this study, I am unable to determine whether direct incorporation of C substrates in new biomass is driving substrate differences in denitrification rates.

The bioavailability of C substrates is an important aspect of their utilization. I included pairs of monomers and polymers to test whether extracellular degradation was rate limiting for denitrifier utilization of C. The only consistent rate limitation of polymers stimulating N_2O production was in the glucose/cellulose and NAG/chitin pairs (Fig. 4.1). However, the difference between NAG and chitin was not always large or significant. With the exception of the vanillyl alcohol/lignin pair, monomers consistently stimulated more C mineralization than their associated polymers. The greater effect of polymers on C mineralization than denitrification suggests that extracellular degradation is not as severely rate-limiting for denitrifiers. This could be because the rate of degradation is sufficient to meet the needs of a relatively small denitrifier community. In addition, denitrifiers may be more competitive for substrate under low C concentrations.

The difference between monomers and polymers was greatest with amino acids and proteins, but this depended on site. Interestingly, proteins stimulated more N_2O production than amino acids at MRC (Fig. 4.1B). Peptide fragments can be more efficiently taken into cells than individual amino acids (Matthews and Payne, 1980; Geisseler et al., 2010), and peptides have also been found to be utilized more readily than amino acids by ruminal microorganisms (Wallace, 1996). Differences between protein and amino acids could also be due to the specific composition of the two mixtures. The amino acid addition was derived from a digestion of casein protein, whereas the protein addition was a soy protein isolate. While these contained similar levels of amino acids, the specific distribution and amounts

of each can differ between the two. For example, tryptophan, cystine, and methionine are often lost in the preparation of digested amino acid mixtures. Different bacteria have their own abilities to utilize amino acids as well as amino acid utilization preferences (Wallace, 1996; Kajikawa et al., 2002; Liu et al., 2020). Indeed, in my follow-up experiment, I found that individual amino acid additions resulted in different rates of denitrification (Fig. 4.5). The provision of particular amino acids in a greater proportion in the protein mixture may have stimulated more denitrifier activity in the MRC soils. On the other hand, this same effect did not appear to be present in the KBS soils.

Diversity effects may have also driven the large amounts of N_2O production following additions of amino acids and protein. The amino acid and protein additions were in reality combinations of many different individual amino acids. These different forms of C can allow for niche partitioning and complementarity in resource utilization, allowing more microbes to be metabolically active at the same time (Goldfarb et al., 2011; Baran et al., 2015). Niche partitioning has been shown to increase overall denitrification rates in synthetic communities of denitrifiers with complementary substrate usage (Salles et al., 2009). My study provides mixed evidence for this possibility. On the one hand, the mixture of six amino acids resulted in only slightly more gross N_2O production than the individual addition of glutamate, but on the other, the casamino-acid mixture produced twice as much N_2O as the six-amino-acid mixture.

Despite most substrate additions stimulating significantly more gross N_2O production than the water-only treatment, net N_2O production rarely exceeded the amount produced in the water-only controls (Fig. 4.2). Whether net N_2O emissions are driven by the production of N_2O or its reduction is an open question (Hallin et al., 2018). In this study,

N_2O reducers appeared highly responsive to varying amounts of N_2O production. In fact, the substrates with the lowest net N_2O concentrations (i.e., amino acids, protein, and organic acids) had the highest amounts of N_2O production. Net N_2O emissions between substrates therefore are apparently driven by N_2O reduction, not production, in the soils I assayed.

Varying N_2O reduction between C substrates could result from the stimulation of microbes with different forms of the gene encoding nitrous oxide reductase, *nosZ*. Previous studies have found that the ability for N_2O to be consumed in a soil is determined more by organisms with *nosZ-II* than those with *nosZ-I* (Jones et al., 2014). In support of this finding, organisms with *nosZ-II* more commonly lack the ability to generate N_2O (Graf et al., 2014), while bacterial isolates possessing *nosZ-II* have been found to have a higher affinity for N_2O than isolates with *nosZ-I*, potentially resulting in lower net concentrations of N_2O (Yoon et al., 2016). Therefore, substrates stimulating *nosZ-II* organisms could be expected to result in lower net production of N_2O. In this connection, at least one study has shown that *nosZ-II* organisms are more responsive to changes in C availability than those with *nosZ-I* (Assémien et al., 2019); however, other studies have shown that both clades of *nosZ* are equally responsive to C availability (Domeignoz-Horta et al., 2015, 2018; Juhanson et al., 2017). In this study, it is possible that the lower net N_2O concentrations with amino acids and proteins results from the greater activity of *nosZ-II* organisms. Nevertheless, since I did not directly determine the activity of individual denitrifier taxa, I cannot directly test this hypothesis.

Apart from the specific response of microbes with different forms of *nosZ*, the availability of substrates to be incorporated into new biomass may be particularly

important for N_2O reduction. The enzymes responsible for producing N_2O are more often constitutively expressed than those responsible for the reduction of N_2O to N_2, which are more sensitive to aerobic conditions (Ka et al., 1997; Morley et al., 2008; Qu et al., 2016). The transition to denitrifying conditions that produce N_2O will require *nosZ* expression to "catch up", and those substrates that allow the most efficient protein synthesis—i.e., by providing biosynthetic building blocks—could ultimately lead to greater rates of N_2O reduction.

When expressed as a percentage of total N_2O production by each substrate, glucose and NAG (in addition to amino acids, proteins, and organic acids) yielded significantly less N_2O than the no-C additions in the KBS soils, while at MRC, NAG and chitin additions resulted in significantly lower dN_2O values (Fig. 4.3). Therefore, with a few exceptions, the most bioavailable substrates tended to stimulate the greatest amount of N_2O reduction. Previous studies have demonstrated dN_2O to be inversely related to the availability of electron donors (Beauchamp et al., 1989; Weier et al., 1993; Miller et al., 2008; Qin et al., 2017b). A greater supply of electron donors increases demand for terminal electron acceptors, thereby driving the reduction of N_2O to N_2 and reducing dN_2O. While all substrates were provided in the same quantity in this study, the more bioavailable ones are immediately available for rapid uptake and metabolism, as opposed to those substrates requiring extracellular depolymerization or those with lengthy degradation pathways. Despite many differences between substrates in N_2O production, generally it appears that more bioavailable substrates drive N_2O reduction and lower dN_2O.

4.5.2. Land-Use Effects on Denitrifier C Preference

To determine the effect of land-use legacies on denitrifier C utilization I utilized different field treatments from two locations in Michigan. Across both locations, I found that the land uses that did not have cover crops tended to have the least potential for N_2O production across most C substrate additions (Fig. 4.1). This agrees with other findings that show cover cropping increases microbial activity in general (e.g., N. Kim et al., 2020). Therefore, the lower denitrification potential in the land uses that did not include cover crops may be due to an overall reduction in microbial activity, rather than an altered response of denitrifiers to substrate additions. It was also interesting to note that, at KBS, denitrifier C utilization in the reduced-input treatment tended to be more similar to C-utilization patterns in the perennial system rather than the conventional treatment, which had the same crop rotation as reduced-input but without cover crops. This suggests that the amount of time that plants cover the soil may be a stronger determinant of land-use effects rather than specific plant composition or diversity, *per se* (Garland et al., 2021).

After accounting for differences in overall microbial activity, I found land-use effects were stronger at KBS than MRC (Fig. 4.4), perhaps reflecting differences in how long these treatments have been established between the two sites. The substrates that had the most differences between land uses were amino acids, proteins, succinate and lignin. With each of these additions (except lignin), the KBS conventional system responded more than the other treatments. This could indicate that the reduction in overall microbial activity is due to a shortage of a particular limiting nutrient provided by those additions, such as easily assimilable organic N. On the other hand, it could be the result of the denitrifiers that utilize those substrates being more abundant and active in soils under conventional agricultural

management (Schmidt and Waldron, 2015). In this connection, opportunistic, fast-growing copiotrophic taxa have been found to be more common in systems with a history of increased N fertilization (Fierer et al., 2012; Ramirez et al., 2012; Leff et al., 2015).

In this study, I found that soils from conventional agriculture treatments also tended to have the lowest rates of net N_2O production across all substrate additions. These lower concentrations of net N_2O likely result from less N_2O being produced in the first place. For instance, denitrifiers in conventional agriculture soils from KBS responded relatively more to amino acid and protein additions than other substrates; accordingly, net N_2O concentrations following the addition of these substrates were as high or higher in the conventional agricultural soils as compared to the other two treatments at KBS. This suggests that differences in N_2O emissions between land uses may be driven by the production of N_2O rather than its consumption. This is in contrast to the patterns observed between C additions within the same soil, as discussed above.

Overall, I did not find a strong effect of land use on the C-utilization profiles of denitrifiers. While representing distinct management strategies, each of the land uses in this experiment were agricultural systems. Other agricultural management factors, such as weed management or aboveground biomass removal, may be more influential in determining denitrifier C-utilization profiles than the chemical composition of plant residue inputs. Previous studies have found that soil type is more important than plant identity in determining denitrification rates (Graf et al., 2016). In another comparison of land use on denitrifier communities, edaphic factors, such as pH and soil organic C, were identified as primary drivers of differences in denitrification rates (Krause et al., 2017). While these land uses at KBS have previously been shown to have differences in C content (Grandy and

Robertson, 2007), these may be too slight to affect the C preference of denitrifiers. On the other hand, distinct communities of denitrifiers may share a common profile of C preferences. Similar studies on a broader range of soils and land uses will be required before drawing general conclusions as to the universality of denitrifier C preference. It is also possible, and likely, that differentiating C preferences between denitrifier communities manifest in the utilization of C compounds not selected for this study.

4.6. CONCLUSION

In this study, my results demonstrate how the chemical identity of C inputs influences N_2O production and reduction in different agricultural soils. To resolve apparently divergent patterns in denitrifier C utilization between different studies, I compared a set of twelve C-addition treatments over five soils under identical assay conditions. I found that amino acids, proteins, and organic acids consistently stimulated the most denitrification and N_2O reduction. While soils from distinct land uses had differing overall rates of denitrification, C-utilization profiles were largely similar between soils, suggesting denitrifier C preferences may be widely held between microbial communities, at least within agricultural soils. The bioavailability of C substrates appears to be a large driver in denitrification and N_2O reduction, with labile substrates stimulating greater activity than polymeric and recalcitrant C additions. The large substrate differences in both gross and net N_2O production indicate the importance of C-substrate identity on process rates in the soil. Given the heterogeneous distribution of different forms of C throughout the soil profile, substrate effects likely contribute to the spatial and temporal variability of N_2O production within soils. The mechanisms underlying these substrate differences constitute an intriguing future line of study; for instance, whether increased activity

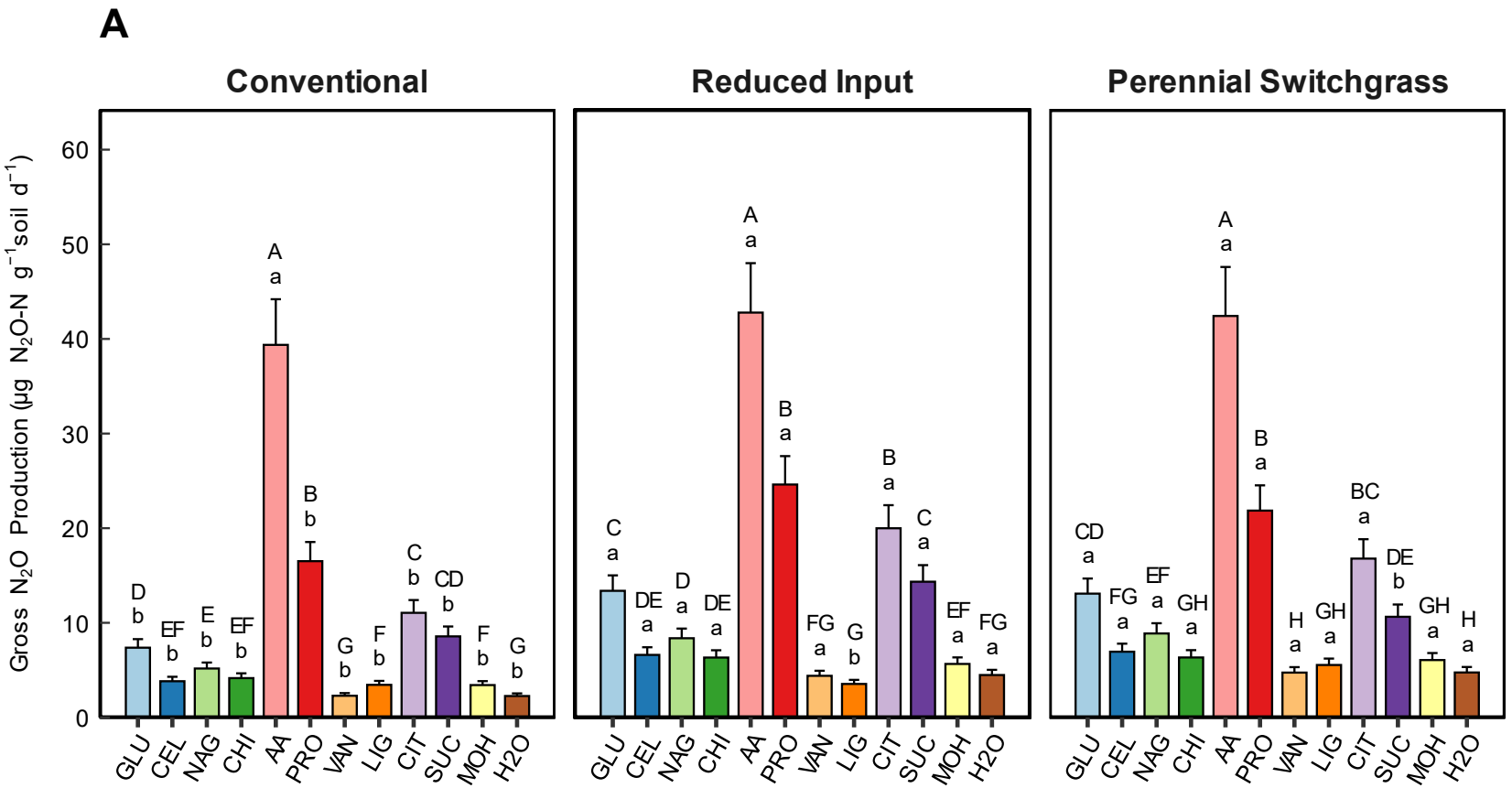
stimulated by some substrate is due to greater incorporation of those compounds in biomass. Moreover, more work is needed to differentiate the physiological effects from the community-level effects, such as whether differences in denitrification rates between substrates are due to phenotypic plasticity in the same group of denitrifiers or whether these differences reflect the activities of phylogenetically distinct organisms, potentially representing niche differentiation between different types of denitrifiers.

APPENDICES

APPENDIX A:

FIGURES

Figure 4.1: Differences in gross N_2O production between substrate additions at KBS (A) and MRC (B)



Cumulative production of N_2O in treatments receiving acetylene. Acetylene inhibits N_2O reduction; thus, N_2O concentrations represent total gross production of N_2O following the addition of glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), methanol (MOH), or no C addition (H2O). Means are shown with error bars representing one standard error (n = 4). Capital letters indicate significantly different means between substrates within each land use; lowercase letters indicate significantly different means between land uses for each substrate ($\alpha = 0.05$).

