**University of Michigan**

*Master's Thesis*

# Cover crop impacts on soil nutrient cycling: effects of functional diversity and management history

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**Abstract**

Interest in increasing agroecosystem diversity through use of cover crops continues to rise. Cover crops are non-harvested crops that provide a range of ecosystem functions, and mixtures of cover crop species with complementary traits, such as legumes and grasses, may increase multiple functions at once. However, the performance of cover crops grown in monocultures and mixtures is expected to vary across farms with different levels of soil fertility, which result from unique management histories. Understanding the interactions of these two factors can help optimize the use of cover crops for more sustainable soil nutrient management. This study therefore addressed the following research questions: (1) Do legume-grass cover crop mixtures alter rates of decomposition compared to legume and grass cover crop monocultures? (2) Are the effects of litter type different in soils with different management histories? We incubated three litter treatments in two soils with contrasting fertility levels for 360 days, and measured decomposition dynamics through respired $CO_2$, microbial extracellular enzyme activity, and inorganic N mineralization. As expected, new carbon inputs to soil increased microbial processes in the short term, but basically had no long-term effect on the measured responses. The lower fertility soil had a greater response to litter addition for both $CO_2$ respiration and enzyme activities for enzymes that degrade labile organic carbon compounds. The total inorganic N release was higher on the high fertility soil. Overall, both cover crop litter addition and farm management history affect

microbial decomposition dynamics. In this study, we found that cover crop litter addition had a stronger effect on soil biological processes compared to management history, however, the difference between litter mixture and monoculture treatments was not significant.

# 1.Introduction

## *1.1 Cover crops and ecosystem function*

Modern agriculture has led to widespread environmental degradation, including soil erosion (Montgomery 2007, Tilman et al., 2002), nutrient leaching and runoff (Blesh & Drinkwater, 2013; Sims et al., 1998; Hart et al., 2004), and the loss of biodiversity (Horrigan et al., 2002; Kremen et al., 2012). It also relies heavily on energy-intensive external inputs, such as fertilizers, pesticides, and herbicides to increase the yield of crops. To improve agricultural sustainability, cover crops (i.e., non-harvested crops grown in rotation with primary crops) are increasingly appealing to farmers for providing agroecosystem services such as erosion control, weed suppression, nitrogen (N) retention, and thus the potential to reduce the environmental costs of industrial agriculture (Snapp et al., 2005; Poffenbarger et al., 2015; Zhang et al., 2007). The introduction of cover crops has also increased agroecosystem biodiversity, and soil organic matter and microbial biomass levels, which has the potential to increase crop yield (Finney, 2016; King & Blesh, 2018; McDaniel et al., 2014; Smith et al., 2014; Wortman et al., 2012).

Cover crops can be divided into functional groups such as grasses and legumes, which have distinct ecosystem functions. Grass species have traits such as fibrous root systems that reduce soil erosion and assimilate and retain soil nutrients. In addition, some cereal species, like rye (*Secale cereale* L.), also have a fast growth rate and high cold tolerance, which can suppress

weeds and reduce the time when temperate crop fields are in a bare fallow (Lawson et al., 2015). However, most grasses cannot provide a supply of new N to the soil, which is essential to balance the N removed from agroecosystems in exported crops (Blesh and Drinkwater, 2013). In contrast, legume cover crops such as hairy vetch (*Vicia villosa* L.) fix atmospheric $N_2$ into plant-available forms through symbiotic relationships with rhizobia bacteria and can reduce the need for synthetic N fertilizer additions. Legumes also have N-rich litter that rapidly decomposes, releasing mineralized N upon incorporation into the soil (Coombs et al., 2017; Fisk et al., 2001).

However, this fast decomposition rate of legume litter can increase the possibility of N losses through leaching or as a gas if N mineralization is not synchronized with the growth of the subsequent crop. As a result, there is a growing interest in the use of cover crop mixtures that combine legume and grass species to simultaneously supply and retain soil N (White et al., 2017).

### *1.2 Increasing functional diversity with cover crop mixtures*

The focus on cover crop mixture research has significantly increased in the past decades due to the expected functional complementarity of legume and grass cover crops. A legume-grass cover crop mixture can combine the contrasting, and desirable, functional traits of legumes and grasses while minimizing their potential shortcomings. For example, legume-grass (e.g., hairy vetch-cereal rye) cover crop mixtures have been shown to "overyield" compared to their respective monocultures (Poffenbarger et al., 2015; Snapp

et al., 2005, Finney, 2016) and to provide better weed suppression (Clark et al., 1997; Creamer, 1996; Teasdale, 1996). Legume cover crops like vetch increase soil N availability (Ranell & Wagger, 1996) for the succeeding cash crops, while grass cover crops produce larger above- and below-ground biomass, effectively taking up excess soil N and reducing N losses through denitrification or leaching (Moller et al., 2008; Kuo & Sainju, 1998; Brophy et al., 1987). Finally, cover crop functional diversity has a more profound effect on the soil N pool than does species diversity (Wortman et al., 2013); a two species legume-grass cover crop mixture had higher N fixation and aboveground biomass compared to a higher diversity cover crop mixture, or a cover crop monoculture (Bessler et al., 2009; Tilman et al., 1997).

*1.3 Decomposition dynamics and organic management history*

In agriculture, increasing N supply from biological N fixation, and from the decomposition of soil organic N pools, can reduce the need for synthetic N fertilizer inputs. By converting $N_2$ gas, which cannot be directly taken up by plants, into plant-available N, legume N fixation and subsequent release of N through decomposition and N mineralization provide an organic, "solar-powered" N source to agroecosystems (Mary et al., 1996). Decomposition is carried out by soil microorganisms. The microbial biomass pool, and associated enzyme activity, are directly related to $CO_2$ flux in the soil (Calderon, 2016, Mbuthia et al., 2015; Nielsen et al., 2002, Balota et al., 2014, Fernandez et al., 2016). Furthermore, decomposition of organic matter

can lead to mineralization of nutrients such as N and phosphorus, increasing their availability to plants.

Different types of enzymes can break down different organic C compounds. Therefore, changes in enzyme activity reflect the types of compounds that the microbial community is decomposing to acquire energy and nutrients, and can also signal nutrient limitation in the soil (Lou et al., 2016; Calderon et al., 2016). Enzymes like b-1,4, -glucosidase (BG), b-1,4,-N-acetyl glucosaminidase (NAG) and acid phosphatase (PHOS) decompose relatively labile organic compounds. In particular, BG releases labile C, NAG releases soil N from proteins, and PHOS releases phosphate groups. In contrast, enzymes like phenol oxidase (PHENOX) degrade more chemically recalcitrant organic C forms like lignin. By analyzing enzyme activities, as well as the ratio between different enzymes, we can better understand the status of the soil nutrient availability and soil nutrient transformation processes.

The decomposition dynamics of cover crop residues are expected to vary with background soil conditions that reflect different soil types as well as farm management histories that drive soil fertility status. Cover crops are grown in windows between primary cash crops, and can, therefore, be integrated into a wide range of farm types. However, they are most commonly planted on organically managed farms, where nutrient sources are organic (e.g., legume cover crops, manure, or compost) rather than synthetic. Long-term studies of organic cropping systems have found that, over time, practices

such as diverse crop rotations, use of cover crops and organic fertility amendments, and lack of synthetic inputs, decrease energy inputs and build stocks of soil organic matter (King & Blesh, 2018; Drinkwater et al., 1998; Clark et al., 1998; Robertson et al., 2014; Pimentel et al., 2005). The length of time under organic management is therefore expected to increase soil organic matter stocks, particularly labile C pools, such as particulate organic matter (Wander et al., 1994; Marriott & Wander, 2006), which are important for internal nutrient cycling, stimulating microbial enzyme activity and organic matter turnover (Gunapala et al., 1998; Cookson et al., 1998).

### *1.4 Previous Studies*

Previous studies have found that crop rotational diversity (including the use of cover crops) influences microbial and nutrient cycling processes, which tend to stimulate decomposition dynamics (Finney et al., 2016; Lawson et al., 2013; McDaniel et al., 2014; Mendes et al., 1999). Furthermore, studies have shown that even though a wide range of legume-grass mixture compositions increase both aboveground biomass and decomposition rates compared to grass monocultures, a certain mixture proportion by weight (80% legume and 20% rye) of legume and grass cover crops is most effective for increasing both factors (Poffenbarger et al., 2015). The interaction of legume and non-legume cover crops is likely to vary with the amount of soil organic matter and the level of N in the soil (Blesh, 2018; Moller et al., 2008; Schipanski & Drinkwater, 2011). However, to date, the interactive effects of

cover crop residue functional diversity and background soil fertility on decomposition dynamics has not been tested. Yet, this is critical information for understanding how conservation practices such as cover cropping will impact soil biological processes in different contexts.