Figure 4.1 (cont'd)

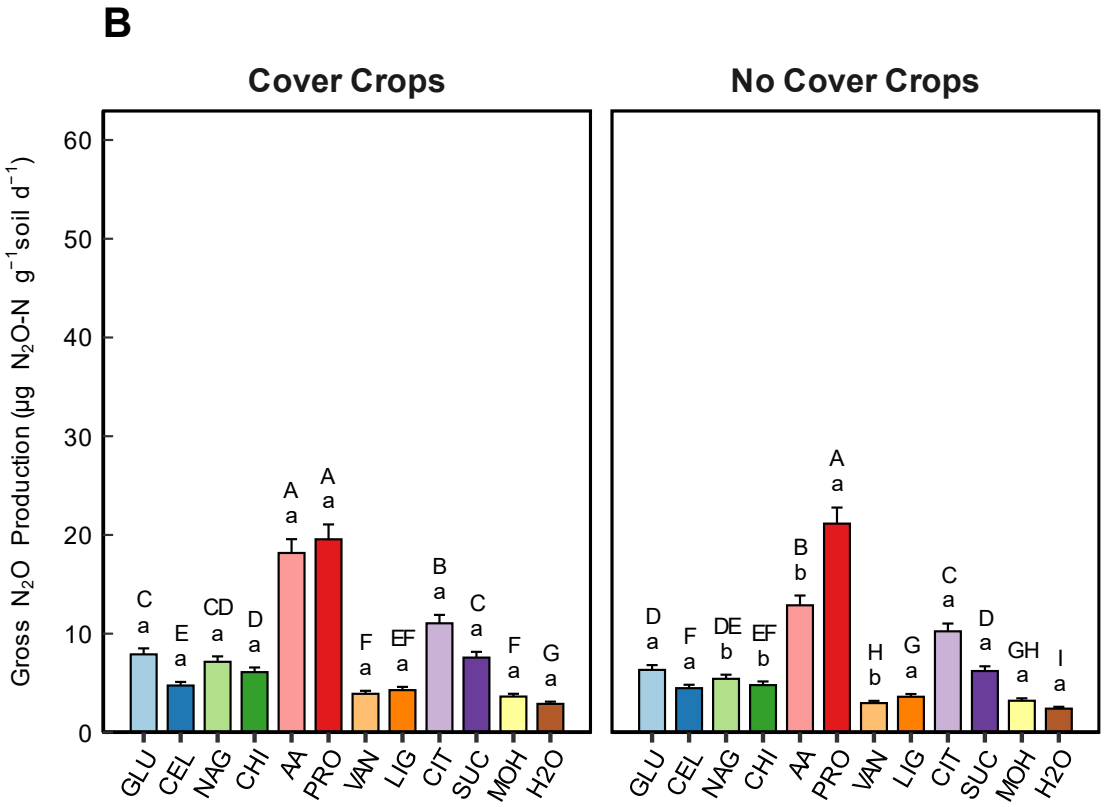
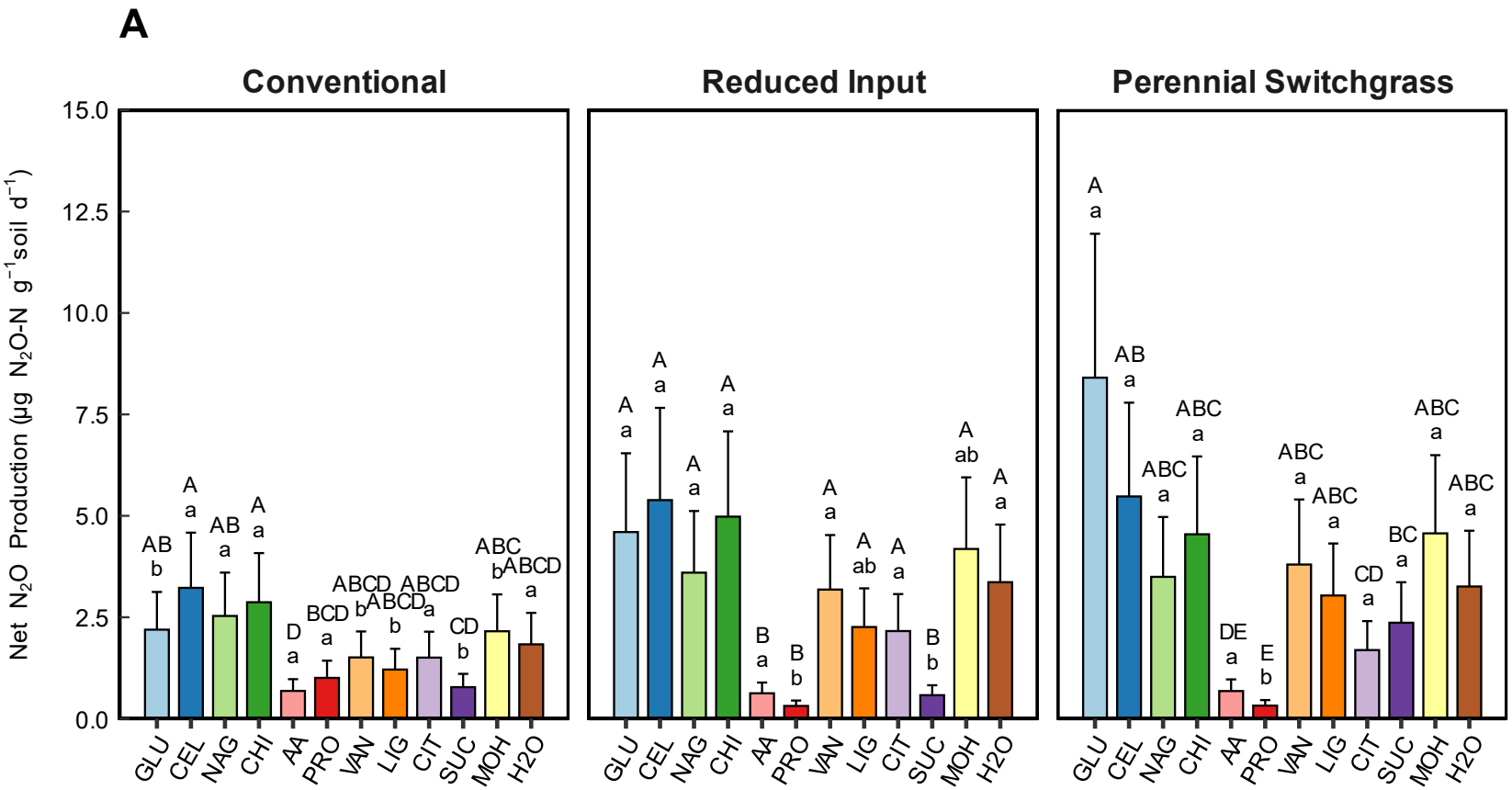


Figure 4.2: Differences in net N_2O production between substrate additions at KBS (A) and MRC (B)



Cumulative production of N_2O in treatments that did not receive acetylene. N_2O values reflect the balance of N_2O production and N_2O reduction following the addition of glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), methanol (MOH), or no C addition (H2O). Means are shown with error bars representing one standard error (n = 4). Capital letters indicate significantly different means between substrates within each land use; lowercase letters indicate significantly different means between land uses for each substrate ($\alpha = 0.05$).

Figure 4.2 (cont'd)

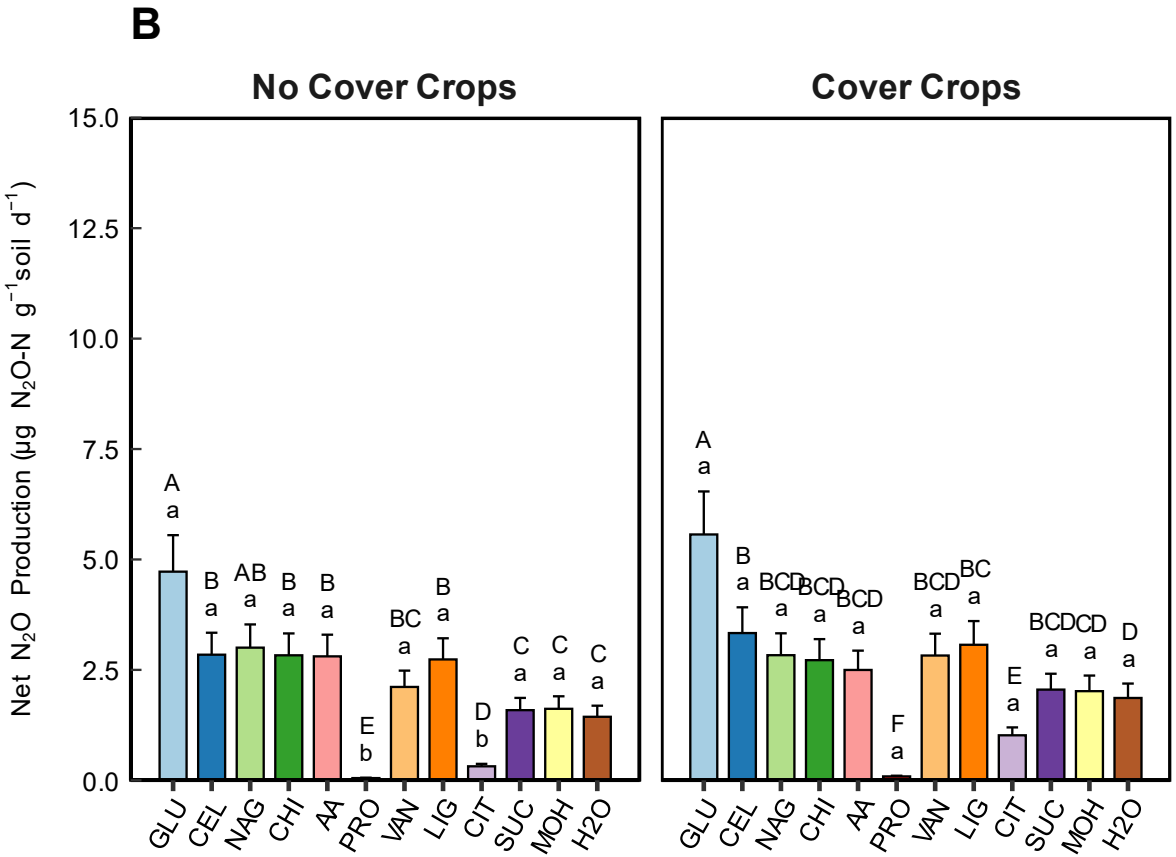
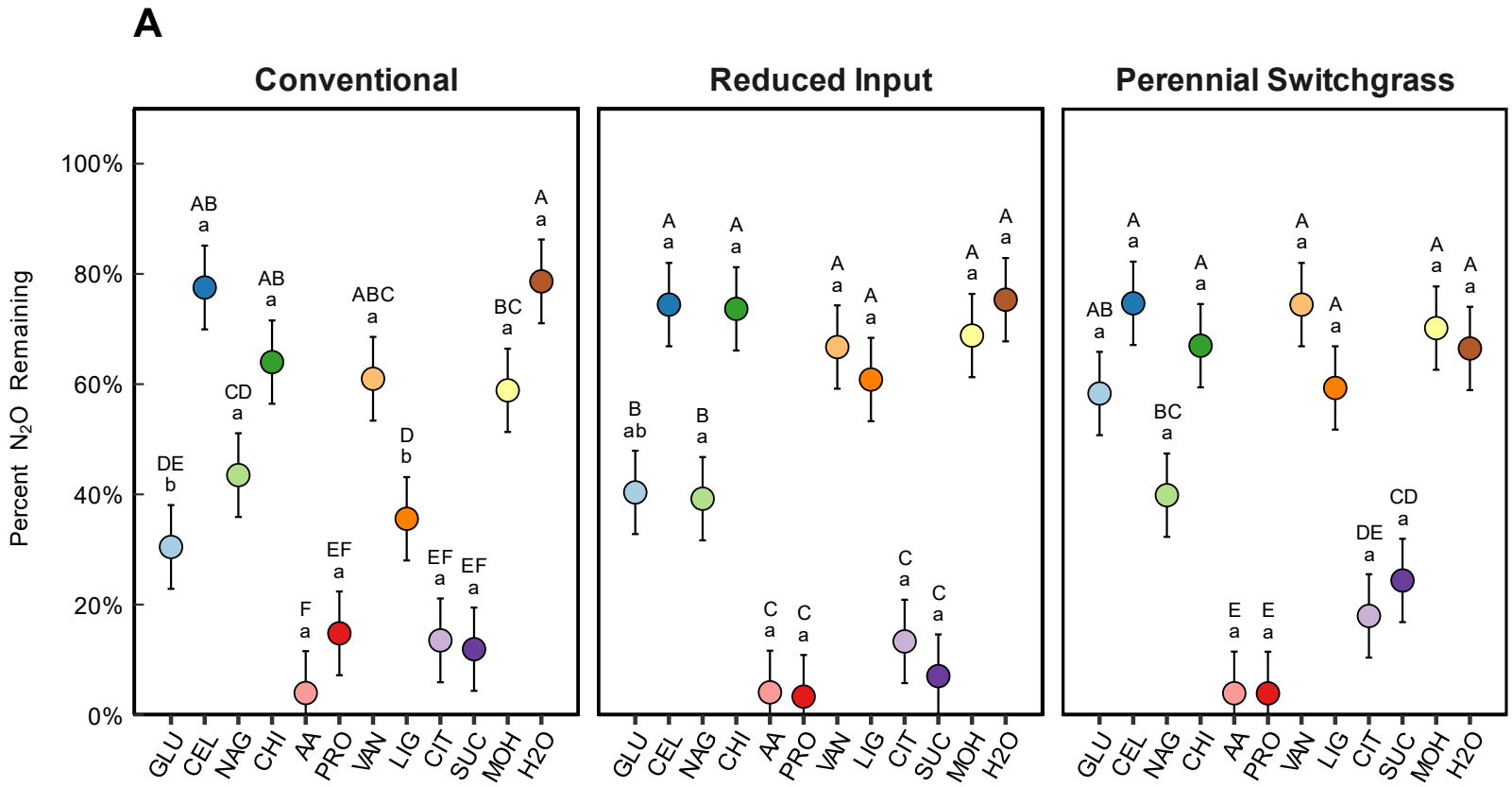


Figure 4.3: Relative amount of N_2O production between substrate additions at KBS (A) and MRC (B)



The percentage of N_2O remaining scales net production of N_2O to total gross production of N_2O. Higher values indicate less N_2O reduction occurred, while values close to zero indicate near complete reduction of all N_2O produced. Values are means of treatments receiving glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), methanol (MOH), or no C addition (H2O). Error bars represent one standard error (n = 4). Capital letters indicate significantly different means between substrates within each land use; lowercase letters indicate significantly different means between land uses at each location for each substrate ($\alpha = 0.05$).

Figure 4.3 (cont'd)

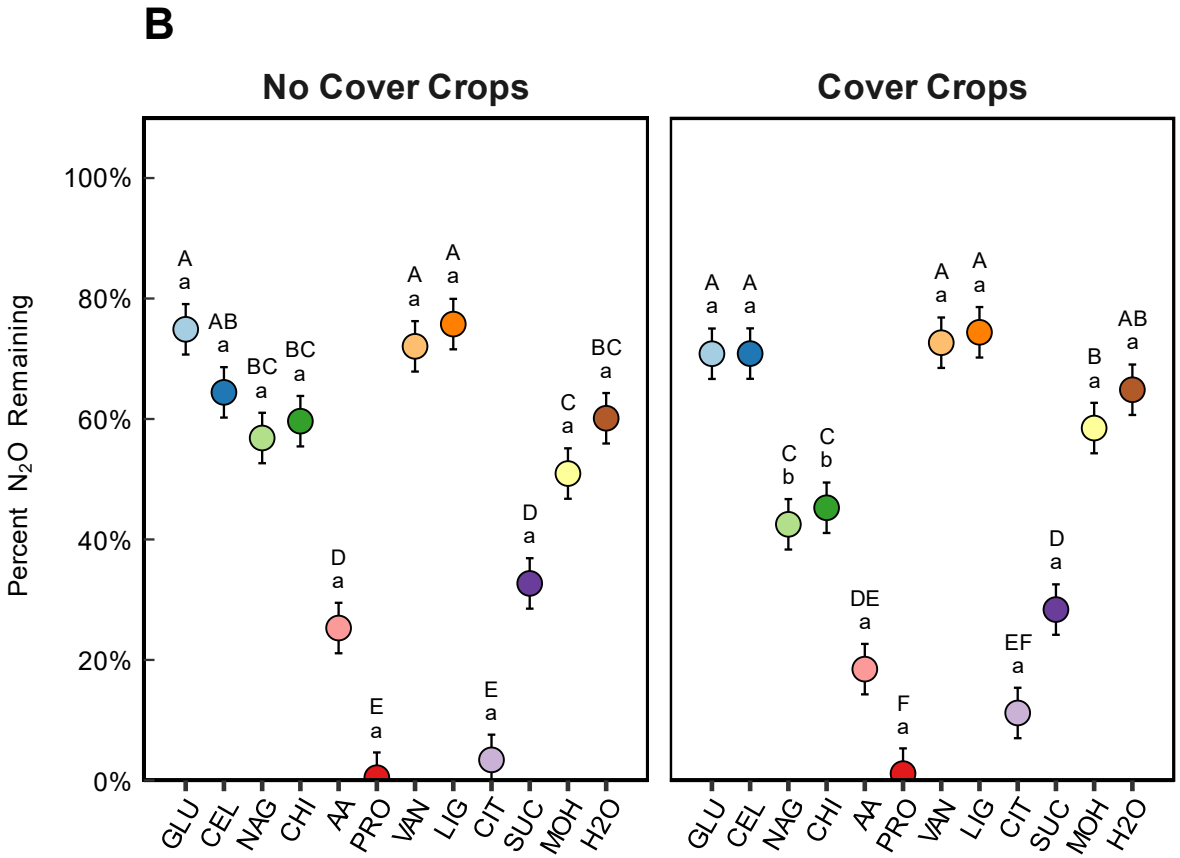
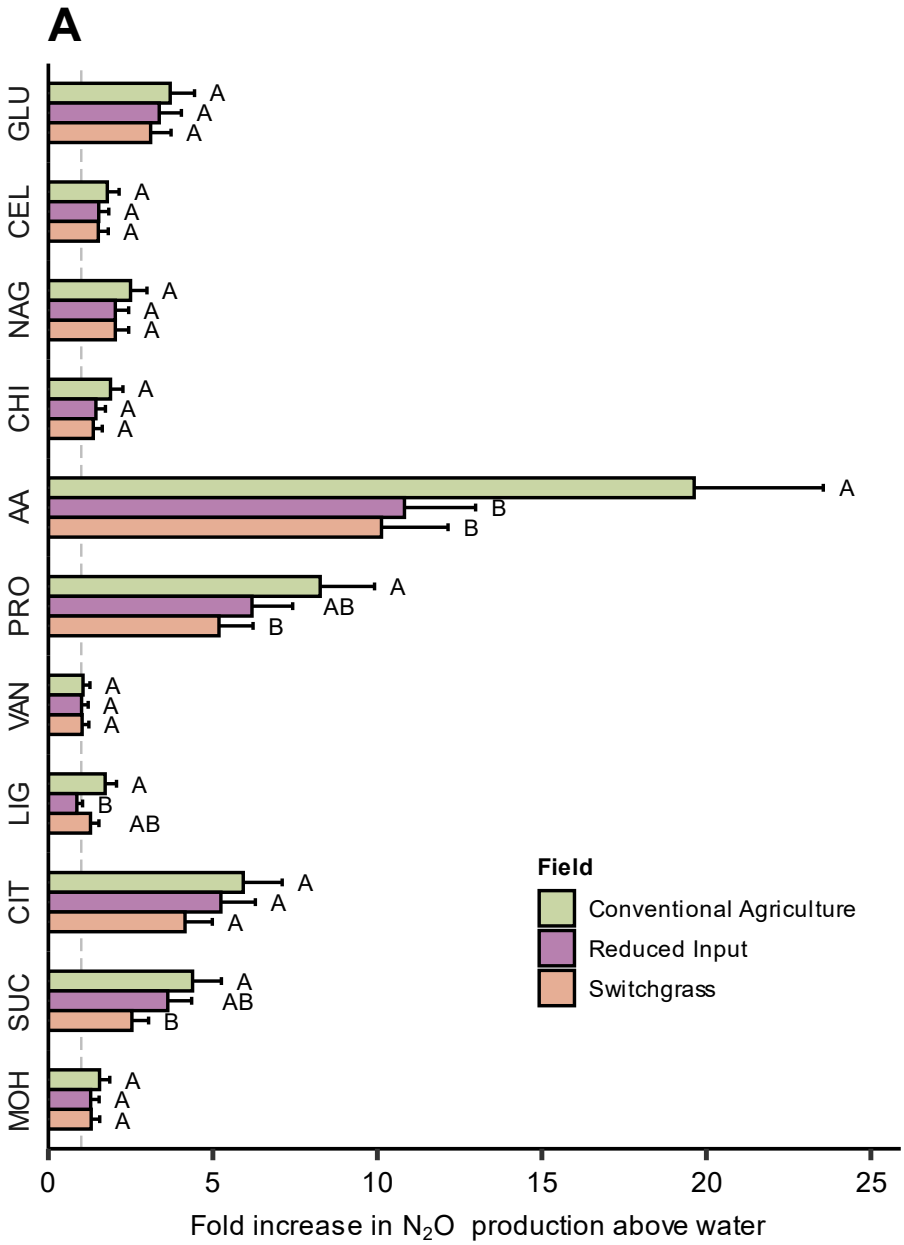