### *1.5 Research Questions*

In this study, we explore how differences in soil organic matter pools resulting from unique long-term management histories impact soil C respiration, inorganic N release, microbial biomass and enzyme activity following addition of cover crop residues. Specifically, we focus on legume-grass cover crop mixtures and component monocultures. Our experiment was designed to address two primary questions: (1) Do legume-grass cover crop mixtures alter rates of decomposition compared to legume and grass cover crop monocultures, as measured through respired $CO_2$, microbial extracellular enzyme production, and inorganic N mineralization? Moreover, (2) are the effects of litter type different in soils with low and high levels of fertility due to distinct management histories? Our hypotheses are that (1) soil $CO_2$ flux rate, microbial enzyme production, and N mineralization will follow the order: legume monoculture > legume-grass mixture > grass monoculture due to the C:N ratio of the different litter treatments, and (2) following litter addition, $CO_2$ production and enzyme activity will be lower in the lower fertility soil.

## 2. Methods and Materials

### *2.1 Research sites and experimental design*

To address our research questions about the effects of cover crop functional diversity and farm management history on decomposition dynamics, we designed a laboratory incubation experiment. Specifically, we compared soils from two different farms that had a different length of time under organic management in Ann Arbor, Michigan. The two farms were selected for their similarity regarding soil physical properties, but significant differences in multiple metrics of soil fertility (Table 1) due to differences in their management histories. The higher fertility farm had more than a decade of organic management, including frequent use of overwintering cover crops with supplemental compost, while the lower fertility farm had been in organic production for just three years. The farms were both parts of a companion study involving an experimental field that had been under the same organic management regime for two years, which included two overwintering seasons of a hairy vetch-cereal rye cover crop mixture, when we began the current study. The experimental fields on the two farms were planted in a mixture of cereal rye (56 kg $ha^{-1}$) and hairy vetch (25 kg $ha^{-1}$) on September 2 and September 14, 2016. Seeds were surface broadcast and lightly incorporated, and the field was divided into four replicate blocks.

Soil samples were collected on April 30$^{th}$, 2017 from the experimental field in the rye/vetch mixture on both farms before cover crop termination. A composite sample of approximately eight - ten 10cm soil cores was collected from each block on both farms and was homogenized, and half of the soil was sieved. About 8 g sieved soil was added to triplicate, 50mL centrifuge tubes

containing 40ml of 2M KCl for extraction of nitrate ($NO_3^-$) and ammonium ($NH_4^+$). The tubes and remaining soil were stored in a cooler on ice until they were brought back to the lab. A subsample of soil was set aside for analysis of baseline microbial biomass and microbial enzyme activities and was refrigerated for up to 72 hours. The remaining fresh soils were sieved to 2mm and divided into 8 mason jars (4 replicate blocks x 2 farms). After measuring soil moisture, soils were brought to 50% Water Holding Capacity and pre-incubated for five days without any plant residue to minimize the effects of disturbance due to our sampling before starting the decomposition incubation (McDaniel et al., 2014).

*2.2 Lab Incubation*

In order to start the incubation study before the 2017 sampling date for vetch and rye in the larger experiment, we used dried and ground vetch and rye litter from the same fields, which had been collected in the first year of the experiment in May 2016. The biomass was sampled to ground level from a $0.25m^2$ quadrat in each block in each field, avoiding soil, immediately before cover crop incorporation. The litter was dried at 60°C for three days, ground to 2mm in a Wiley mill, and stored until use. To establish the incubation treatments, 50ml Falcon tubes were labeled and their weights recorded. We took 500 g of pre-incubated soil (400 g dry soil equivalent) from each block on each farm and divided it equally among four, 1L mason jars. To the mason jars, except for the control, we added 1.2 g of the dry plant litter for each

treatment (rye, vetch, and rye-vetch mixture). For the mixture treatment, we used 0.54g vetch and 0.66g rye litter to reflect the mixture proportion of the cover crop biomass in the field. After soils were well-mixed with litter, each soil-residue treatment was then divided evenly into three 50mL tubes (for three destructive sampling dates), and the weight of each tube was recorded. The three tubes for each treatment (and the control with no plant residue) were placed in a Mason jar and incubated in a dark room at approximately 25° C for 360 days. We used the tube weights to track and maintain the soil at 50% water holding capacity using DI water.

*2.3 Soil Inorganic N*

We extracted soil inorganic N ($NH_4^+$-N + $NO_3^-$-N) on day 0 from the composite soil sample per block, and on day 30 of the incubation period, with 2M KCl. On day 30, one tube was destructively sampled from each Mason jar. We randomly took 16g of soil from each tube and evenly divided it into two replicate test tubes. We added 40 ml of 2M KCl to each tube. Tubes were placed on a shaker for one hour, centrifuged for 10 minutes and filtered into 20 ml vials and frozen until analysis. The amount of $NH_4^+$ and $NO_3^-$ in each sample was analyzed colorimetrically on a continuous flow analyzer (AQ2; Seal Analytical, Mequon, WI). Remaining soil from the sampled tubes was processed for microbial biomass and enzyme analysis.

*2.4 $CO_2$ Flux Measurements*

Soil respiration rates were measured by the amount of $CO_2$ produced in a given period. We first uncapped the lids of all Mason jars and let them sit for a half hour so that the $CO_2$ concentration inside the jar would equilibrate with the concentration in the lab. The Mason jars were then recapped using lids fitted with rubber septa and incubated in 25℃ in the dark environment. $CO_2$ was measured for a total of 20-time points over the incubation. The $CO_2$ concentrations at each time point were corrected using a 1.01% $CO_2$ standard. Over the first two weeks, the incubation time was approximately 3 hours. After the respiration rate decreased, the incubation time increased to 6 hours and eventually to 48 hours. Accordingly, the interval between tests slowly increased from once per day, to once per month after four months. A syringe with a needle was used to extract gas from each Mason jar through the rubber septa and was immediately injected into a LiCor-820 for analysis of $CO_2$ concentration. The respiration rate was calculated by subtracting the concentration of $CO_2$ in the jar immediately after covering, and after 3-48 hours (depending on the time course of the incubation), divided by the headspace in the jar and the grams of dry soil equivalent in the jar. The headspace (HS) was calculated with the following equation with the number of tubes per jar (n=3 initially; n decreased on day 90 and 360), the height of the soil in the tubes (h) in cm, the inner diameter of each test tube (d), the amount of water that each Mason Jar could hold without any tubes (V), and the volume of one empty tube (v).

$$\text{Headspace(HS)} = \text{V} - \text{n} * \left[\text{v} + \left(\frac{\text{d}}{2}\right)^2 * h * \pi\right]$$

*2.5 Microbial Biomass*

Along with soil inorganic N, we measured microbial biomass on days 0 and 30 due to a large decline in microbial activity after day 30, following the protocol from previous studies (Rinkes et al., 2011, McDaniel et al., 2014). Microbial biomass was estimated using the chloroform-fumigation extraction method (Vance et al., 1987, Gregorich et al., 1990). We divided 10 g soil evenly into two replicate tubes, added 40 ml 0.5M $K_2SO_4$ to both replicates and 0.5ml chloroform to one of the replicates and capped the tubes immediately under the fume hood. All tubes were shaken at 150 revs for 4 hours and then centrifuged at 1500 RPM for 10 minutes. The solution was filtered using #1 Whatman filter papers into 20ml vials, frozen at -20°C, and analyzed on a Shimadzu TOC-TN (Shimadzu Scientific Instruments, Columbia, MD).

*2.6 Enzyme Activity*

To decompose organic matter, microbes release extracellular enzymes. We measured the extracellular enzyme activity (EEA) of four enzymes produced by microbes, which reflect the type of organic C compounds they are breaking down, and release of different nutrients like N or P.
We selected four enzymes for measurement: B-1,4,-glucosidase (BG) cleaves glucose from cellobiose; b-1,4,- N-acetyl glucosaminidase (NAG) cleaves N-

acetyl glucosamine from chitin and peptidoglycan oligomers; acid phosphatase (PHOS) cleaves phosphate groups from organic phosphorus, and phenoloxidase (PHENOX) is a lignin-degrading enzyme. Briefly, BG releases labile C; NAG releases soil N from proteins, and PHOS releases phosphate groups; PHENOX is used degrade more chemically recalcitrant organic C compounds.