Figure 4.4: Substrate-induced increase in N_2O production between substrate additions at KBS (A) and MRC (B)



Values represent the fold increase in gross N_2O production following substrate addition compared to no C addition. Substrate additions included glucose (GLU), cellulose (CEL), N-acetyl-glucosamine (NAG), chitin (CHI), casamino acids (AA), soy protein isolate (PRO), vanillyl alcohol (VAN), lignin (LIG), citrate (CIT), succinate (SUC), and methanol (MOH). The gray dashed line indicates no stimulation to N_2O production. Error bars represent one standard error (n = 4). Capital letters indicate significantly different means between land uses at each location for each substrate ($\alpha = 0.05$).

Figure 4.4 (cont'd)

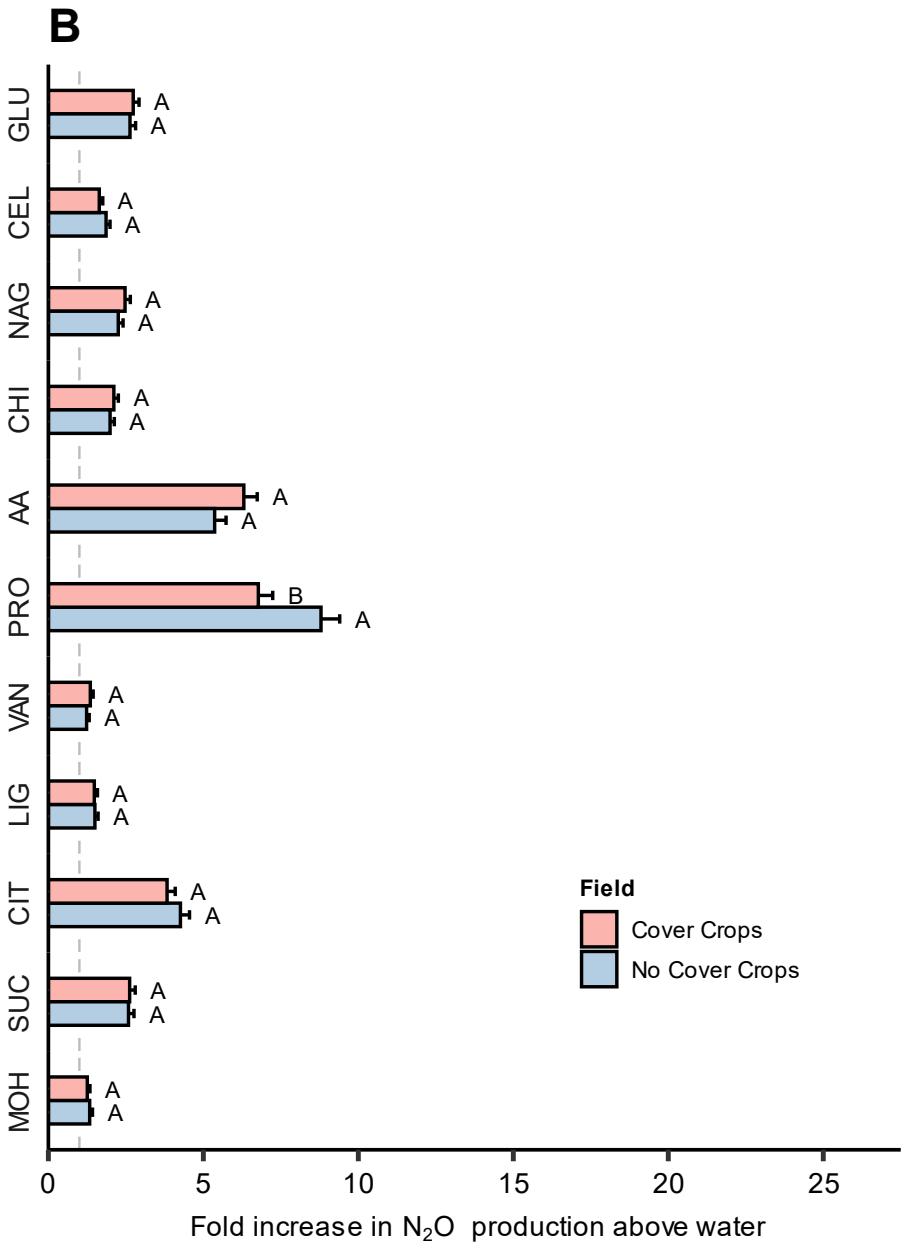
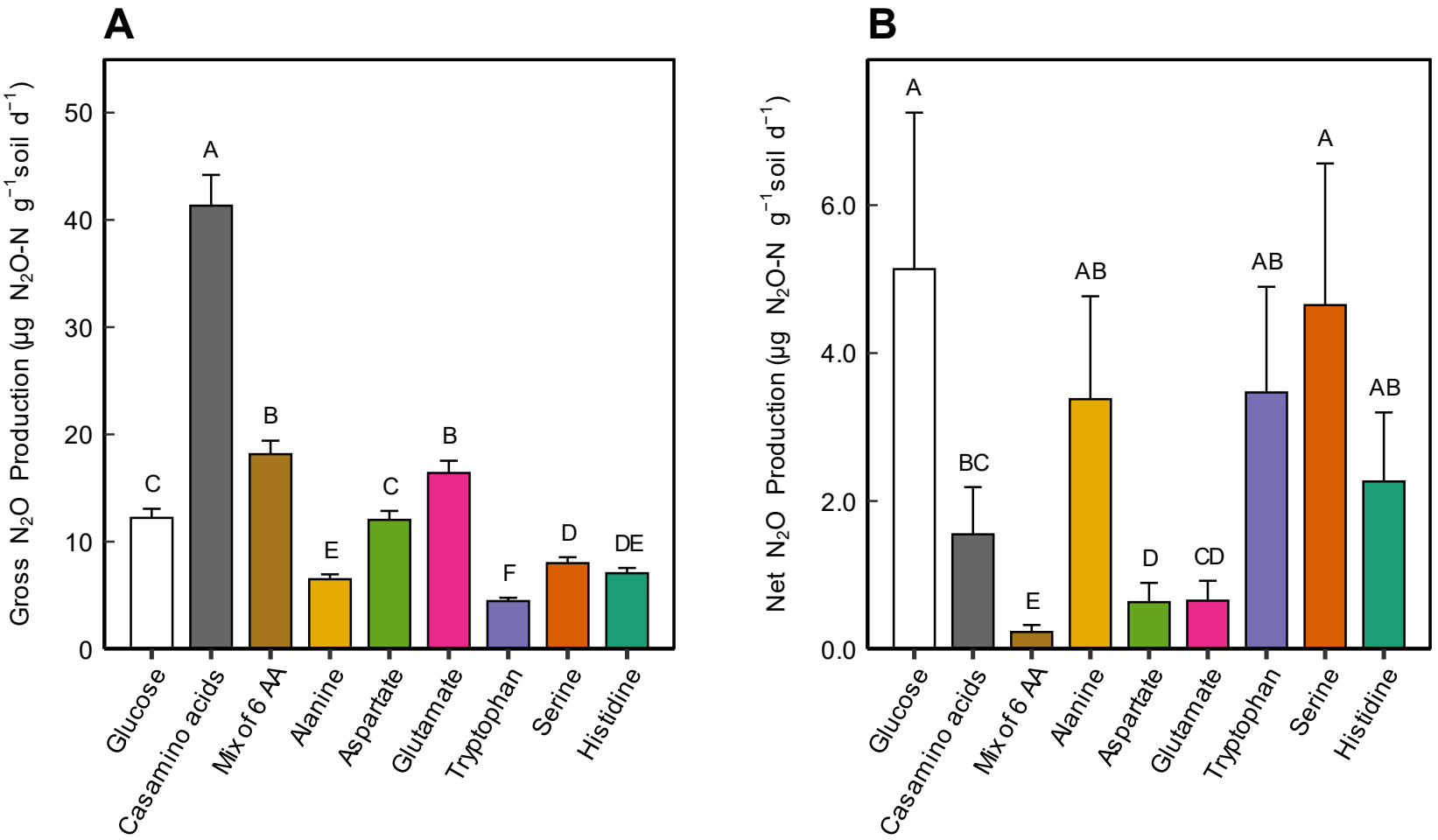


Figure 4.5: Differences in gross (A) and net (B) N_2O production between amino acid additions



Cumulative production of N_2O in treatments with acetylene (A) or without (B). Substrate were added to soils from the reduced input treatment at KBS. Means are shown with error bars representing one standard error (n = 4). Capital letters indicate significantly different means between substrates ($\alpha = 0.05$).

APPENDIX B:

TABLES

Table 4.1: Edaphic factors of study sites

	Total C (%)[c]	pH	CEC (cmol kg^{-1})
Kellogg Biological Station[a]			
Conventional Row Crop	0.91 ± 0.08	6.12 ± 0.03	8.13 ± 0.23
Reduced Input Row Crop	1.09 ± 0.05	6.28 ± 0.01	7.95 ± 0.11
Perennial Switchgrass	1.42 ± 0.06	6.12 ± 0.03	8.13 ± 0.23
Montcalm Research Center[b]		6.5	10
Maize-Potato with No Cover	0.87 ± 0.03		
Maize-Potato with Vetch and Rye	1.15 ± 0.19		

Total soil carbon, pH, and cation exchange capacity of the soil utilized in the study. [a]Soil data for Kellogg Biological Station was obtained from Robertson and Simmons (2020). [b]Cation exchange capacity and pH for soils sampled from Montcalm Research Center were obtained from the Web Soil Survey (USDA NRCS). [c]Soil carbon data for KBS was obtained from Grandy and Robertson (2007).

Table 4.2: Type III ANOVA table of fixed effects

	Gross N_2O		Net N_2O		dN_2O		Substrate-Induced N_2O	
	F	P	F	P	F	P	F	P
Kellogg Biological Station								
Substrate	93.9	< 0.001	5.5	< 0.001	33.4	< 0.001	115.8	< 0.001
Land Use	89.1	< 0.001	3.5	0.100	0.7	0.546	2.9	0.133
Substrate × Land Use	2.0	0.013	3.5	< 0.001	1.4	0.140	1.9	0.025
Montcalm Research Center								
Substrate	207.0	< 0.001	85.3	< 0.001	70.7	< 0.001	173.9	< 0.001
Land Use	6.0	0.092	12.1	0.040	0.5	0.520	0.1	0.820
Substrate × Land Use	1.9	0.081	1.9	0.074	2.1	0.048	1.9	0.081

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CHAPTER 5:

LIFE-HISTORY STRATEGIES CREATE TRADE-OFFS IN DENITRIFIER CARBON-USE EFFICIENCY AND NITROUS OXIDE REDUCTION ACROSS LAND USES

5.1. ABSTRACT

Denitrification is an anaerobic microbial metabolism that closes the nitrogen (N) cycle and is responsible for the production of nitrous oxide (N_2O), a potent greenhouse gas. As most denitrifiers are heterotrophic, denitrification couples carbon (C) and N cycles, but beyond the effect of C abundance, the influence of C-compound identity on denitrification is poorly understood. To determine how the biochemistry of C compounds affects denitrification, I supplied eight C substrate treatments to soils from three land uses and determined rates of N_2O production and reduction. The chemical identity of C substrates exerted a strong control on potential rates of N_2O production and N_2O reduction. I also found that the response to C substrates varied widely between land use, with agricultural soils having the greatest response to C substrates while denitrifiers from native forest soils were nearly unaffected by C additions. To explore the mechanisms of these C substrate effects on denitrification, I determined the C-use efficiency (CUE) for each of these substrates. Across land uses, I found that CUE and denitrification rates, including both the production and reduction of N_2O, were negatively correlated. Moreover, CUE values were much higher than expected, exceeding often cited theoretical maximums. I suggest that this was due to the production of C storage compounds by non-denitrifiers. As this experiment was one of the few to determine the CUE of soil microbial communities under anaerobic

conditions, it offers critical insight to the environmental parameters that can influence this important microbial trait.

5.2. INTRODUCTION

Intensive agriculture relies on large inputs of nitrogen (N), which often results in major environmental impacts, such as the production of nitrous oxide (N_2O), a potent greenhouse gas (Robertson and Vitousek, 2009; Shcherbak et al., 2014). Understanding the drivers of denitrification, the primary process for generating N_2O in agricultural systems, is essential for increasing the sustainability of agricultural production (Butterbach-Bahl et al., 2013; Hallin et al., 2018). The sustainable intensification of agriculture is increasingly focused on managing the soil microbial community to provide ecosystem services while minimizing ecosystem disservices (Bardgett and Van Der Putten, 2014; Fierer, 2017). For example, increasing the diversity of agricultural systems improves the functionality of the soil community, leading to increasing soil carbon (C) sequestration and greater N retention (Tiemann et al., 2015; Venter et al., 2016; Chapter 1). With management decisions being tailored to improve soil health through the functioning of the soil microbial community, we need to understand the mechanistic basis by which management decisions impact microbial functions in the soil.

Minimizing N_2O emissions will require a detailed understanding of the drivers behind the processes that generate and consume N_2O. Net emissions of N_2O are often linked to the ratio of available C and N, with greater available N tending to yield more net N_2O (Weier et al., 1993; Miller et al., 2008; Morley and Baggs, 2010; Senbayram et al., 2012, 2018; Köster et al., 2015). By providing the fuel for denitrification, C availability is often linked to greater reduction of N_2O (Weier et al., 1993; Miller et al., 2008; Qin et al., 2017b),

and the chemical identity of available C also affects the end-product ratio (N_2O:N_2) of denitrification (Morley et al., 2014; Giles et al., 2017). The effect of C substrate identity on denitrification can be mediated through substrate chemistry, such as the provisioning of organic N (Huang et al., 2004; Toma and Hatano, 2007); physiological effects determined by the utilization of the substrate by individual denitrifiers (Salles et al., 2009); and ecological drivers, which determine denitrifier substrate preference and access to different forms of C (Tiedje et al., 1983; Salles et al., 2012).