Microbial enzyme activity was measured at four time points: Day 0 (i.e., baseline), Day 30, Day 90 and Day 360. We adjusted the soil pH with a sodium acetate buffer to accurately reflect the pH of soil from both farms (6.7 and 7.2). We used a blender to homogenize 1 g of soil with 80 g of sodium acetate buffer and pipetted the mixture into 96-well plate. Soils were incubated in a dark space for 6 hours for BG, NAG and PHOS and 24 hours for PHENOX. The enzyme plates were read on a plate reader using 365nm for BG, NAG and PHOS and 460 nm for PHENOX.

### *2.7 Data Analysis*

#### 2.7.1 Enzyme activity

Enzyme data were analyzed using two-way analysis of variance (ANOVA) models, with treatment and farm as fixed effects, and replicate block as a random effect. We used Tukey's HSD for post-hoc comparisons of least square means. Statistical analysis was conducted using R version 3.4.3 (The R Foundation for Statistical Computing, 2017) and SAS version 9.4 (SAS Institute, 2017).

We tested the activity of the four enzymes at three-time points separately using two ANOVA models. Bartlett's Test and Levene's Test were used to examine the homogeneity of variance. In model 1, we used the extracellular enzyme activity of each enzyme (BG, NAG, PHOS, PHENOX) as the response variable; farm and treatment as the main effects; and replicate block as the random variable for all three time points. Model 2 was the same as model 1 except that we included both times and replicated blocks as random effects. We also used Tukey's HSD to calculate post hoc mean comparisons.

### 2.7.2 Soil inorganic N and microbial biomass

Inorganic N and microbial biomass data were first checked for normality using Levene's and Bartlett's tests. Lab replicates that were outliers were re-run to exclude the possibility of experimental errors. Because we had four replicates for each plot, three outliers that were caused by measurement errors were removed before analysis. Data were then analyzed in R using the same ANOVA model (model 1) used for enzyme activity. We also used pairwise comparisons to compare differences between all treatments in both farms.

### 2.7.3 Carbon flux

The raw carbon flux data were initially processed by using the concentration difference between $CO_2$ measured by the gas analyzer (Li820,

LI-COR) at time 1(start) and time 2(end). The $CO_2$ concentration at each time point was first corrected and converted to mass unit ($C_m$) using the ideal gas law equation with the concentration of $CO_2$ ($C_v$), the molecular weight of $CO_2$-C (M), the barometric pressure in atmosphere (P), the universal gas constant (R) and the incubation temperature in °K (T)

$$C_m = C_v * M * \frac{P}{R * T}$$

The $CO_2$ flux (F) was then calculated with the following equation with the change of the $CO_2$-C mass over the incubation period ($C_m$), the headspace volume (L), the volume of DI water added to the tubes ($W_v$) and the weight of soil in the mason jar before adding water (m), the actual incubation time (t).

$$\mathrm{F} = \frac{C_m * (L - \frac{W_v}{1000})}{m * t}$$

The $CO_2$ flux was then adjusted with the standard reading (1.01% $CO_2$) to get the actual flux for each sample (µg $CO_2$-C/g/day). The data for all 20 time points was entered into an Excel spreadsheet.

We then carried out an individual ANOVA test for each specific date. We use flux as the response variable, farm, treatment, farm*treatment as the main effects and replicate block as the random variables in R. The cumulative $CO_2$ flux ($C_c$) during the whole incubation was then calculated using the

trapezoidal function in Excel with the total number of time points (N), the flux at two adjacent time points ($f_{d-1}$ and $f_d$), and the number of days between these two time points (g).

$$C_c = \sum_{k=1}^{N} \frac{f_{d-1} + f_d}{2} * g$$

We also used a 3-pool, six parameter exponential decay equation to fit the cumulative C respiration curves to calculate the amount of $CO_2$ flux derived from each of three soil organic matter pools (active or labile C, slow C, and the stable C pool) over the course of the incubation (Sigma Plot, Systat Software). (Paul et at., 1999; Sanford & Kucharik, 2013, McDaniel et al., 2014):

$$C_c = M_a e^{-k_a t} + M_s e^{-k_s t} + M_r e^{-k_r t}$$

In this equation $M_a$, $M_s$, and $M_r$ are the measured respiration rates from active, slow and stable C pools, respectively, while $k_a$, $k_s$, and $k_r$ are the respiration decay constants for these three different soil organic C pools; and t is the day of the incubation. The model was used to calculate the M's and k's in the equation above because these values have not been previously determined for the particular soil-residue combinations in the incubation. As a result, we excluded the control (no litter) from this analysis. We then used the M and k values to calculate the proportion of $CO_2$ originating from each pool (e.g., active = $\frac{\mathrm{Ma}}{\mathrm{ka}} * \mathrm{C}_c$).

## 3. Results

### *3.1 Soil and Litter Chemistry*

In 2016 of the on-farm experiment the cover crops were incorporated relatively early in the spring (on 4/27 and 4/28) because of the timing of planting the following crop. As a result, rye plants were still in a vegetative growth stage, and the rye and vetch litter did not have the contrasts in C: N ratio, and %N that we were expecting based on their plant functional types (Table 2). However, rye did have the highest hemicellulose content, and lowest lignin and cellulose percentage, while vetch litter was the opposite. The rye-vetch mixture had similar litter chemistry properties as the rye monoculture. (Table 2).

Regarding soil properties, the two soils had significant differences in soil fertility measures as expected based on their management histories. The soil from the farm that had a long history of organic management had higher total soil organic matter (3.7%), while the farm that had only recently transitioned to organic management had lower organic matter (1.7%). In terms of labile organic matter pools expected to be important for microbial decomposition and nutrient cycling, free particulate organic matter (Free POM) in the higher fertility soil (32.19 Mg $ha^{-1}$) was seven times higher than in the lower fertility soil (4.68 Mg $ha^{-1}$). Moreover, the N content of the physically protected POM was 85.2% higher in the high fertility soil, and plant-available phosphorus content was 42.8% higher for the higher fertility soil than the lower fertility soil. The soil organic C pool

was also 64.8% higher for the higher fertility soil (64.1 Mg $ha^{-1}$) than in the lower fertility soil (38.9 Mg $ha^{-1}$), mirroring the result of total soil organic matter.

**Table 1.** Initial soil chemical and physical properties for two the farms.

| Soil Origin | Chemical Properties | | | | | Physical Properties | | | |
|---|---|---|---|---|---|---|---|---|---|
| | OM | Free POM | Protected POM N | Phosphorus | Total organic C | Sand | Silt | Clay | Soil series |
| | (%) | (Mg $ha^{-1}$) | (kg $ha^{-1}$) | (ppm) | (Mg $ha^{-1}$) | | (%) | | |
| Higher Fertility Farm | 3.7 | 32.2 | 276.1 | 40 | 64.1 | 64 | 10 | 26 | Miami B |
| Lower Fertility Farm | 1.7 | 4.7 | 149.1 | 28 | 38.9 | 70 | 16 | 14 | Fox sandy loam A |

**Table 2.** Initial litter chemistry for the three different treatments in the incubation study.

| Treatment | C: N | C | N | Lignin | Cellulose | Hemicellulose |
|---|---|---|---|---|---|---|
| | | | | % | | |
| Rye | 22.38 | 42.13 | 1.92 | 1.79 | 23.22 | 22.76 |
| Vetch | 16.07 | 36.11 | 2.37 | 3.83 | 41.15 | 4.17 |
| Mixture | 21.69 | 39.44 | 1.96 | 2.81 | 27.53 | 16.3 |

*3.2 Inorganic N*

The concentration of soil $NH_4^+$ was negligible. We therefore summed the soil $NO_3^-$ and $NH_4^+$ concentrations as the total extractable inorganic N pool ($N_i$). Compared to the baseline levels on day 0, litter addition led to a significant increase in $N_i$ on day 30 (Figure 1). The $N_i$ in the controls for both lower and higher fertility farms increased by 30% and 150% respectively, with a significant difference in $N_i$ concentrations between the lower and higher fertility farms ($p < 0.0001$). Averaging the $N_i$ across treatments, the higher fertility soil had ten times the $N_i$ compared to the lower fertility soil. On the higher fertility soil, there was significantly lower $N_i$ in the control group compared to the rye-vetch mixture treatment. However, on the lower fertility soil, there were no significant differences in $N_i$ between all four treatments ($p > 0.05$).

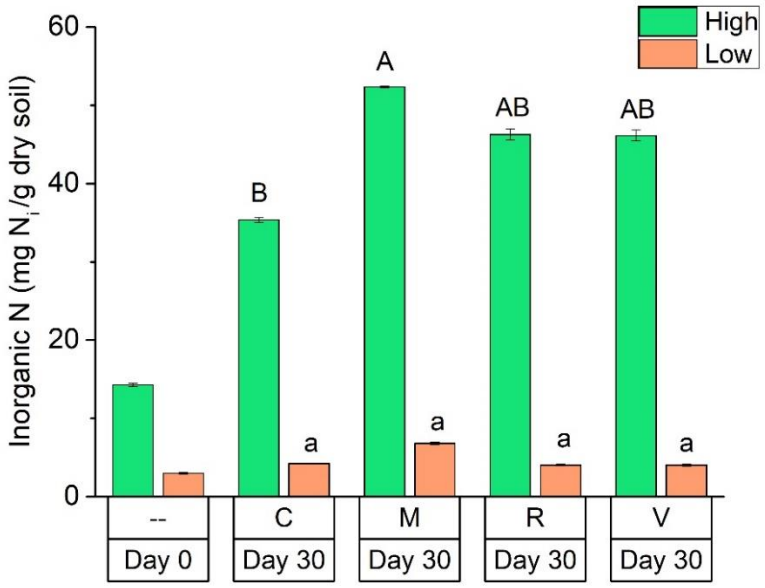



**Figure 1**. Total extractable soil inorganic N ($NO_3^-$ + $NH_4^+$) measured on Day 0 and Day 30 of the incubation. C = Control, M = Rye-Vetch mix, R = Rye, V = Vetch. High = the high fertility soil and Low = the low fertility soil. Same letters indicate no significant differences between litter treatments at $P<0.05$.

*3.3 Carbon Dynamics*

Without litter addition, soils with different fertility levels showed a very different $CO_2$ flux through the entire study (Figure 2), as expected. Without litter addition, the higher fertility soil had 34.43% greater $CO_2$ flux over the course of the incubation compared to the lower fertility soil. Because the two farms had similar soil physical properties, and the fields had the same crop species and organic management practices for two years during the companion on-farm experiment, the difference in $CO_2$ respiration was driven by differences in the biological and chemical properties of the two soils, which resulted from distinct management histories (Table 1).

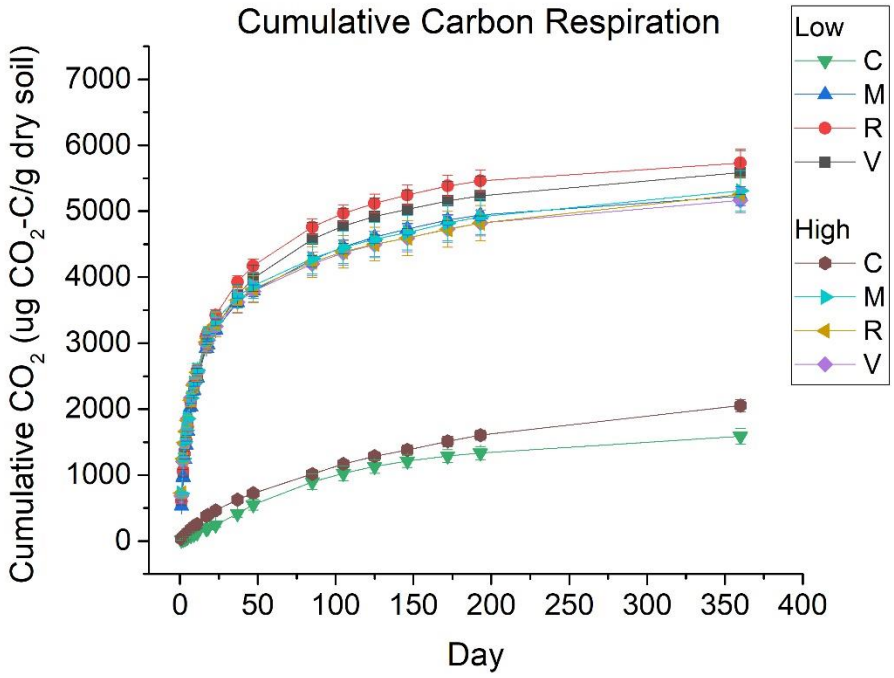



**Figure 2.** Cumulative $CO_2$ respiration for all treatments on the two farms. The y-axis is the cumulative $CO_2$ produced over the 360-day incubation per gram of dry soil. The x-axis is the day of the incubation. Low = lower fertility soil. High = higher fertility soil. C= control; M = rye-vetch mixture; R = rye monoculture; and V = vetch monoculture.

Regarding the cover crop litter treatments, cumulative $CO_2$ fluxes ranged from 5394 to 5720 µg $CO_2$-C/g dry soil. Overall, the $CO_2$ flux was much higher with the added plant litter than without, indicating a significant response of microbial activity to the fresh C input (Figure 2). In contrast to our hypothesis, the soil from the lower fertility farm with rye litter tended to produce the greatest amount of $CO_2$ during the incubation, while the higher fertility farm with rye-vetch mix produced the lowest $CO_2$ flux. However, differences in $CO_2$ production among the three cover crop residue treatments were not statistically significant. The lower fertility soil without litter had 700

µg $CO_2$-C/g lower $CO_2$ flux in the 360-day period compared to the higher fertility soil, but following cover crop litter addition, $CO_2$ flux from the two farms were not different. Even though treatments were not significantly different, Figure 2 also shows more separation among treatments for the lower fertility farm compared to the higher fertility farm, potentially reflecting the greater response to new litter inputs on the lower fertility farm.

The first three weeks of the experiment following litter addition were the optimal time for microbial enzyme production, plant residue decomposition, and $CO_2$ respiration. Therefore, most of the respiration differences between the farm and litter treatments were expected during this period. Figure 3 shows the cumulative $CO_2$ in the first 25 days of the experiment. The $CO_2$ respiration for the first 25 days ranged from 3220 to 3410 µg $CO_2$-C/g dry. After 25 days, the C respiration rate for all treatments slowed down. However, ANOVA results indicate there was no significant difference in cumulative $CO_2$ for the three treatments with cover crop residue addition, on either on day 25 or day 360.

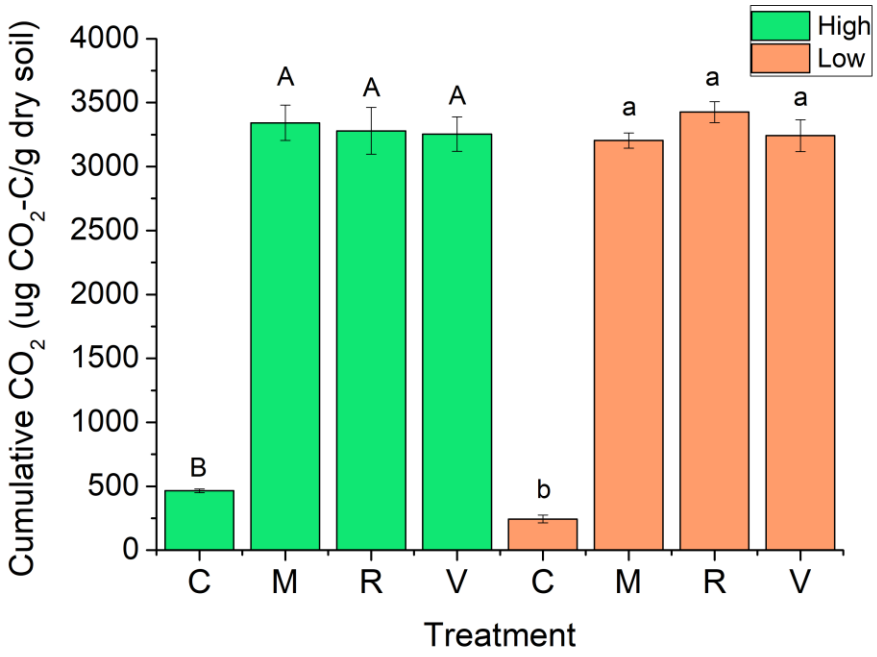
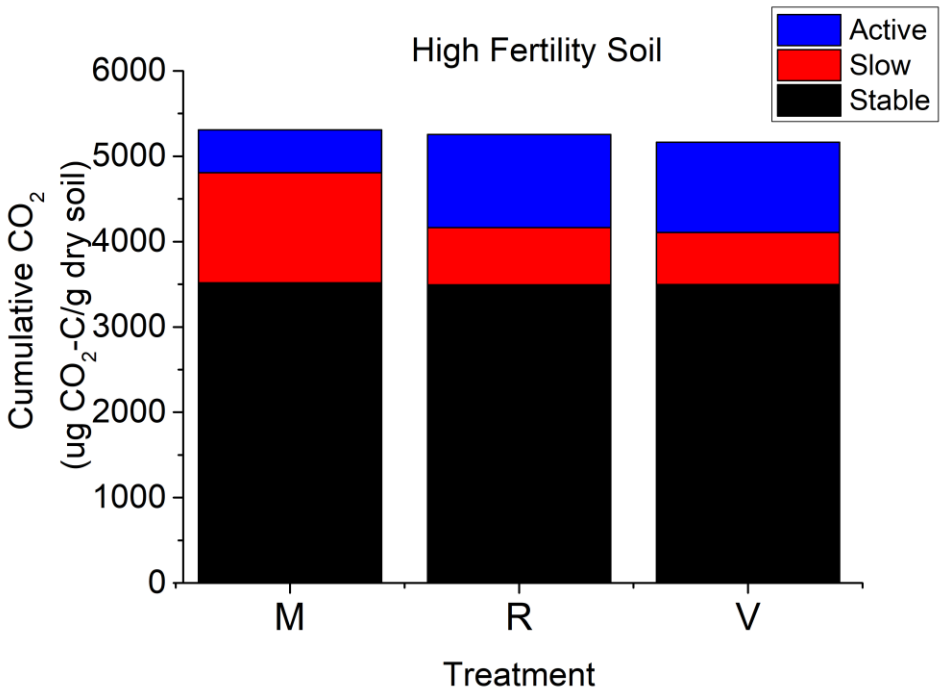