Carbon substrate identity may also be linked to other microbial traits, such as C-use efficiency (CUE). CUE expresses the fraction of total microbial C uptake that is partitioned towards microbial biomass relative to C respired as CO_2 (Manzoni et al., 2012; Sinsabaugh et al., 2013). Therefore, microbes with a higher CUE mineralize relatively less C to CO_2 and can potentially help build soil C stocks (Six et al., 2006; Kallenbach et al., 2016). However, the effect of substrate chemistry on CUE is variable, and only a handful of studies have investigated how the CUE of soil microbes differs between types of available C (Gommers et al., 1989; Brant et al., 2006; Frey et al., 2013; Jones et al., 2018). Moreover, C utilization is coupled to denitrification in that denitrifiers—which are mostly heterotrophic—reduce N-oxides through electrons obtained by oxidizing organic C compounds (Shapleigh, 2013). However, we don't know how the partitioning of C towards anabolic and catabolic processes is linked to denitrification, including the relative production of N_2O and N_2.

Trait-based approaches are becoming increasingly adopted as a way of describing the functional differences of soil microbial communities (Krause et al., 2014; Wieder et al., 2014; Malik et al., 2020). Microbial traits are a tractable way of characterizing microorganisms and their interactions within the complex soil environment. A broad

simplification of one trait—life-history strategies—is the oligotroph-copiotroph continuum (Fierer et al., 2007; Ho et al., 2017). This describes the relative trade-offs in microbial growth strategies between fast, inefficient growth with low resource affinity for success in high-resource environments, versus the ability to more efficiently take up nutrients at low resource densities at the expense of slow growth. CUE is often used as a proxy for characterizing fast-growing copiotrophs and slower growing oligotrophs with lower and higher efficiencies, respectively (Fierer et al., 2007; Beardmore et al., 2011; Blagodatskaya et al., 2014; Kallenbach et al., 2015). An important area in trait-based approaches is to understand how different microbial traits are related to one another. Such links between life-history strategies and denitrification have rarely been explored.

Land use alters the structure and functioning of microbial communities (Jangid et al., 2011; Barnett et al., 2020). Agricultural and natural systems have vastly different C and nutrient inputs, shifting microbially mediated transformations of C and N and how these cycles are coupled to one another (Grandy and Robertson, 2007; de Vries et al., 2013). As an example, land use can influence the way that microbes utilize different forms of C (McDaniel et al., 2014a; Jones et al., 2018). Differences in C utilization by denitrifiers from distinct land uses can help to unravel how management decisions that influence C inputs change C and N cycling.

To investigate the mechanisms behind the variable effects of C-substrate identity on denitrification, and to determine how microbial life-history traits and denitrification are related, I conducted an experiment using soils collected from a long-term ecological research site and eight C-substrate additions under denitrifying conditions. My previous research has demonstrated that denitrifiers from this site respond differently to various C

additions (Chapter 4). To further unravel the mechanisms behind the denitrifier response to C chemistry, I utilized ^{13}C-labelled substrates to track the partitioning of substrate C between anabolic and catabolic metabolism. I hypothesized that the CUE of substrates that are more readily incorporated into biomass, such as amino acids, will have a higher CUE than analogous compounds without an amine group. Since the reduction of N_2O requires the production of additional enzymes, I further hypothesized that compounds that are more readily incorporated into biomass will be associated with greater N_2O reduction, resulting in a positive relationship between CUE and N_2O reduction. Finally, I expected that the effects of land management on the type and abundance of soil C inputs would affect the structure and functioning of the soil microbial community by altering denitrifier C preference and CUE.

5.3. METHODS

5.3.1. Soil Sampling

I sampled soils from the Kellogg Biological Station's Long-Term Ecological Research site in southeast Michigan, USA (42° 24′ N, 85° 24′ W), in October 2021. Soils here are a mixture of Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs) sandy loams (Crum and Collins, 1995). Three treatments from the Main Cropping System Experiment were sampled, representing different plant compositions and management regimes including an annual crop rotation of corn (*Zea mays*)-soybean (*Glycine max*)-wheat (*Triticum aestivum*), a perennial switchgrass (*Panicum virgatum* L.) crop, and a late-succession deciduous forest, which has never been in agriculture. For corn-soy-wheat and switchgrass, I sampled from one-hectare plots in four replicate blocks. The deciduous forest is replicated three times, so one of the blocks was

sub-divided into two sub-blocks to have an equal number of field replicates across all field treatments. Three soil cores (1.9 cm diameter to a depth of 10 cm) from each of five sampling stations were taken from each block. Samples were kept on ice until returned to the lab for processing. Soil cores from each plot were sieved through a 2 mm mesh, homogenized, and kept refrigerated until assays were completed within three weeks.

5.3.2. Denitrification Potential Assays

From each replicate block, 8 g of soil were weighed into four sets of eight jars. Each jar within a set received a different C-substrate treatment. Substrate treatments included glucose, casamino acids, acetate, glycine, succinate, L-glutamate, vanillin, and a control that did not receive C. Two sets of jars were utilized to determine denitrification potential. Denitrification potential assays were performed by modifying a standard method (Groffman et al., 1997). Briefly, each jar received additions of nitrate (0.200 mg NO_3-N g^{-1} soil) and one of the eight C-substrate treatments (0.100 mg C g^{-1} soil). Carbon and nitrate additions were supplied in solution (3 mL g^{-1} soil). Following C and N additions, jars were evacuated and flushed three times with N_2. Following the final evacuation, jars were filled with N_2 to atmospheric pressure, and acetylene (C_2H_2) was added to one set of jars (10% v/v). Acetylene inhibits nitrous oxide reductase and allows for the estimation of gross N_2O production (Yoshinari and Knowles, 1976). The other set of jars received an equal volume of N_2. The N_2O in this set of jars reflected net N_2O production. Jars were incubated on an orbital shaker, and headspace samples were collected at 4, 6, and 8 hours during the first day and after 24, 26, and 28 hours during the second day. Headspace samples were analyzed on a TRACE 1310 gas chromatograph (Thermo Scientific, Waltham,

Massachusetts) equipped with an ECD and TCD to determine concentrations of N_2O and CO_2.

5.3.3. Carbon-Use Efficiency

The remaining two sets of jars were utilized to estimate C-use efficiency (CUE) by tracking the incorporation of ^{13}C-labelled substrates into microbial biomass and CO_2 (Frey et al., 2013; Geyer et al., 2019). Seven substrate solutions of 99 atom% ^{13}C-labelled compounds were prepared at a concentration of 0.250 mg C mL^{-1}. Labelled substrate solutions were diluted with non-labelled substrate solutions to yield a final enrichment of 20 atom% ^{13}C. In addition, a no-C control was included for each soil type to determine background soil and microbial ^{13}C. CUE assays were meant to mimic the conditions of the denitrification potential assays; therefore, the same amounts of C and nitrate were provided in solution as in the denitrification potential assays (0.200 µg NO_3-N g^{-1} soil and 0.100 mg C g^{-1} soil). After the addition of the C and nitrate solution (3 mL g^{-1} soil), jars were evacuated and flushed with N_2 three times. Jars for determining CUE did not receive C_2H_2, but N_2 was provided to match the slightly positive headspace pressure. Jars were incubated on an orbital shaker for 28 hours.

At the conclusion of the incubation, headspace samples were taken from one set of jars and placed into evacuated Exetainer vials for $\delta^{13}C$-CO_2 determination. K_2SO_4 solution (0.05 M) was added to these jars (5 mL g^{-1} soil), and they were shaken for one hour. The other set of jars was fumigated to determine microbial biomass C (MBC). A simultaneous fumigation and direct extraction method (Gregorich et al., 1990) was adopted since jars contained soil slurries. Briefly, 1 mL of chloroform was added to each jar. Jars were then re-capped and incubated on an orbital shaker for 24 hours. Jars were then vented for one hour,

followed by extraction with 0.05 M K_2SO_4, as described above. Dissolved organic C (DOC) and total dissolved N content of fumigated and non-fumigated soil extracts were determined with an elemental analyzer (vario TOC select, Elementar Americas, USA). The difference in DOC between fumigated and non-fumigated samples was taken as MBC. No correction for extraction efficiency was utilized, since preliminary data showed that the wet-fumigation method resulted in much greater efficiency of MBC extraction (Appendix A).

To determine ^{13}C enrichment, soil extracts were lyophilized and placed into aluminum tins. Lyophilized extracts and headspace samples were sent to the UC Davis Stable Isotope Facility (Davis, California) for ^{13}C quantification. Enrichment of gas samples was determined using a Thermo Scientific GasBench II coupled to a Thermo Finnigan Delta Plus XL isotope-ratio mass spectrometer. Analysis of lyophilized soil extracts was completed using an Elementar vario MICRO cube elemental analyzer (Elementar Analysensysteme GmbH) interfaced to a Sercon Europa 20-20 IRMS (Sercon Ltd., Cheshire, United Kingdom).

5.3.4. Calculations and Statistics

The reduction of N_2O was estimated by subtracting net N_2O production from gross N_2O production. I expressed N_2O reduction as a fraction of the total N_2O produced, providing a measure of relative N_2O reduction (rN_2O). The rate of CO_2 production was calculated for the first and second day of the assay. I compared the increase in C mineralization over the course of the assay between substrate additions by dividing the rate of CO_2 production at the end of the assay by the rate of CO_2 production that occurred between four and eight hours after addition of each substrate.

The following equations were used to estimate CUE. First the atom% ^{13}C enrichment (at%) of microbial biomass (at% MBC) was estimated from DOC concentrations in fumigated (DOC_{fum}) and non-fumigated (DOC_{non}) extracts and at% in fumigated (at% DOC_{fum}) and non-fumigated (at% DOC_{non}) extracts:

$$at\%\ MBC = \frac{\left(at\%\ DOC_{fum} \times DOC_{fum}\right) - \left(at\%\ DOC_{non} \times DOC_{non}\right)}{DOC_{fum} - DOC_{non}}$$

The relative proportion of MBC that was derived from substrate (pMBC) was then calculated using a two-pool mixing model, with at% MBC from non-labelled treatments serving as a control (at% MBC_{ctrl}):

$$pMBC = \frac{at\%\ MBC_{sub} - at\%\ MBC_{ctrl}}{at\%\ sub - at\%\ MBC_{ctrl}}$$

The total amount of MBC derived from substrate (MBC_{sub}, μg MBC-C g^{-1} soil) was obtained by multiplying the proportion of substrate-derived MBC by total MBC:

$$MBC_{sub} = pMBC \times MBC$$

Similarly, the amount of CO_2 derived from substrate (pCO_2) was calculated from at% of CO_2 samples receiving substrate (at% $CO_{2\text{-}sub}$) or water (at% $CO_{2\text{-}ctrl}$) as follows:

$$pCO_2 = \frac{at\%\ CO_{2_{sub}} - at\%\ CO_{2_{ctrl}}}{at\%\ sub - at\%\ CO_{2_{ctrl}}}$$

The total amount of CO_2 derived from substrate ($CO_{2\,sub}$, μg CO_2-C g^{-1} soil) was calculated from pCO_2 and the total amount of CO_2 produced in each treatment:

$$CO_{2_{sub}} = pCO_2 \times CO_2$$

CUE was then calculated as:

$$CUE = \frac{MBC_{sub}}{MBC_{sub} + CO_{2_{sub}}}$$

Statistical analysis of all data was performed in R. The *lme4* package (Bates et al., 2015) was utilized to create mixed-effects models with substrate and field as fixed effects and field block as a random effect. The significance of fixed effects was assessed with Type III ANOVA tables with degrees of freedom estimated by the Kenward-Roger method using the *lmerTest* package (Kuznetsova et al., 2017). When fixed effects were significant at a Type I error rate of 0.05, means were separated by substrate and land-use treatment using Fisher's LSD. Data were log transformed as needed to obtain homoscedasticity and normality of residuals.

When constructing models for estimating treatment effects on CUE, I omitted all data from the vanillin additions since such low amounts of this substrate were utilized by the microbial community. Many experimental units had negative values for vanillin-derived MBC. Such values are methodologically impossible and were due to experimental and technical variation in the DOC content of fumigated and non-fumigated extracts combined with extremely low enrichment in the vanillin additions. This resulted in severely biased models for the estimation of treatment effects on CUE. In addition, over half of the added vanillin was unaccounted for by the end of the assay, suggesting systematic issues with this treatment.

5.4. RESULTS

Gross nitrous oxide (N_2O) production varied significantly between C substrate additions (Fig. 5.1A; Table 5.1). Moreover, the effect of C substrate depended on land-use type. Amino acids stimulated the most N_2O production in corn-soy-wheat (12.9 μg N_2O-N g^{-1} soil d^{-1}) and switchgrass systems (10.8 μg N_2O-N g^{-1} soil d^{-1}), and glucose stimulated similar amounts of denitrifier activity in the switchgrass system (10.3 μg N_2O-N g^{-1} soil d^{-1}).

Succinate and glutamate elicited similar responses from denitrifiers in corn-soy-wheat and switchgrass soils, with these treatments producing between 7 and 8 μg N_2O-N g^{-1} soil d^{-1}. These additions resulted in significantly greater gross N_2O production than the no-C addition (3.5 and 4.9 μg N_2O-N g^{-1} soil d^{-1} in the corn-soy-wheat and switchgrass soils, respectively). Acetate and vanillin inhibited gross N_2O production (i.e., significantly less N_2O than no C addition; as low as 1.9 μg N_2O-N g^{-1} soil d^{-1} in the corn-soy-wheat soils), while across all land uses, glycine was not significantly different from the no-C treatment. In general, the deciduous forest soils were far less affected by C additions; no substrate addition yielded significantly more gross N_2O production than the no-C addition (3.0 μg N_2O-N g^{-1} soil d^{-1}). Acetate was the only substrate that resulted in significantly less gross N_2O production compared to the no-C treatment.

Net N_2O production (Fig. 5.1B) and relative N_2O reduction (Fig. 5.1C) were also significantly affected by C-substrate identity and land use (Table 5.1). Denitrifiers in the corn-soy-wheat had the most complete denitrification pathways, with almost no net N_2O production following the addition of amino acids, succinate, and glutamate (less than 0.5 μg N_2O-N g^{-1} soil d^{-1}; Fig. 5.1B). In this system, vanillin also had significantly less net N_2O production than the no-C treatment. Although vanillin stimulated less gross N_2O production, relative N_2O reduction was still significantly higher than in the no-C treatment (Fig. 5.1C). In the switchgrass system, none of the substrate additions resulted in significant changes in net N_2O production compared to the no-C control, which produced 3.5 μg N_2O-N g^{-1} soil d^{-1} (Fig. 5.1B); however, the relative reduction of N_2O, taking into account gross N_2O production, was significantly higher in switchgrass soils following the addition of amino acids, succinate, and glutamate (Fig. 5.1C). Within the forest soils, only amino acids

resulted in significantly lower net N_2O production (Fig. 5.1B). In general, very little N_2O reduction occurred in forest soils compared to the other land uses (Fig. 5.1C). Most substrates did not stimulate significant amounts of N_2O reduction, with less than 25% of N_2O production being reduced to N_2.

The effect of C substrates on rates of CO_2 production was similar to their effect on N_2O production (Figs. 5.2 & 5.3). Amino acids stimulated a doubling of CO_2 production by the end of the assay across all land-use treatments. Within the corn-soy-wheat treatment, succinate and glutamate additions resulted in a similarly large increase in CO_2 production. These substrates also tended to increase CO_2 production in the other two land uses, but not to the same extent. The glucose treatment also produced about a 50% increase in CO_2 production across all land uses. CO_2 production tended to decrease by up to 60% by the end of the assay in the acetate and vanillin across all land uses. CO_2 production also declined slightly in the agricultural soils that received glycine. The reductions in CO_2 production with these substrates were not significantly different from the treatments that did not receive a C addition.

CUE values were very high across all substrates and land uses, always greater than 80% (Fig. 5.4). Across all land uses, acetate and glycine elicited the highest CUE, with values upwards of 95%. Succinate, glutamate, and amino acids yielded similar CUE in the deciduous forest (~93%), where the glucose addition resulted in the lowest CUE. The CUE was more variable in the agricultural soils. In the corn-soy-wheat, the 89% CUE of glucose was higher than amino acids and succinate (both 85%), and glutamate was the lowest among all substrates (81%). In switchgrass, succinate and glutamate both had 88% CUE, which was higher than amino acids (84%), but only succinate had a significantly higher

CUE than glucose (89% versus 85%). Between land uses, CUE of amino acids was significantly higher in deciduous forest (92%) compared to both agricultural land uses, and the CUE of succinate was significantly higher in the forest soils (93%) than in the corn-soy-wheat (85%).