**Figure 3.** Cumulative $CO_2$ for four treatments and two farms on Day 25. Same letters indicate no significant differences between litter treatments at $P<0.05$.

The 3-pool, 6-parameter exponential decay equation identified the amount of $CO_2$ produced over the course of the incubation that was derived from each of the three C pools in the model (active, slow, and stable) for the six different soil-plant residue combinations. The $R^2$ values for all six farm-treatment combinations were 0.99. In the first 30 days, when the $CO_2$ flux was the fastest, more $CO_2$ came from microbes decomposing the active C pools, including the recently added litter. However, not all of the added litter C gets consumed, and some is stabilized in soil C pools that persist for a long time. Further, the $CO_2$ that is respired by microorganisms comes from all of these soil C pools, especially in later stages of the incubation.

Over the course of the incubation, most of the cumulative $CO_2$ came from the stable soil C pool, followed by the slow and active C pools, reflecting the different sizes of these C pools in soil. Although the cumulative $CO_2$ flux for farms and litter treatments were not significant, we found a significant difference in the $CO_2$ derived from slow C pools between the two farms ($p = 0.04$). For the higher fertility soil, only 1/10 to 1/5 of the $CO_2$ came from the slow pool, while for the lower fertility soil, about ½ of total $CO_2$ respiration come from the slow pool. Moreover, the litter mixture treatment in both soils had the lowest $CO_2$ respiration from the active pool compared to the soil with both litter residue monocultures.



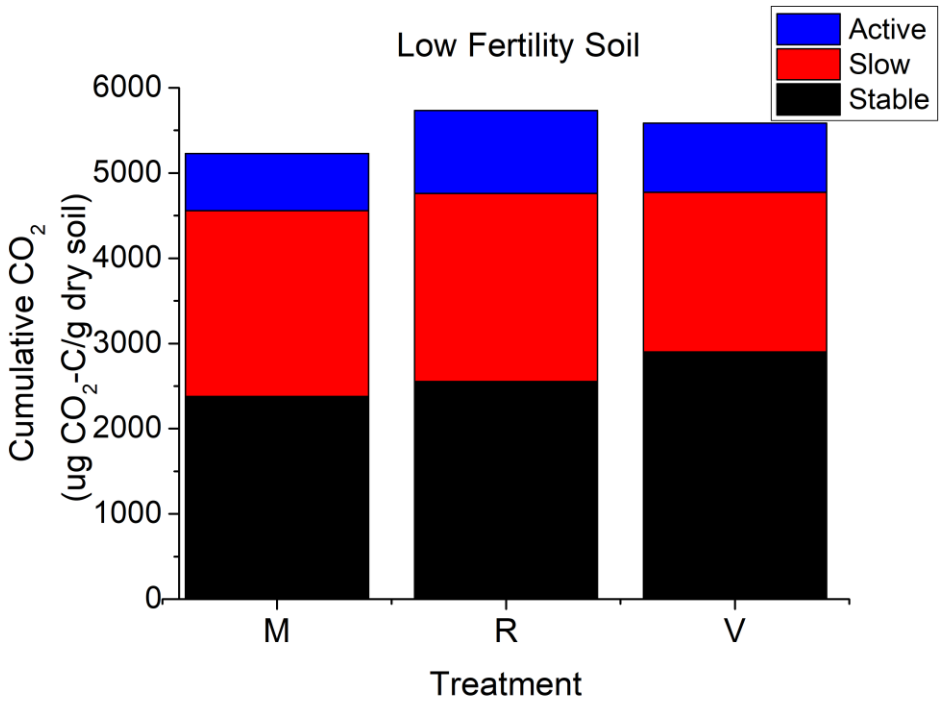



**Figure 4.** Three pool C respiration model for soils with litter addition.

*3.4 Microbial biomass*

Microbial biomass carbon (MBC) generally increased over the first 30 days of the incubation (Figure 5). In contrast to our expectations, the soils with rye monoculture on both farms had the highest MBC, followed by the mixture and the legume monoculture ($p < 0.05$). Also, the three plant litter treatments had 50% to 80% higher MBC than the no litter control groups, indicating the strong effect of cover crops on increasing the soil dissolved organic C pool in the short term.

We also analyzed the ratio of MBC to microbial biomass N (MBC: MBN) on days 0 and 30 of the incubation. At day 0, the lower fertility soil had a similar MBC: MBN ratio to the higher fertility soil (7.33 vs. 8.32),

indicating there was no significant difference ($p = 0.91$) in the initial microbial biomass C: N between the high and low fertility soils (Figure 6). The MBC: MBN ratio for soils from both farms slightly increased on day 30 following litter addition. In the control group where no litter residue was added, the C: N ratio on day 30 was still similar to day 0. On the lower fertility farm, the mixture litter treatment had a significantly lower C: N ratio compared to the two monoculture treatments at day 30. Similar to the results for C respiration, the lower fertility soil showed a greater response to the residue addition and had a more significant change in C: N ratio than the higher fertility soil did. For the soils with rye and vetch monoculture treatments, the difference in the C: N ratio between the high and low fertility soils was significant. The lower fertility soils with monoculture litter had a 31%-45% higher C: N ratio compared to the higher fertility soil, likely reflecting the lower soil N availability in the lower fertility soil. In the lower fertility soil, the litter monoculture addition barely changed the C: N ratio compared to control, while for higher fertility soils, the vetch monoculture tended to have a lower C: N ratio compared to the no litter control.

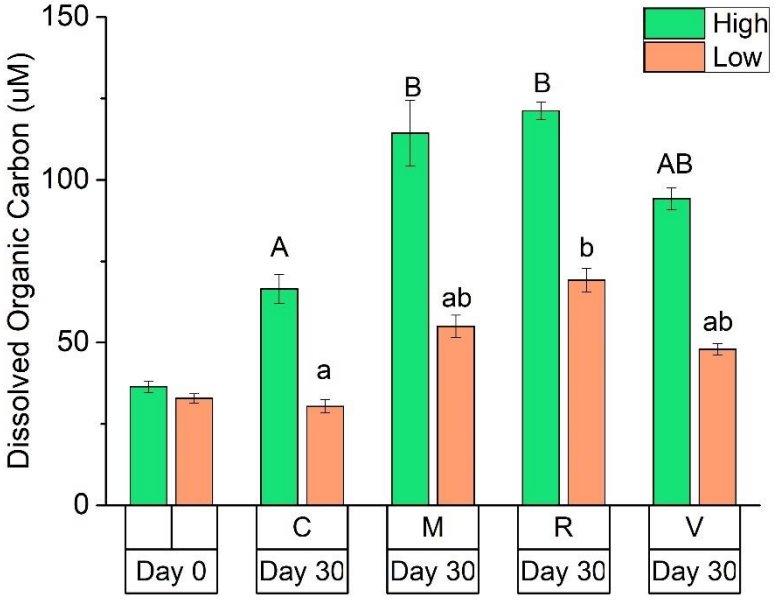



**Figure 5**. Microbial Biomass Carbon between farm and treatment on Day 0 and Day 30 Same letters indicate no significant differences between litter treatments at P<0.05.

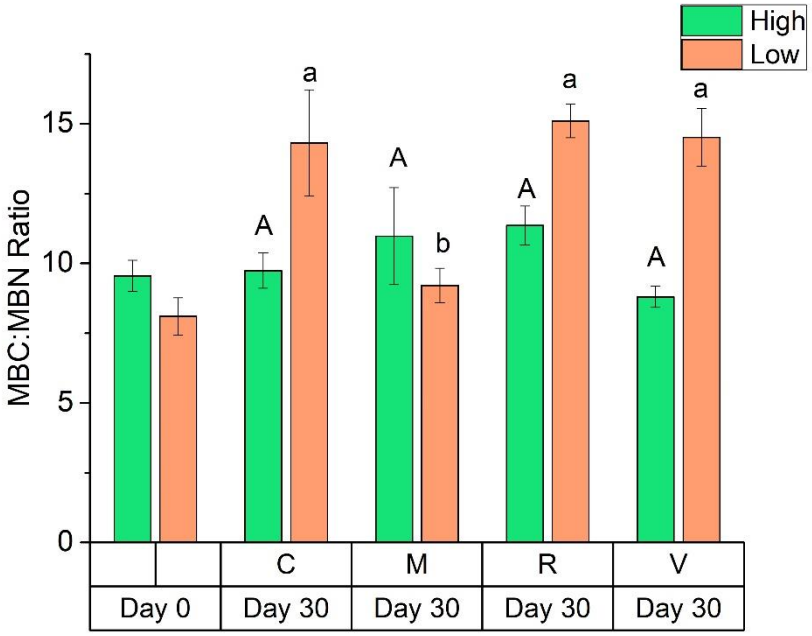



**Figure 6.** Microbial Biomass Carbon to Microbial Biomass Nitrogen ratio on Day 0 and Day 30. Same letters indicate no significant differences between litter treatments at P<0.05.

*3.5 Enzyme Dynamics*

For all three enzymes that degrade labile compounds (NAG, BG, PHOS), the higher fertility soil had significantly higher initial enzyme activities compared to the lower fertility soil (Figure 7). However, enzyme activities on the lower fertility farm caught up to those in the higher fertility soil by day 30. The difference between farms was significant on day 0, but not on day 30. Labile enzyme activities on the higher fertility soil increased less from baseline (day 0), and the plant litter treatments were not different from the control. However, the difference in labile enzyme activities between the three plant litter treatments was not significant through the whole incubation study, even though the activities on the lower fertility soil tended to have a greater response to plant litter addition compared to the higher fertility soil.

Conversely, for the enzyme that reflects the breakdown of recalcitrant C compounds (PHENOX), the lower fertility soil had a higher enzyme activity at day 0 (Figure 7). PHENOX activity was much lower at day 30 than at baseline for all treatments on the lower fertility farm. In contrast, PHENOX activity increased during the first 30 days on the higher fertility soil in all treatments including the control. On the lower fertility soil, there was slight suppression of PHENOX activity compared to the control, although it was not significant.

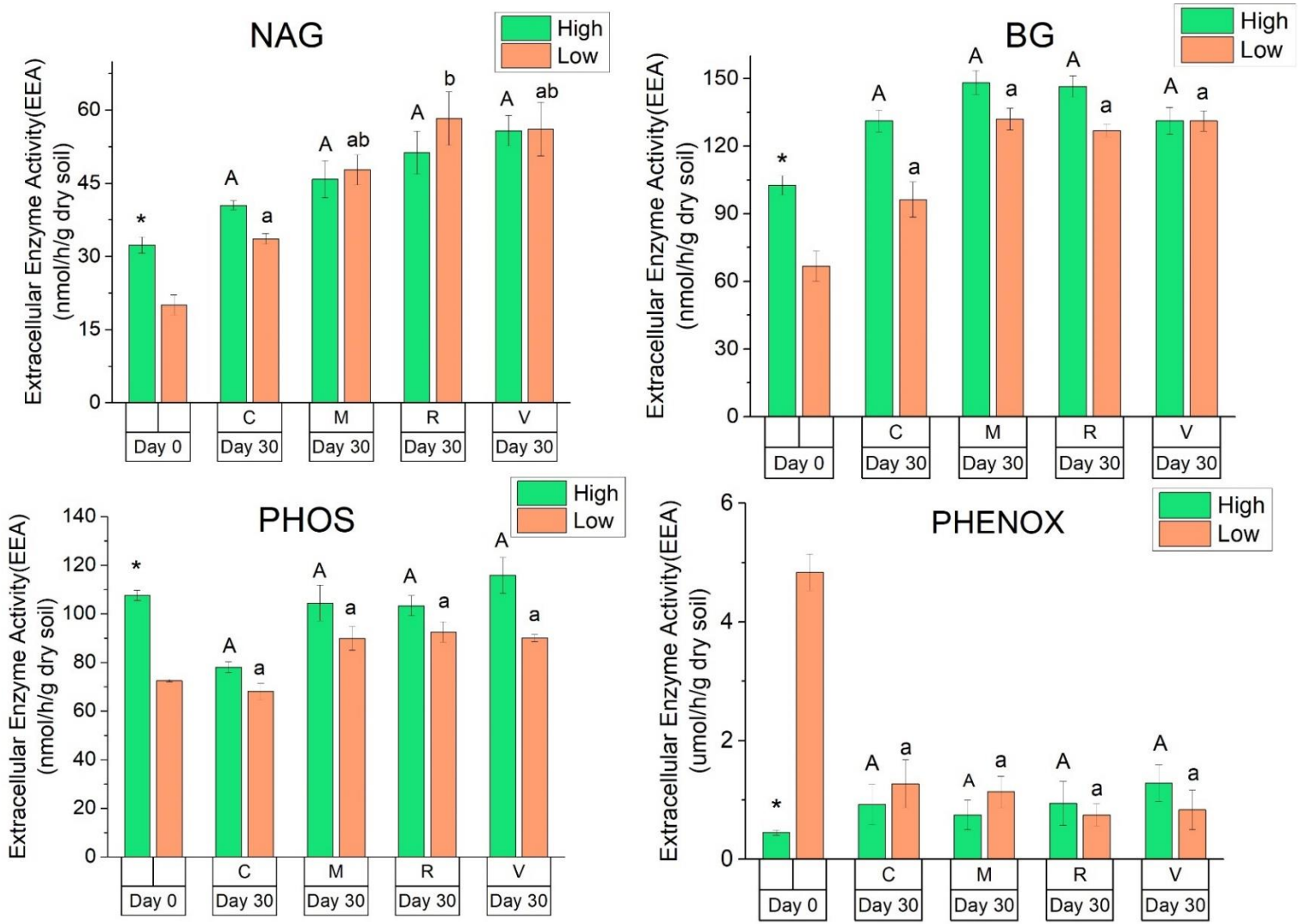



**Figure 7.** Extracellular enzyme activity (EEA) for day 0 and day 30 for all treatments on high and low fertility soils. Same letters indicate no significant differences between litter treatments, and an asterisk indicates significant difference among farms at $P<0.05$.