Variation in CUE was primarily due to differences in the mineralization of substrate-derived C (Fig. 5.5A). Across all soils, excluding vanillin, between 3.2 and 39 μg CO_2-C g^{-1} soil d^{-1} was produced from the mineralization of added substrates. I found up to ten times more ^{13}C-CO_2 produced after glucose, amino acids, succinate, and glutamate additions compared to acetate and glycine. Vanillin only produced 0.2 to 1 μg CO_2-C g^{-1} soil d^{-1}. Within the agricultural soils, I saw more ^{13}C-CO_2 from amino acids compared to succinate and glutamate. Between land uses, the mineralization of amino acids, glucose, and glutamate was significantly greater in the agricultural soils than the forest soils, which only produced half as much substrate-derived CO_2 compared to the corn-soy-wheat with those additions. Between the agricultural land uses, the corn-soy-wheat produced about 50% more succinate- and glutamate-derived CO_2 than the switchgrass. Within each land use, apart from vanillin, there were few differences in the amount of substrate-derived C being incorporated into MBC (Fig. 5.5B). Excluding vanillin, the amount of substrate-derived C found in MBC was between 100 and 250 μg MBC-C g^{-1} soil, which was between 10- to 100-fold more than the amount of substrate-derived C identified in CO_2. Only 8.5 to 26 μg MBC-C g^{-1} soil was incorporated from vanillin. Apart from vanillin, only glutamate had significantly less incorporation into MBC than glucose in the corn-soy-wheat, and there were no significant differences in substrate MBC within the switchgrass treatment. In the forest soils, acetate was less incorporated into biomass than amino acids, glycine, and

succinate. Between land uses, glucose and acetate were incorporated into biomass more readily in the corn-soy-wheat than in the deciduous forest.

CUE was strongly and negatively correlated with gross N_2O production (r = -0.724) and both overall and relative reduction of N_2O (Table 5.2). There was also a less strong, but still significant, positive correlation between CUE and net N_2O production (r = 0.247). The correlations of denitrification with substrate utilization were even stronger when only looking at substrate-derived CO_2 production (r = 0.882). The various measures of denitrification were only weakly or not at all correlated with the amount of substrate incorporated into microbial biomass (Table 5.2).

5.5. DISCUSSION

5.5.1. Substrate Effects on Denitrification

I sought to identify the mechanisms behind the differential effect that C substrate identity has on the activity of denitrifiers. In agreement with my previous findings (Chapter 4), I found that amino acids stimulated the greatest denitrifier activity in the agricultural soils, with more N_2O production and reduction (Fig. 5.1). In addition, previous studies have also reported differential effects of various C substrates on denitrifier activity (de Catanzaro and Beauchamp, 1985; Morley et al., 2014; Giles et al., 2017). Across all land uses, the substrates that stimulated the greatest amount of denitrification and N_2O reduction also had the lowest CUE (Table 5.2), suggesting possible cooccurrence and trade-offs between these microbial traits. The greater allocation of C toward dissimilation likely drove demand for terminal electron acceptors and increased the relative portion of N_2O being reduced (Miller et al., 2008; Köster et al., 2015; Senbayram et al., 2018). This study provides insight to the physiological and ecological factors that determine rates of denitrification.

The stimulation of both N_2O production and reduction by succinate indicates that substrate C:N ratio does not drive denitrification rates across different substrate additions (Huang et al., 2004; Millar and Baggs, 2004; Toma and Hatano, 2007). Further, I observed widely varying rates of gross and net N_2O production with N-containing substrates. Compound differences may instead be driven by their utilization in biochemical pathways as opposed to their chemical properties. For instance, succinate is a direct intermediate in the TCA cycle (Finan et al., 1981; Hederstedt and Rutberg, 1981; Morley et al., 2014), while glutamate only requires a simple transformation catalyzed by a ubiquitous enzyme before it can be integrated into the TCA cycle as either 2-oxoglutarate or fumarate (Hudson and Daniel, 1993). In contrast, glycine is fermented to acetate through the glycine-cleavage system or indirectly incorporated into central metabolism by first being converted to serine (Andreesen, 1994; Hong et al., 2020). In turn, the complete oxidation of acetate under anaerobic conditions is relatively restricted and often depends on the carbon-monoxide dehydrogenase pathway (Thauer et al., 1989). Utilization of substrates thus reflects the presence and activity of microorganisms able to utilize those substrates, with a lack of denitrification following the addition of acetate and glycine suggesting that denitrifiers utilizing those substrates were not present in these soils. I expected acetate to stimulate denitrification, since many denitrifiers are able to utilize that substrate (Pichinoty et al., 1979; Thauer et al., 1989; Morley et al., 2014). Nevertheless, the lack of acetate-utilizing denitrifiers in these soils indicates that the ability to utilize certain forms of C is not universal within functional guilds.

In agreement with my previous findings, I found that agricultural soils possessed similar C preferences (Chapter 4); however, here I found that forest soils had a notably

distinct response to C additions, with no significant increase in potential gross N_2O production compared to not adding any C (Fig. 5.1). Since similar levels of C and N were provided to all soils in this assay, the variation in substrate-induced denitrification between land uses is likely driven by differences in microbial communities. Previously, studies have found land use to affect the composition and functioning of soil microbial communities, both in general and with respect to denitrification (Cavigelli and Robertson, 2000; Barnett et al., 2020). These changes in C preference can be linked to denitrification potential through niche differentiation of organisms with particular denitrification genes (e.g., Assémien et al., 2019; Maul et al., 2019). For instance, nitrate reducers with the copper-containing NirK enzyme tend to be more sensitive to C availability (Laurent Philippot et al., 2009; Bárta et al., 2010; Chen et al., 2010; Assémien et al., 2019). At the same time, the ability to reduce N_2O tends to cooccur with denitrifiers possessing the cytochrome-containing NirS enzyme (Graf et al., 2014). Therefore, land-use induced changes in denitrification potential could be mediated by the effects of land use on C availability and the C preference of the microbial community.

5.5.2. The Relationship between CUE and Life-History Strategies

Links between CUE and life-history strategies rely on the assumption that microbial yield is inversely related to growth rate. Such links have been amply demonstrated in studies of pure cultures as well as complex communities (Monod, 1942; Beardmore et al., 2011). Many mechanisms have been described for this trade-off (for a thorough description, see Russell and Cook, 1995). Simply put, all else being equal, fast growth will require additional cellular machinery for taking up nutrients and synthesizing proteins, all of which requires the expenditure of energy that a slow-growing organism would not have to

sacrifice. Such links, however, have been thrown into doubt by studies finding a positive correlation between CUE and growth rate (e.g., Sinsabaugh et al., 2013).

Positive relationships between growth rate and efficiency can be observed as a mathematical consequence of measures of CUE. Often CUE is calculated as the fraction of C put towards anabolic processes divided by total C uptake, often assumed to be the sum of microbial biomass and respiration. At low growth rates, non-dormant microbes still have energy requirements, and this will generate CO_2 in what is often termed "maintenance respiration". Conceptually, if a microbe is not growing but is still respiring, then CUE will be close to zero. As a microbial population increases its growth rate from zero, CUE will always increase because of how "efficiency" is calculated and how it encompasses non-growth maintenance costs. However, as a greater proportion of resources is put to growth relative to maintenance, the expected inverse relationship between efficiency and growth rate appears. This has been well illustrated conceptually in a diagram by Lipson (2015), who shows an initial positive relationship between biomass yield and growth rate followed by a sigmoidal-shaped downward curve that is based off empirically derived inverse relationships between yield and growth rate.

All microbes will demonstrate such dynamics in their growth-yield relationship as growth rate increases. The use of efficiency as an indicator of life-history strategy needs to take this into account. For instance, a near-dormant oligotroph may exhibit a very low CUE due to maintenance costs representing the majority of its respiration, while a copiotroph in rich growth media may have a relatively higher CUE because maintenance costs are only a minor share of total respiration. Therefore, different communities or populations would need to be compared under similar conditions in order to infer differences between life-

history strategies on the basis of CUE. These conceptual issues are effectively described by Kallenbach et al. (2019). Here, they use the term "trait moderating" to describe how a positive environmental change can cause a population to increase its CUE due to the release from nutrient limitation. On the other hand, "trait filtering" is the mechanism by which an environmental change shifts the microbial community to one with a different growth-efficiency curve. These different curves also help to reconcile why different studies have observed that nutrient additions result in faster growing populations with both greater efficiency (Manzoni et al., 2012; Sinsabaugh et al., 2013) and lower efficiency (Noah Fierer et al., 2012; Leff et al., 2015; Silva-Sánchez et al., 2019). In the first case, with greater efficiency, the alleviation of nutrient limitation increases the growth rate of a resource-limited community or population. In the second case, nutrient addition is described as having direct and indirect effects on community composition, which shifts towards less efficient and fast-growing populations.

So, is CUE an adequate proxy for life-history strategy? Links between CUE and life-history strategy should certainly not be automatically assumed. When differences in CUE are used to infer differences in life-history strategies, the conditions should be carefully considered. For instance, observing CUE at a community or population's maximum growth rate will ensure that comparisons are made at equivalent points on the growth-efficiency curve. In the case of this study, I have supplied non-limiting quantities of both C and N. Therefore, differences in CUE are likely to reflect differences in the utilization of these C compounds by different microbes and are not overly influenced by maintenance costs.

5.5.3. Differences in CUE

Relatively few studies have looked at the importance of substrate identity on the CUE of soil microbes (Gommers et al., 1989; Brant et al., 2006; Frey et al., 2013; Jones et al., 2018). I found that differences in CUE between substrates were mostly consistent between land uses (Fig. 5.4). Such patterns may be caused by the utilization of certain compounds by organisms with different life-history strategies. For instance, oligotrophs are associated with a higher CUE, due to slower but more efficient growth, than copiotrophs with fast growth rates and a lower CUE (Fierer et al., 2007). Therefore, the higher CUE following the addition of acetate and glycine could indicate preferential utilization by oligotrophs. These compounds in particular are commonly sequestered by microbes to form storage compounds under environmental stress, such as anaerobic conditions (Oehmen et al., 2007; Nguyen et al., 2015; Dorofeev et al., 2019; Qiu et al., 2020). Storage-compound formation is a trait of oligotrophs which allows them to adapt to low-resource environments (Hirsh et al., 1979; Tecon and Or, 2017). In this study, despite large amounts of acetate- and glycine-derived C being taken up into microbial biomass (Fig. 5.5B), I saw no increase in respiration rates over the course of the assay (Fig. 5.2), indicating that these compounds were not being put towards growth *per se*. In contrast, the low CUE of other substrates was largely driven by increased respiration rates, more than likely indicating microbial growth. Studies tracking the utilization of labeled C have demonstrated that the addition of different C compounds can stimulate subsets of the oligotrophs and copiotrophs within the same soil (Eilers et al., 2010; Pepe-Ranney et al., 2016; Barnett et al., 2021). Preference for certain C compounds between copiotrophs and oligotrophs could reflect the conditions under which those substrates are typically available in the environment (Killham and Prosser, 2015). For

instance, many anaerobic metabolisms end in the production of acetate, since it is a non-fermentable compound (Thauer et al., 1989). In addition, glycine is often found in soils since it is the primary constituent of betaine, an osmoprotectant (Andreesen, 1994). The presence of these compounds therefore typically occurs under stressful conditions, not suited to copiotrophic activity. On the other hand, amino acids and organic acids are commonly supplied in the rhizosphere (Jones, 1998; Sasse et al., 2018), which is a microbial hotspot favoring copiotrophic life-history strategies (Fierer et al., 2007; Ling et al., 2022).

Across all substrate additions, CUE was very high and exceeded theoretical maximums that are based off the energetic costs of converting substrate into biomass compounds, such as proteins, cellular membranes, and nucleic acids (Roels, 1981; Gommers et al., 1989). However, such theoretical maximums are based on a number of assumptions, which may not always hold depending on how CUE is calculated. For instance, microbes may utilize one substrate for biomass assimilation and a different substrate as an energy source (Gommers et al., 1989), skewing estimates of CUE based on the utilization of a single compound. Moreover, methods for calculating CUE that rely on quantifying ^{13}C in MBC and CO_2 do not account for fermentation. In these calculations, fermentation byproducts will not be "counted" once they have been exported from the cell—it will be as if the microbe never utilized the substrate to begin with. However, prior to extracellular transport, fermentation byproducts will appear as microbial biomass, leading to overestimations of CUE. Fermentation byproducts, such as ethanol following the fermentation of glucose, can build up within the cell to appreciable levels, up to 10% (w/v) (D'amore et al., 1989).

Initial CUE values that are inordinately high can also arise when C uptake is temporally decoupled from mineralization (Hill et al., 2008; Geyer et al., 2016). As microbes utilize C, the CUE value will decrease to more commonly observed values (Geyer et al., 2019). A prolonged instance of such decoupling is when microbes take up excess amounts of C in the environment and store it for later utilization (Nguyen and Guckert, 2001). The production of such storage compounds is a common phenomenon in the microbial world, especially within environments such as soil, which encounters feast-or-famine regimes (Wang and Bakken, 1998; Kadouri et al., 2005). The dormancy strategies of microbes rely on the build-up of storage compounds when nutrients are abundant so that microbes can sustain minimal functions at more limiting times (Lennon and Jones, 2011). Previous studies that have found elevated CUE have attributed this to the production of storage compounds (e.g., Nguyen and Guckert, 2001), but this mechanism has been contested on the basis of metabolic modeling by Dijkstra et al., (2015). However, that rebuttal only accounted for a very particular pathway of storage compound synthesis, where the same substrate being incorporated into storage compounds also provided the energy for the assimilation. Mechanistic studies on the production of poly-β-hydroxy butyrate (PHB) from acetate have found that the energy and reducing power for the production of these storage compounds more often comes from the endogenous metabolism of polyphosphate and glycogen (Arun et al., 1988; Smolders et al., 1994). Therefore, acetate-derived C used to produce PHB would show up almost exclusively in microbial biomass, resulting in CUE values near unity. Follow-up studies can determine whether the high CUE was due to storage compound formation, such as by determining the concentration of PHB (Mason-

Jones et al., 2018) or by coupling ^{13}C tracing with other measures of CUE, such as the ^{18}O method (Geyer et al., 2019).

The unexpectedly high CUE values in this study highlight the importance of determining how various environmental factors, such as anaerobicity, affect CUE (Manzoni et al., 2012). The CUE of soil microbial communities subjected to anaerobic conditions has only been investigated in a handful of previous studies. One study found that the recycling of fermentation byproducts led to an increase in CUE relative to aerobic systems (Devêvre and Horwáth, 2000). Other studies concluded that the utilization of recycled C was less efficient (Parsons and Smith, 1989; Santruckova et al., 2004). The type of substrate and timeframe differed between these studies, demonstrating how estimates of CUE depend on a variety of environmental conditions and experimental factors. Therefore, the effects of reduced oxygen availability shouldn't be assumed to only decrease efficiency by increasing fermentation (Chakrawal et al., 2020; Colombi et al., 2022); rather a host of metabolic processes, including a variety of anaerobic respirations, fermentation, and storage strategies, are likely to proceed concurrently (Keiluweit et al., 2017).

The CUE of substrates differed among land uses, with forests having the highest CUE across most substrate additions (Fig. 5.4). As with the differences in denitrification rates, these differences in CUE are also likely determined by microbial community structure and the effects of land management on community composition. Observed differences in CUE between sites have been driven by changes to microbial community structure (Silva-Sánchez et al., 2019). Previous work at KBS has found that copiotrophic denitrifiers were positively correlated with the degree of land use intensity (Schmidt and Waldron, 2015). Overall, less of the added substrate was utilized by the microbes in the forest soils. While

these microbes may have been more efficient in their use of C, the microbial community was slower to respond to the pulsed addition of C and nutrients, representing a potential trade off. For example, a higher CUE indicates that less C will be mineralized in the forest soils, but the relatively inefficient utilization of N by denitrifiers led to greater net N_2O emissions.

Because of the potency of N_2O as a greenhouse gas, it is important to understand trade-offs between increasing the efficiency of C and N cycling. I expected that substrates that are more readily incorporated into biomass would drive greater N_2O reduction, yielding a positive relationship between CUE and relative N_2O reduction. Others have also proposed that the ability of certain compounds to be incorporated into biomass would be beneficial for N_2O reduction (Giles et al., 2017). However, I found that CUE and both gross N_2O production and relative N_2O reduction were actually negatively related (Table 5.2). In other words, with low CUE I saw relatively low rates of N_2O production and N_2O reduction. These results may be linked to previous observations that show N_2O reduction is favored when there is a high demand for terminal electron acceptors, such as during rapid growth (Miller et al., 2008; Köster et al., 2015; Senbayram et al., 2018). Moreover, past studies have found that the community composition of N_2O reducers responds more quickly to fluctuating environmental conditions than N_2O producers (Domeignoz-Horta et al., 2015, 2018; Maul et al., 2019; Tao et al., 2022). The existence of a trade-off between CUE and N_2O reduction could imply that microbial communities that more efficiently utilize C and respire less CO_2 may favor non-N_2O-reducing denitrifiers, resulting in a net increase in greenhouse gas emissions. Therefore, relating N_2O reduction to other microbial traits—such as life-history strategies—will be important for understanding the ecological significance of

denitrification and for mitigating N_2O emissions in managed ecosystems (Hallin et al., 2018).

APPENDICES

APPENDIX A:

MICROBAL BIOMASS EXTRACTION OPTIMIZATION

Overview

I performed an optimization experiment to determine whether I could reduce the concentration of K_2SO_4 without affecting the determination of microbial biomass. In addition, I wanted to see whether performing a chloroform fumigation in a soil slurry (8 g soil in 24 mL solution) would impact MBC estimates. I used three concentrations of K_2SO_4: 0.5 M, 0.2 M, and 0.05 M. For each of these, I performed chloroform fumigations in both soil slurries and dry soil. Chloroform fumigations of soil slurries were performed as described in the methods of Chapter 5. Soils were taken from the Kellogg Biological Station Main Cropping System Experiment T1 rep 6. These soils had been sampled in April 2021, sieved through 2 mm, and kept at 4^{o}C. Each of four replicates in this K_2SO_4 experiment used the same soil.

Non-fumigated Extracts

I found clear differences in the amount of dissolved organic carbon (DOC) extracted at the different K_2SO_4 concentrations (Fig. 5.6). The 0.5 M solution has more than twice as much DOC as the 0.2 M solution, which in turn has about twice as much as the 0.05 M solution. The differences in total dissolved nitrogen (TN) extracted are much more slight. Only the difference between the 0.5 M and 0.05 M solutions are significant. The variability within each concentration of K_2SO_4 is similar.

Fumigated Extracts

Within each concentration of K_2SO_4, fumigation was performed two ways: via the addition of chloroform to dry soil or the addition of chloroform to a soil slurry (8 g soil in 24 mL solution). For both fumigation techniques, fumigations were performed in sealed serum bottles. All bottles were shaken for 24 hours then vented for one hour to remove

chloroform. Following fumigation, additional K_2SO_4 solution was added to a final ratio of 5 mL of solution per g soil. Soils were then shaken for an additional hour before being filtered. During the experiment, I became concerned about the potential for residual chloroform to inflate measures of DOC. Therefore, I took one set of replicates (rep 2) and bubbled them under N_2 for 30 minutes.

Among wet-fumigated samples, all concentrations of K_2SO_4 resulted in similar amounts of DOC (Fig. 5.7). However, there was increased variability in the wet-fumigated samples with 0.05 M K_2SO_4. While reps three and four were nearly identical, reps one and two were equally distant from the overall mean. Rep two had the lowest DOC in the 0.05 M and 0.2 M K_2SO_4 extracts. Rep two had been bubbled in case of residual chloroform, but this rep also had lower N, so the higher DOC concentrations in the other reps does not appear to have been caused by left-over chloroform. Microbial biomass carbon (MBC) estimates were highest in the soils extracted with 0.05 M K_2SO_4, but these differences were slight: 15.63 mg MBC-C L^{-1} in the 0.05 M concentration versus 10.56 and 9.86 mg MBC-C L^{-1} in the 0.2 M and 0.5 M concentrations, respectively (Fig. 5.8). Since concentrations of DOC in fumigated extracts were similar in all wet-fumigated soils, these differences in MBC were caused by lower levels of DOC in the non-fumigated extracts at 0.05 M K_2SO_4.

Within the dry-fumigated samples, the concentration of DOC was roughly equal across all concentrations of K_2SO_4, with a slight increase from 6.3 to 6.9 to 7.9 mg DOC-C L^{-1} as the solution increased in concentration. The concentration of K_2SO_4 did not significantly alter estimates of MBC, despite significantly different levels of DOC in the non-fumigated extracts. Here, MBC tended to decline as the concentration of K_2SO_4 increased; this was similar to the pattern observed in the wet-fumigated extracts, but the pattern was less

pronounced. The variability between replicates was also more consistent across all concentrations of K_2SO_4.

The biggest differences were between the fumigation methods. Wet fumigation had consistently higher concentrations of DOC than dry-fumigated extracts. This carried over to wet-fumigated extracts resulting in higher estimates of MBC than dry fumigation. These differences among fumigation methods were similar across the 0.2 M and 0.5 M concentrations of K_2SO_4, where the wet-fumigation method produced MBC estimates roughly twice that of the dry-fumigation method. This difference was more pronounced with the 0.05 M solution. Here, MBC was 15 mg MBC-C L^{-1} in the wet-fumigated extracts compared to about 5 mg MBC-C L^{-1} in the dry-fumigated extracts.

I also looked at the MBC:MBN ratio to determine whether the higher concentrations of MBC in wet-fumigated samples were due to improved extraction of microbial biomass or to other factors, such as residual chloroform. Within each concentration of K_2SO_4, there was virtually no difference in MBC:MBN ratio between wet- and dry-fumigated samples. This suggests that the higher MBC estimates in wet-fumigated extracts is due to improved extraction of microbial biomass. There are a couple of factors that may improve the extraction efficiency with the wet fumigation. First, the wet fumigated soils were, in effect, extracted for 24 hours after the addition of chloroform followed by another hour of extraction after venting. This is in contrast to the 1-hour extraction following the dry fumigation. Second, the wet-fumigation method could facilitate the disruption of aggregates and soil homogenization, allowing the chloroform to come into contact with a greater portion of the soil microbial community.

Interestingly, within each fumigation method, the MBC:MBN ratio tended upwards as the strength of the K_2SO_4 solution decreased. This could be due to the reduced ability of the lower concentrated solutions to extract N. The absolute amounts of TN were lower in the fumigated samples extracted with 0.2 M solution compared to 0.5 M solution. However, the TN values were higher in the 0.05 M solution compared to the 0.2 M.

Conclusions

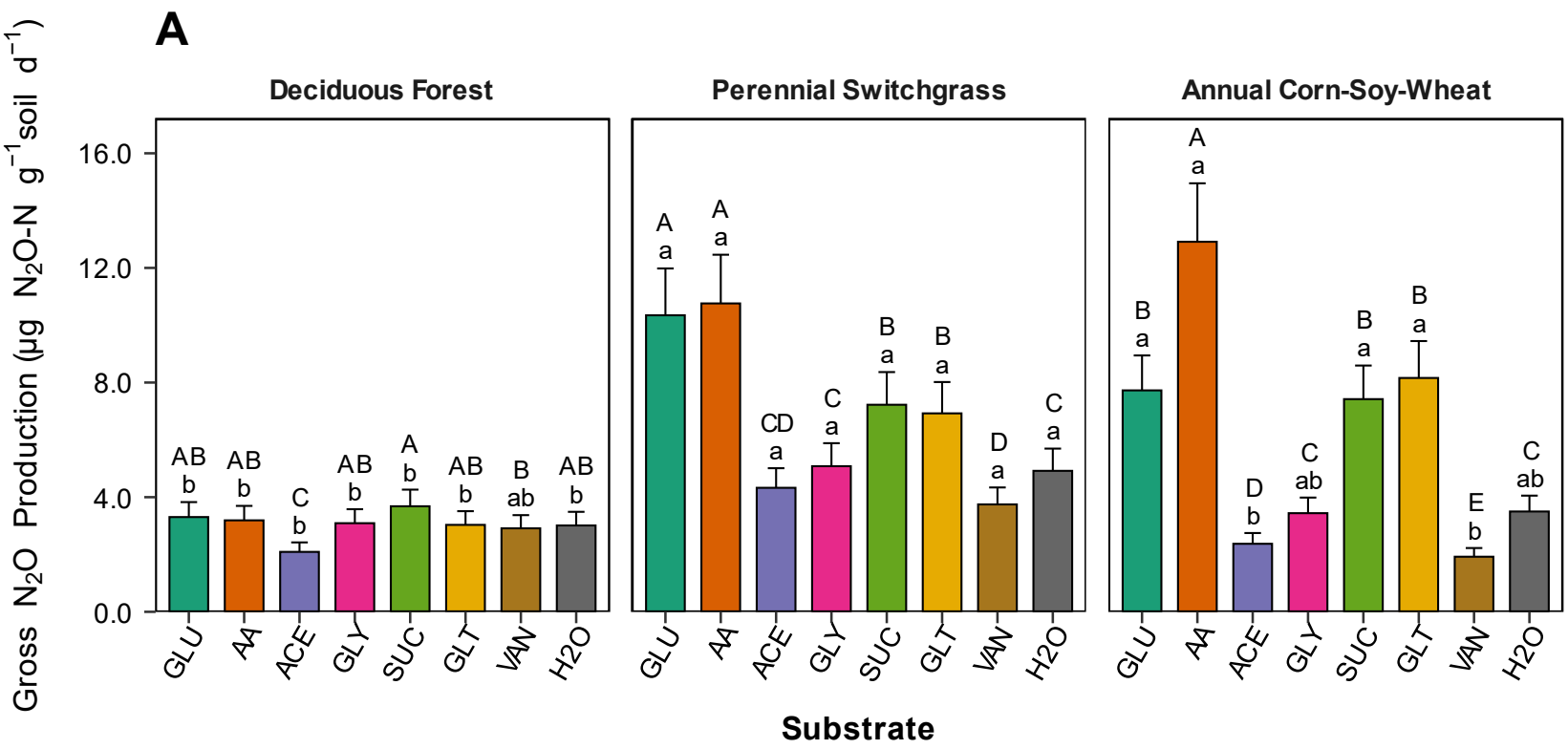
Taken together, it seems that the 0.2 M solution provides estimates of MBC that are not significantly different from those obtained from 0.5 M solution. In addition, the wet-fumigation method increases the estimation of MBC, but this is likely due to increased extraction efficiency rather than systematic error, since the MBC:MBN values are similar between wet- and dry-fumigated samples. The wet-fumigation method appears to be highly effective, and I am comfortable using it. To compensate for the increased extraction efficiency, I will not apply a correction factor during my MBC calculations.

With respect to the concentration of the K_2SO_4 solution, a lower concentration of K_2SO_4 is required for downstream applications. The only concerning aspect of the wet-fumigated soils extracted with 0.05 M K_2SO_4 is the high variability of DOC. However, this does not seem to be due to either the solution concentration or fumigation method, *per se*. It is possible that there is some sort of interactive effect that renders this method highly variable, but it is also likely that this is experimental error. Since the lower solution concentration is required for this experiment, and the 0.05 M solution did not affect the variability of non-fumigated soils, I still feel comfortable using this 0.05 M solution.

APPENDIX B:

FIGURES

Figure 5.1: Denitrification activity following substrate additions across soils from three land uses: gross N_2O production (A), net N_2O production (B), and relative N_2O reduction (C)



Gross N_2O was measured in jars receiving acetylene. Net N_2O was measured in jars that did not receive acetylene and thus represent the balance of N_2O production and reduction. Relative N_2O reduction is the percentage of total gross N_2O production that was reduced to N_2. Within land uses, significant differences between substrates are indicated by capital letters. Significant differences between land uses for each substrate are indicated by lowercase letters. Error bars indicate standard error (n = 4). GLU, glucose; AA, amino acids; ACE, acetate; GLY, glycine; SUC, succinate; GLT, glutamate; VAN, vanillin; H2O, no-C addition.

Figure 5.1 (cont'd)

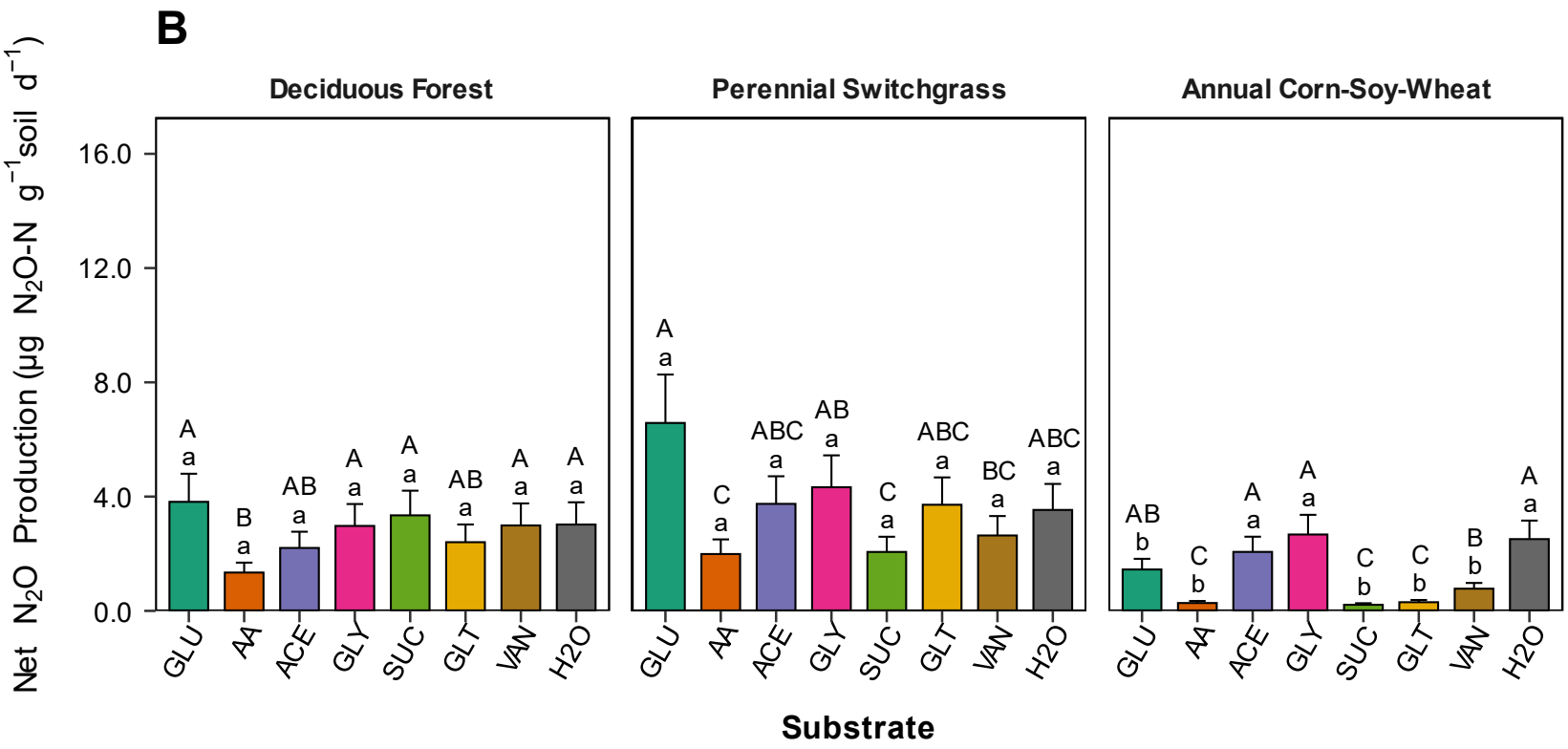


Figure 5.1 (cont'd)