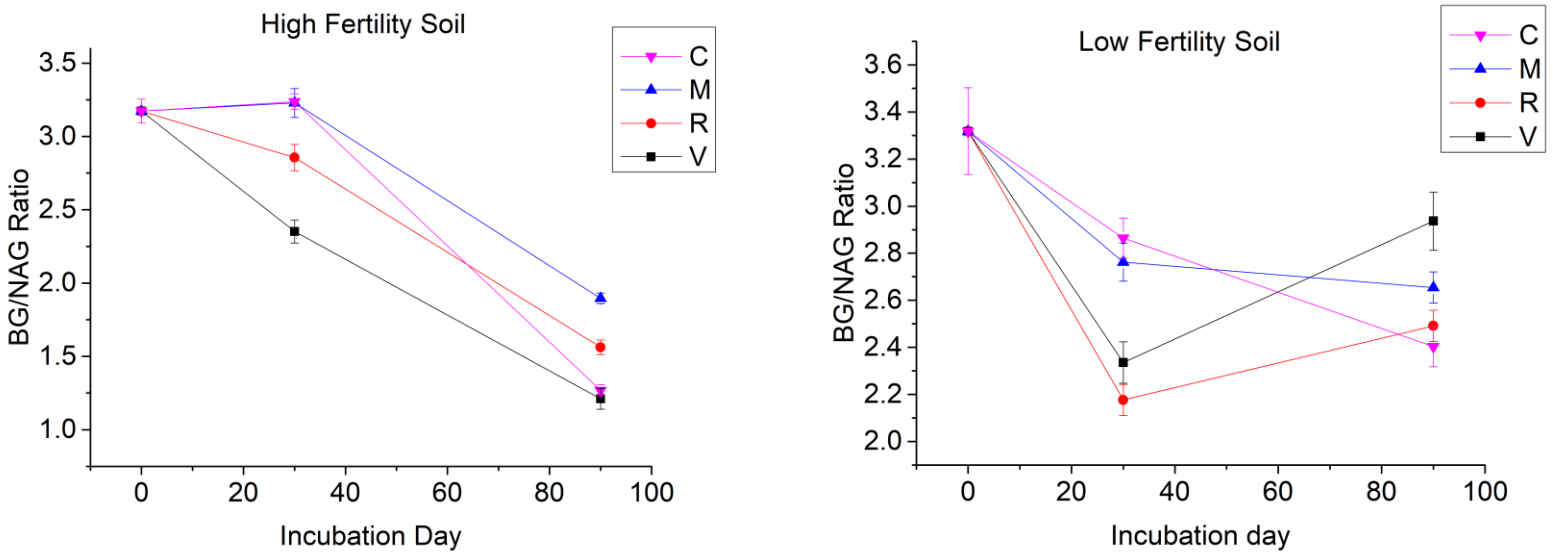



**Figure 8.** BG: NAG ratio (C acquiring enzyme vs. N acquiring enzyme) ratio during the 90-day incubation for all treatments on high and low fertility soils.

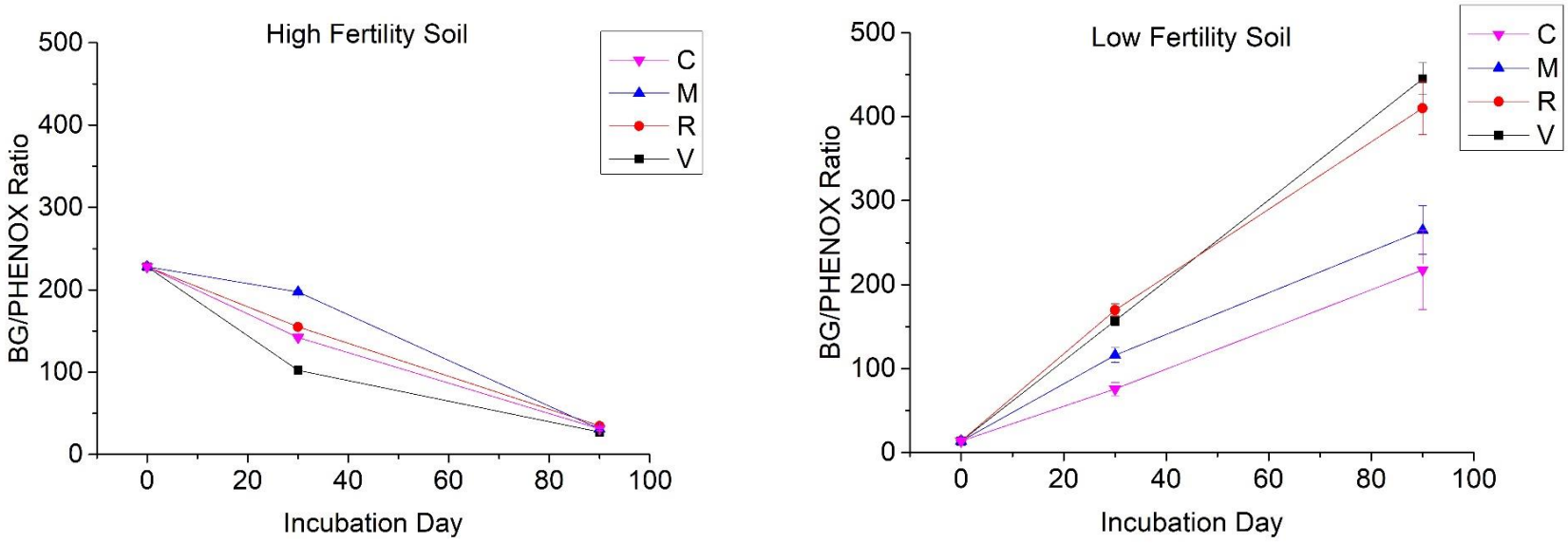



**Figure 9.** The ratio of cellulose- to lignin-degrading enzymes on high and low fertility soils for four treatments in a 90-day incubation.

We also considered ratios of enzyme activities over the course of 90 days, at which time we expected decomposition of the added litter material to be much lower than at the start of the incubation. Figure 8 shows the ratio of BG: NAG (i.e., C-acquiring to N acquiring enzyme ratio) and Figure 9 shows the ratio of BG: PHENOX (i.e., labile C to recalcitrant C degrading enzymes) separated by high and low fertility soil treatments.

For the higher fertility soil, the ratio of the C acquiring to N acquiring enzymes (BG: NAG) changed little between day 0 and day 30, similar to the MBC:MBN ratio. After day 30 the ratio dramatically decreased, so BG and NAG production became similar at day 90. The effect was weakest for the litter mixture and the rye. At day 30, the lower fertility soil responded more to litter addition with NAG production, potentially reflecting N limitation of the microbial community. The ratio for vetch and rye treatments increased again at day 90, which may reflect a shift back toward C limitation.

For BG: PHENOX the microbial community in the lower fertility soil produced relatively more BG over time, in response to greater access to more labile C following litter addition. At the baseline sampling, microorganisms in the low fertility soil were likely C-limited based on the high activity of PHENOX. In the high fertility soil, the BG: PHENOX ratio slowly declined over time indicating that microbes had to invest more energy in PHENOX production later in the incubation. Overall, there appeared to be a smaller response to the litter C addition in the higher fertility soil.

## 4. Discussion

Cover crop mixtures of legumes and grasses are promoted as part of an ecological nutrient management approach that can supply an organic N source to fields through biological N fixation, alongside greater N retention from soil N assimilation by the intercropped grass (Snapp et al. 2005; Finney and Kaye, 2017; Blesh 2018, White et al. 2017). However, the impacts of cover crop mixtures on decomposition dynamics and N availability likely vary with different soil fertility levels that result from distinct land management histories. While the effects of cover crop residue type (e.g., legume, non-legume) and soil properties on decomposition dynamics have been tested separately in previous studies, to our knowledge no studies have tested the interactive effect of these two factors on soil biological processes. In this study we aimed to test if: (1) legume-grass mixtures alter decomposition rates compared to legume monocultures; and (2) the impacts of cover crop residues on decomposition dynamics vary with contrasting levels of soil fertility.

Through a long-term lab incubation study, we found that the addition of any kind of cover crop litter to soil increased extractable inorganic N, soil C respiration, dissolved organic C, microbial biomass and the activities of three enzymes (BG, NAG, PHOS), compared to the no litter control. Similar to the previous studies comparing decomposition dynamics between conventional management and organic management, we found that the management history of a farm affects how microbial decomposition dynamics respond to new C inputs to soil, with cascading impacts on soil C and N

cycling processes. (Stark et al., 2008; Berthrong, Buckley & Drinkwater, 2013). Overall, we observed a stronger effect of cover crop residue addition on the lower fertility soil compared to the higher fertility soil for C respiration, microbial biomass C: N ratio, and microbial enzyme activity, which may have resulted from lower soil organic matter pools in the lower fertility soil that limited microbial activity (Sparling, 1992; Stark et al., 2008). In contrast, for extractable inorganic N and dissolved organic C, the higher fertility soil had a larger response due to higher initial soil organic C and N pools.

Rye and vetch litter residues typically have contrasting biochemical properties and were therefore expected to impact soil decomposition dynamics differently. However, the litter chemistry (i.e., C: N ratio) of the two species was similar due to the early termination date of the cover crop in the companion field experiment (i.e., when rye was still in a vegetative growth stage with low C: N ratio). Therefore, we predict that this may explain why we did not find significant differences in decomposition dynamics for the three plant litter treatment groups. Overall, understanding how different cover crop types impact microbial activity and N mineralization rates across different soil conditions is critical information for optimizing management of organic N sources for crop productivity and environmental sustainability in different contexts.