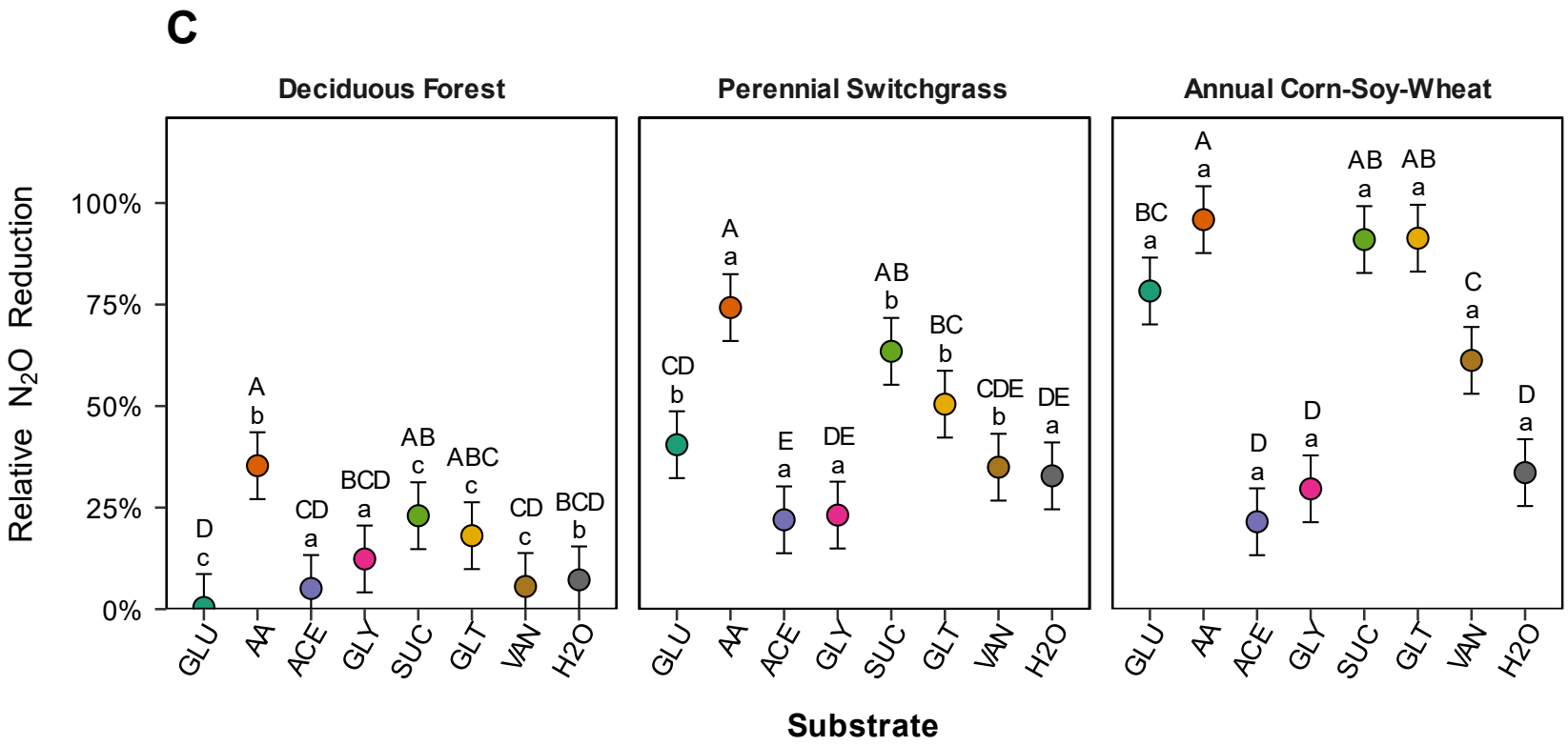
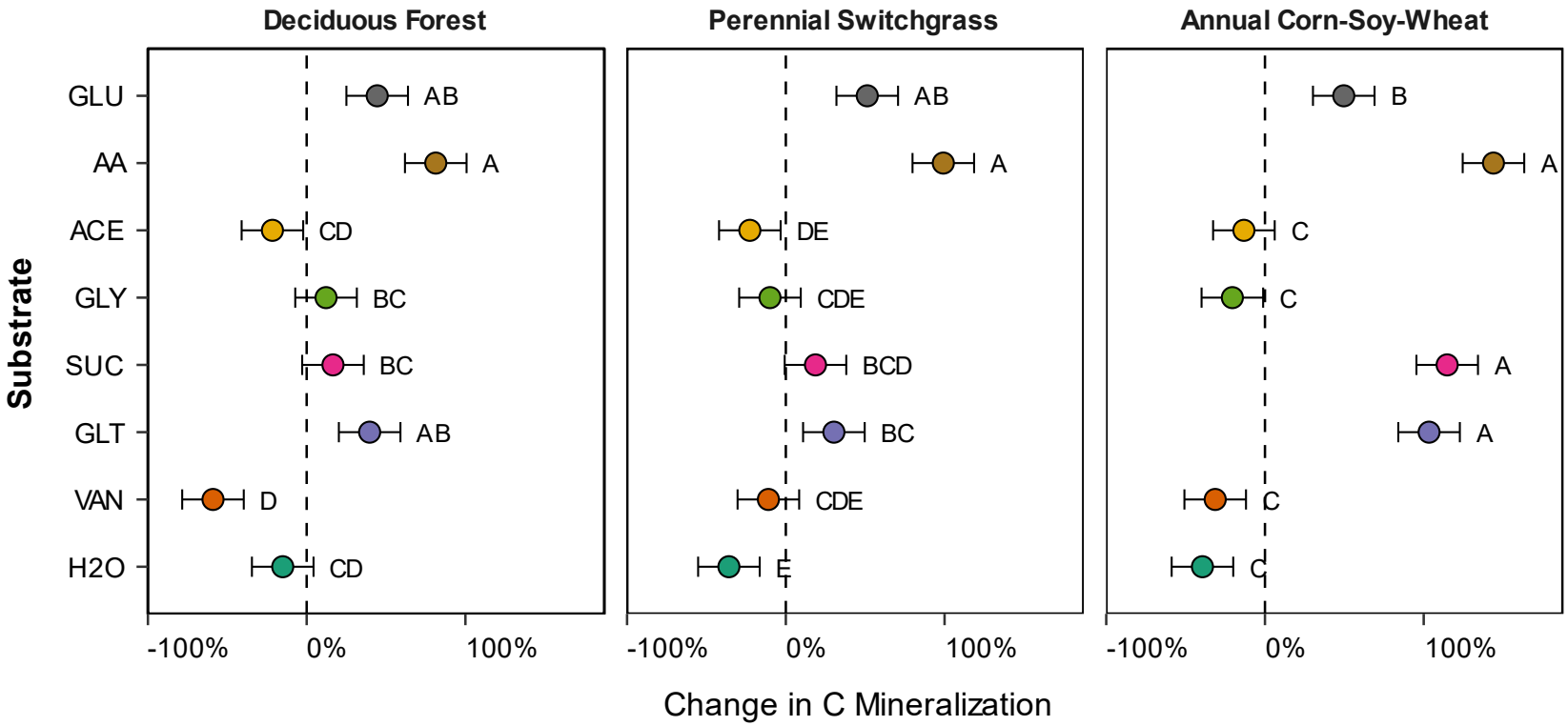
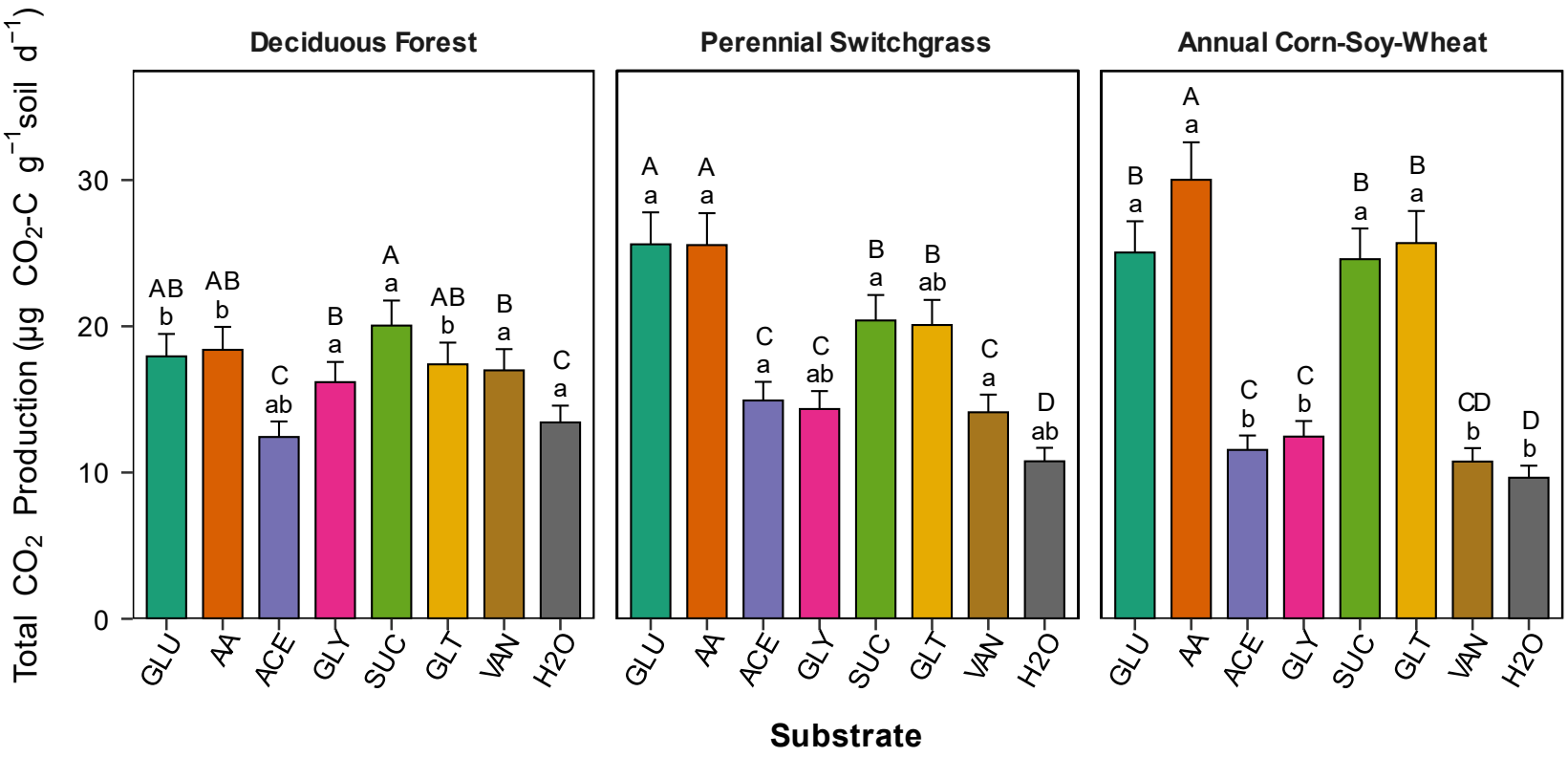


Figure 5.2: Change in rates of C mineralization between the start and end of the denitrification potential assays



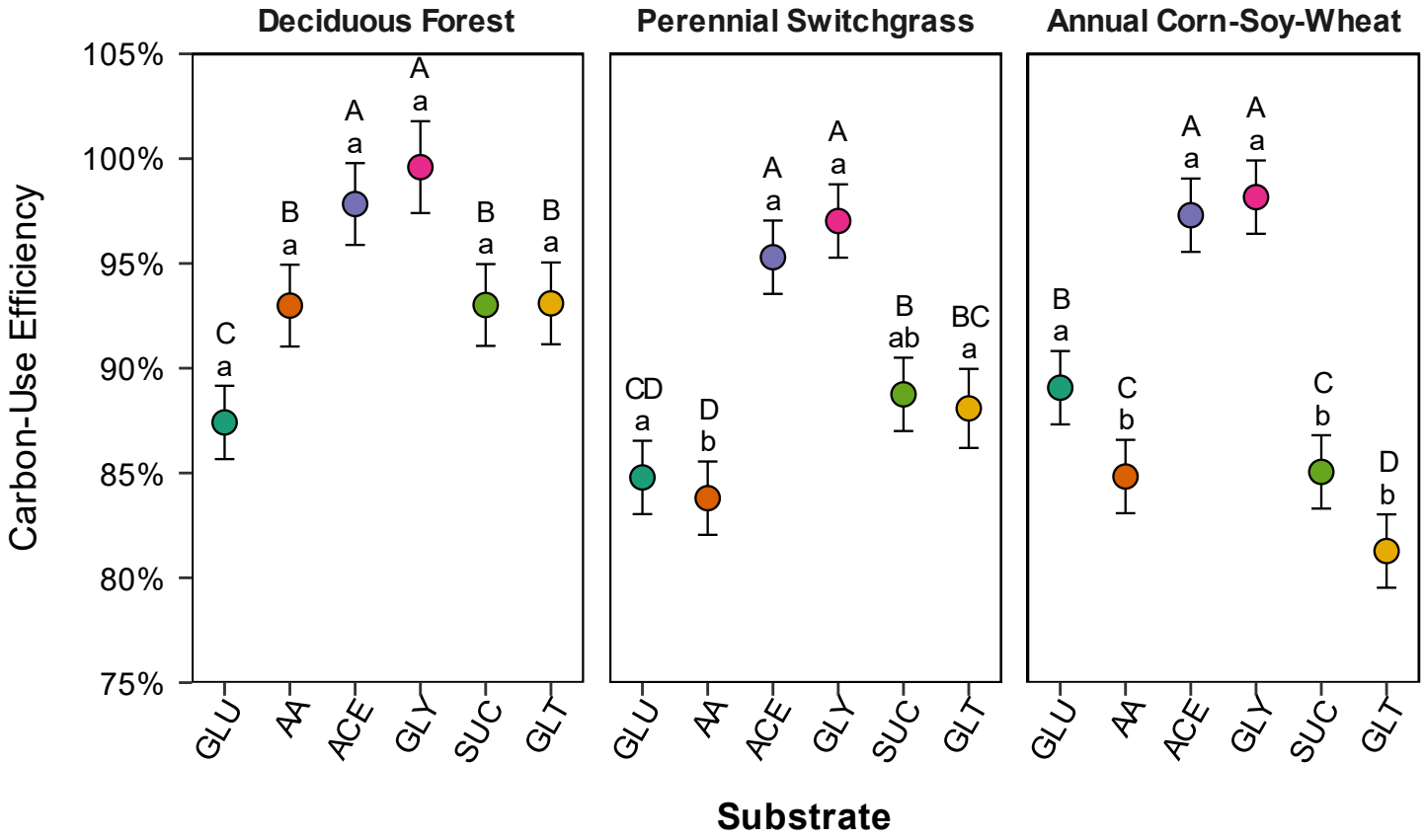
Change in C mineralization is the rate of CO_2 production between 24 and 28 hours divided by the rate of CO_2 production between 4 and 8 hours. Capital letters indicate significant differences between substrates within each land use. Error bars are one standard error (n = 4). GLU, glucose; AA, amino acids; ACE, acetate; GLY, glycine; SUC, succinate; GLT, glutamate; VAN, vanillin; H2O, no-C addition.

Figure 5.3: Total CO_2 production between substrates and land uses



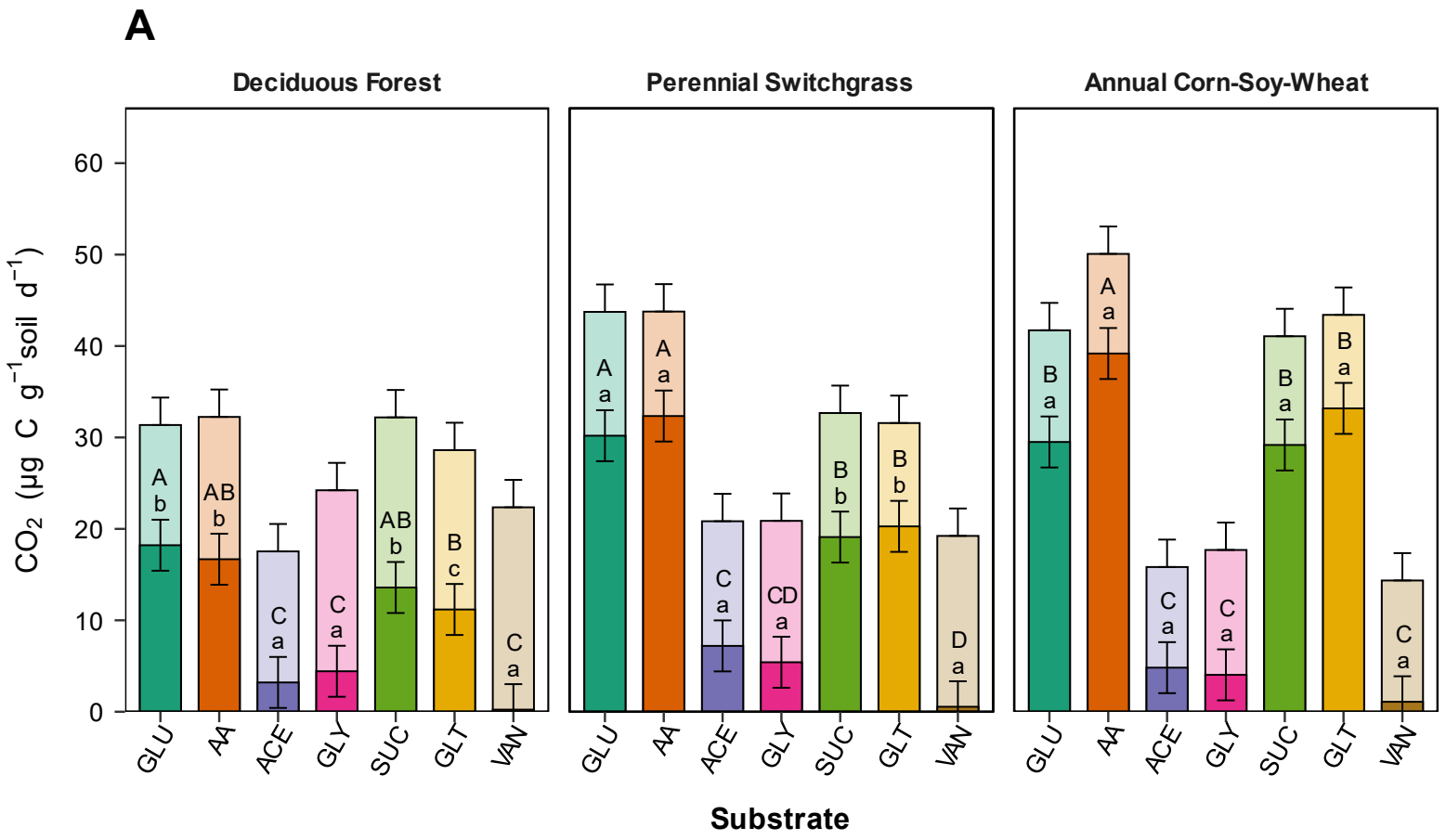
Cumulative production of CO_2 after one day. Capital letters indicate significant differences between substrates within each land use. Error bars are one standard error (n = 4). GLU, glucose; AA, amino acids; ACE, acetate; GLY, glycine; SUC, succinate; GLT, glutamate; VAN, vanillin; H2O, no-C addition.