### *4.1 Carbon Respiration.*

Soil respiration is a key process of the global C cycle that releases $CO_2$ to the atmosphere. At the scale of agroecosystems, respiration and is an indicator of soil health and fertility status (Schiesinger, 2000). Carbon dioxide respiration in soil is positively correlated with intensity of microbial activity, which, in agroecosystems, is strongly affected by management practices like tillage (Alvarez et al., 1995; Fernandez et al., 2010) and the use of cover crops (Hendrix, Han & Groffman, 1988; Steenwerth & Belina, 2008). From the perspective of soil chemistry, soil nutrients and water also play a vital role in the respiration of microorganisms (Bowles et al., 2012; Davidson et al., 1998; Nordgren et al., 1988).

Comparing the two soils without litter addition, we observed a higher $CO_2$ respiration rate for higher fertility soil, indicating a strong effect of the size and quality of soil organic matter pools on decomposition (Fog et al., 1988). Although length of time under organic management had a positive effect on soil decomposition dynamics, it was less than the effect of litter residue treatment (Figure 3) (McDaniel et al., 2014). We observed a greater response to the fresh C input in the lower fertility soil where microbes were likely more C limited throughout the experiment. These findings suggest that farmers with lower fertility soils could realize short-term benefits to soil biological processes following cover crop adoption, even though increasing the size of the total soil organic matter pool can take 5-10 years or longer (Drinkwater et al., 1998; Robertson et al., 2014).

Although the total soil $CO_2$ respiration was not significantly different between farms and treatments with litter addition, we noticed that the proportion of $CO_2$ from different pools of organic matter was different. Our experimental results are consistent with previous studies: only a small proportion of $CO_2$ flux originated from the active and slow C pools following fresh C addition, while the vast majority of $CO_2$ came from the large, stable C pool (Collins et al., 2010; Parton et al., 1994). The higher fertility soil had about 67% of cumulative $CO_2$ flux from the stable pool because it had a much larger stock of soil organic C to begin with, whereas the lower fertility soil had 49% of cumulative $CO_2$ flux from the active and slow pools, indicating a larger response to litter addition and a larger contribution of new litter C to microbial decomposition.

### *4.2 Inorganic N*

Inorganic N (e.g., $NO_3^-$, $NH_4^+$) is an essential nutrient for both plants and microorganisms, and soil N availability therefore affects both microbial biomass and enzyme activity (Ocio et al., 1991; Waldrop et al., 2004). Compared to sole-planted legume cover crops, mixtures of legumes and grasses have the potential to better balance the timing of N release through mineralization with the N demand of the following crop, reducing N losses (Kuo & Sainju, 1998; Moller, Stinner & Leithold, 2008; Snapp et al.,2005).

In terms of N mineralization, we observed a large incubation effect on the high fertility farm, reflecting the larger pools of potentially mineralizable

N in the higher fertility soil (Table  (Andraski et al., 2000; Dinnes et al., 2002).  Previous studies have shown that vetch monoculture cover crops have the greatest impacts on short-term soil N availability, while vetch-rye mixtures, and rye monocultures, have only 80% and 50% of the effects of vetch, respectively (Poffenbarger et al., 2015). In our study, there was only a small effect of residues on soil inorganic N, which might be due to the similar chemical properties of litter residues. We found almost no N mineralization on the lower fertility farm, suggesting that the microbial community was N limited in the lower N soil. At day 30, soil inorganic N levels had only slightly increased from day 0, and none of the treatments were different from the control. The N added in the plant litters could be immobilized in the microbial biomass, however at day 30 the MBC: MBN ratio was higher in the lower fertility soil than in the higher fertility soil. It is possible that the N added in the cover crop residues had already cycled through microbial biomass and was either stored in more stable N pools or was lost via denitrification. Thus, in this experiment, background soil fertility was the greatest driver of N mineralization rates, and cover crop residues had little effect.

### *4.3 Microbial Biomass Carbon and Carbon/Nitrogen Ratio*

Microbial biomass is responsive to litter addition, which provides a high quality C source for microbial decomposition and associated soil nutrient cycling processes (Moore et al., 2010; Wardle 1993; Xu et al.,2013). In this

incubation study, the significant difference between microbial biomass C with litter treatments, and the no litter control, reflects the increase in the microbial biomass pool in response to the addition of an external energy source (Vance et al., 1987). Although there was no significant difference between the litter residue treatments, the soil with rye monoculture residue did contain the highest amount of dissolved organic carbon, followed by the rye-vetch mixture and the vetch monoculture. This is the expected pattern based on the C: N ratio of the added cover crop residues.

For the microbial biomass C: N ratio, the lower fertility farm appeared to be more N limited (in terms of MBC: MBN) compared to the higher fertility farm, which was supported by the soil inorganic N data (Ajwa et al., 1999). On the higher fertility farm, vetch residue led to a lower MBC: MBN, which was the hypothesized result, because vetch has higher N content and lower C content than rye monoculture or rye-vetch mixture. It is also possible that there was a time lag for the added litter C to impact microbial decomposition dynamics on the higher fertility farm (relative to the incubation effect, given the large background level of soil organic C).

### *4.4 Enzyme Dynamics*

Enzymes are closely related to soil decomposition dynamics, which can more accurately reflect microbial C and nutrient demand (Bandick & Dick, 1999; Bowles et al., 2012, McDaniel et al., 2014). The initial activities between the two farms were as expected; the higher fertility soil had higher C

and N pools, so the microbial community can invest in 'less expensive' enzymes that degrade labile compounds (Bandick et al., 1999; Dick, 1994). In contrast, the lower fertility farm had lower total organic matter levels, along with lower levels of high-quality organic matter pools such as particulate organic matter and lower levels of plant available phosphorus (Table 1). Therefore, the lower fertility soil had greater PHENOX enzyme activity to decompose more chemically recalcitrant organic carbon forms such as lignin (Burke et al., 2011). However, as the experiment progressed, there was no difference between enzyme activities for treatments and farms on day 30. For instance, the BG dynamics corresponded with the $CO_2$ respiration results, showing a greater response to litter addition on the lower fertility soil.

The BG:NAG ratio suggests that the higher fertility farm became more N limited by day 90, with the mixture having the lowest N availability. For the lower fertility soil, the BG: NAG ratio also confirms that at day 30 the lower fertility soil had a stronger response to litter addition with NAG production (i.e., BG and NAG activities became more similar), suggesting the microbial community was more N limited in that soil and invested more in NAG production (Stark et al., 2008). At day 30, the BG:NAG ratio for the mixture on the lower fertility soil changed less than the two monoculture treatments, which corresponds to the microbial biomass C: N analysis.

For BG: PHENOX, the lower fertility soil had a greater change in enzyme ratio over time, suggesting a stronger response to the new C input. The community produced relatively more BG over time as they had access to

more labile C. At the baseline measurement (day 0) the microbial community was more C-limited, as shown by their greater investment in PHENOX compared to the community in the higher fertility soil. In the higher fertility soil the ratio of BG: PHENOX slowly declined over time as the higher quality C was decomposed and microbes had to invest more in the production of PHENOX over time to access C from more stable pools. These results for enzyme activities correspond with findings from the 3-pool model, where more C came from the stable pool on the higher fertility soil.

## 5. Conclusion

Results from our experiment show that new C inputs to soil from any cover crop residues (monoculture or mixture) increase soil microbial processes in the short term (e.g., 30 days). However, over one year, in the high fertility soil, added cover crop residues had little impact on decomposition dynamics. On the lower fertility soil, added cover crop residues stimulated decomposition, releasing more $CO_2$ for two of the treatments compared to the higher fertility soil. The mixture on the lower fertility soil had the lowest respiration rate, perhaps indicating that more of the added C was retained in the soil, which could contribute to organic matter stabilization over time. For all enzyme activities except for PHENOX, the lower fertility soil responded more strongly to new litter addition than the higher fertility soil. Overall, N release was higher on the high fertility soil, showing the importance of management history to internal nutrient cycling processes, particularly the

benefits of regular cover crop use over time. Future studies should test cover crop residues with greater contrasts in C: N ratio to adequately address the question about litter residue type. Taken together, our results highlight the need to understand how organic nutrient sources impact decomposition dynamics, and associated ecological outcomes, in different soil conditions, in order to strategically target incentives for conservation practices to where they will be most effective.

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