Figure 5.4: Carbon-use efficiency (CUE) of microbial communities from three different land uses amended with six ^{13}C-labelled C-substrate treatments



CUE is the percentage of substrate C taken up by microbes that was incorporated into biomass. Total substrate uptake was estimated as the sum of CO_2 production and microbial biomass C. Within land uses, significant differences between substrates are indicated by capital letters. Significant differences between land uses for each substrate are indicated by lowercase letters. Error bars indicate standard error (n = 4). GLU, glucose; AA, amino acids; ACE, acetate; GLY, glycine; SUC, succinate; GLT, glutamate; VAN, vanillin; H2O, no-C addition.

Figure 5.5: Sources of CO_2 production (A) and microbial biomass C (B) in soils amended with seven ^{13}C-labelled C-substrate treatments



Darkened portions of the bars indicate substrate-derived C, while the lighter bars indicate the utilization of endogenous C. Within land uses, significant differences between substrate-derived pools of C are indicated by capital letters. Significant differences between land uses for each pool of substrate-derived C are indicated by lowercase letters. Error bars indicate standard error (n = 4). GLU, glucose; AA, amino acids; ACE, acetate; GLY, glycine; SUC, succinate; GLT, glutamate; VAN, vanillin.

Figure 5.5 (cont'd)

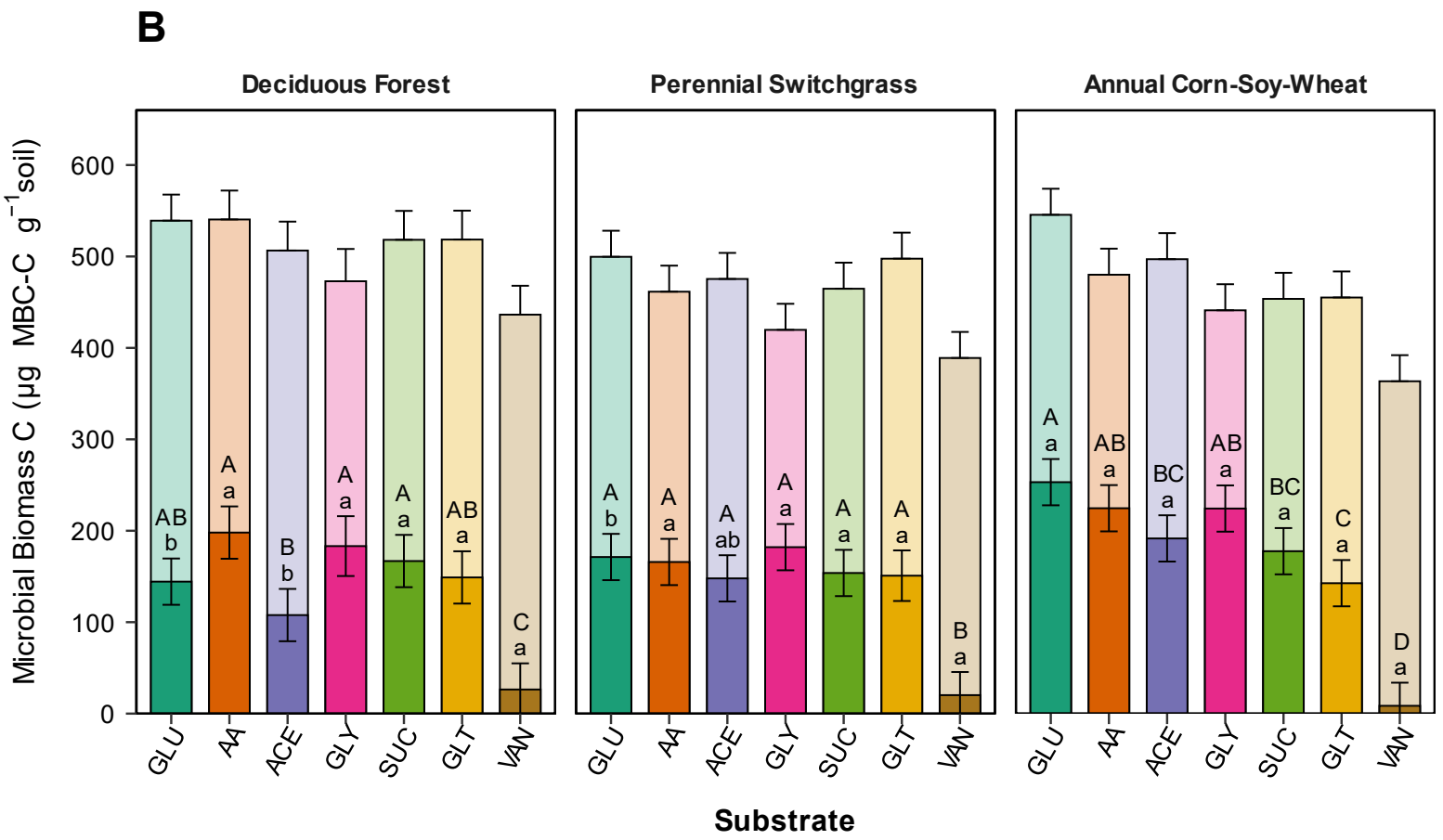
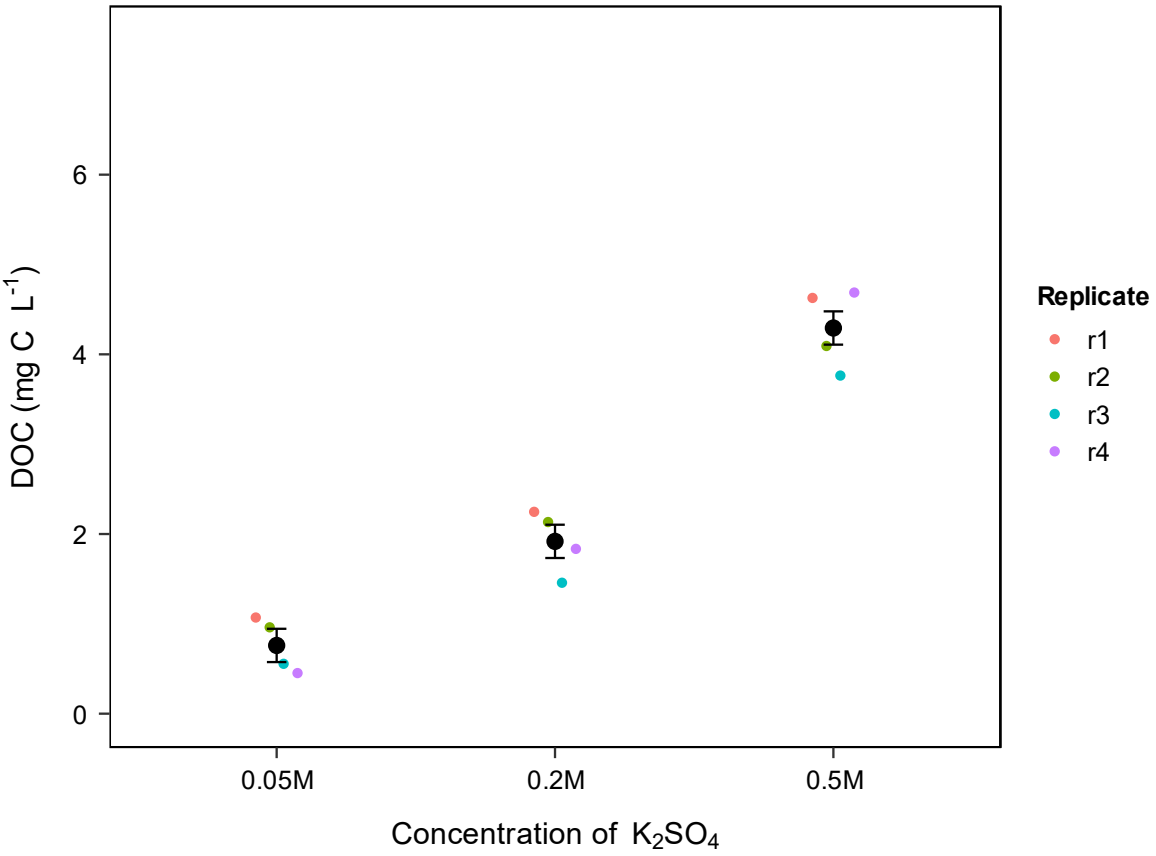
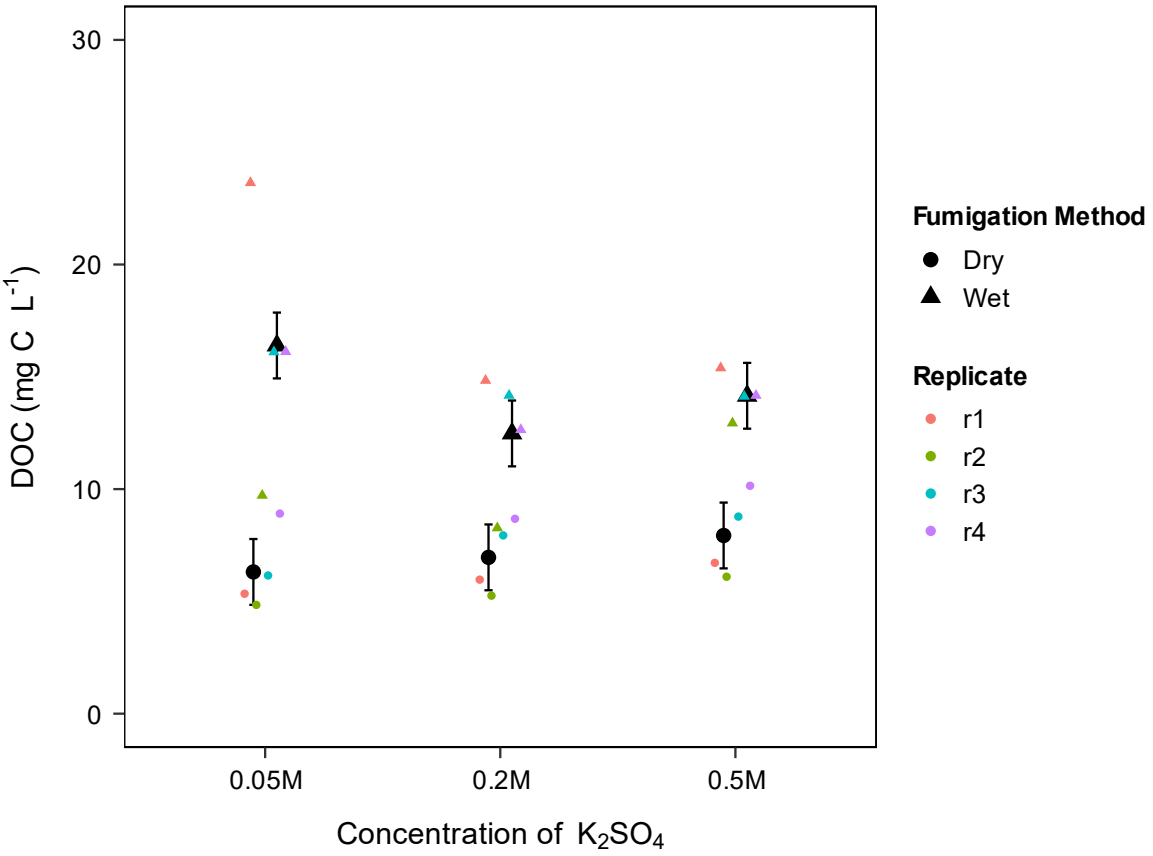


Figure 5.6: Microbial biomass optimization, non-fumigated extracts



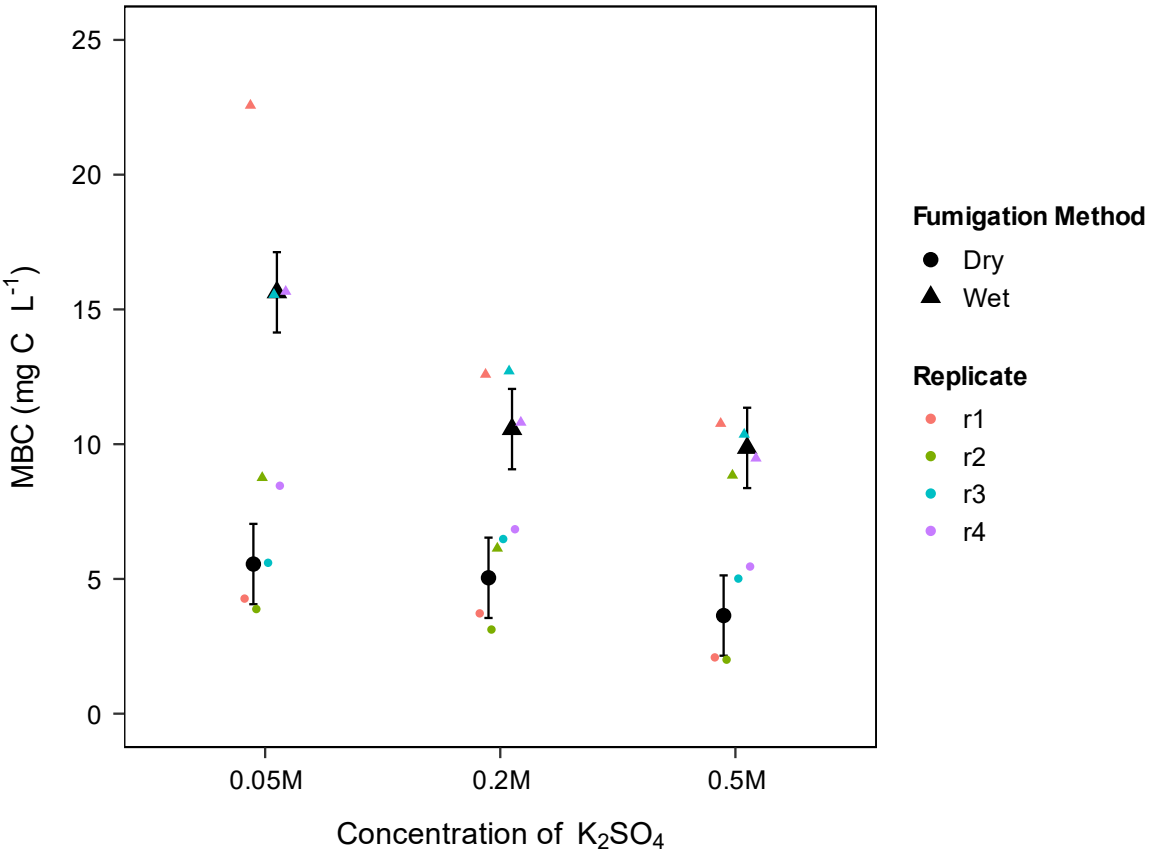
Dissolved organic carbon (DOC) content of soils extracted with three different concentrations of K_2SO_4. Individual replicates are indicated by colored points, and black points and error bars represent overall means with one standard error (n = 4).

Figure 5.7: Microbial biomass optimization, fumigated extracts



Dissolved organic carbon (DOC) content of soils fumigated with two different fumigation methods and then extracted with three different concentrations of K_2SO_4. Individual replicates are indicated by colored points, and black points and error bars represent overall means with one standard error (n = 4).

Figure 5.8: Microbial biomass optimization, microbial biomass carbon



Microbial biomass carbon (MBC) content of soils fumigated with two different fumigation methods and then extracted with three different concentrations of K_2SO_4. Individual replicates are indicated by colored points, and black points and error bars represent overall means with one standard error (n = 4).

APPENDIX C:

TABLES

Table 5.1: Type III ANOVA table of fixed effects

	Gross N_2O		Net N_2O		rN_2O		CUE		ΔCO_2	
	F	P	F	P	F	P	F	P	F	P
Substrate	74.9	< 0.001	11.9	< 0.001	22.6	< 0.001	47.2	< 0.001	19.5	< 0.001
Land Use	7.8	0.021	41.2	< 0.001	18.3	0.003	3.3	0.109	5.5	0.007
Substrate × Land Use	19.6	< 0.001	5.2	< 0.001	5.2	< 0.001	7.3	< 0.001	2.5	0.010

Table 5.2: Correlation coefficients between measures of denitrification activity and substrate C utilization

	CUE	$CO_{2\text{-sub}}$	MBC_{sub}
Gross N_2O	-0.724***	0.882***	0.259*
Net N_2O	0.247*	-0.269*	-0.052
Reduced N_2O	-0.719***	0.865***	0.246*
rN_2O	-0.704***	0.794***	0.218

Values are Pearson correlation coefficients. rN_2O, relative N_2O reduction as a percentage of gross N_2O production; CUE, carbon-use efficiency; $CO_{2\text{-sub}}$, substrate-derived CO_2; MBC_{sub}, substrate-derived microbial biomass C. Significance of the correlations is indicated by asterisks (*, P-value < 0.05; **, P-value < 0.01; ***, P-value < 0.001).

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