THESIS

COVER CROP EFFECTS ON THE SOIL MICROBIOME AND MICROBIALLY MEDIATED SOIL FUNCTIONS

Submitted by

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ABSTRACT

COVER CROP EFFECTS ON THE SOIL MICROBIOME AND MICROBIALLY MEDIATED SOIL FUNCTIONS

Cover crops are often grown to improve soil health through changes in soil physical, chemical, and biological properties. Currently, there is growing interest in identifying how soil microbes may mediate some of the benefits provided by cover crops. However, most studies analyzing cover crop effects on the soil microbiome and soil health have been conducted in controlled settings such as greenhouses for short-term effects and field studies have focused on accumulated, longer-term effects. We conducted a short-term field experiment in northern Colorado with four different cover crop species: cereal rye (*Secale cereale*), hairy vetch (*Vicia villosa*) rape seed (*Brassica napus*), and sorghum *(Sorghum bicolor*) planted in August 2022 as monocultures and a no cover crop control to research these effects within a field environment at the time of cover crop termination in May 2023 and into the subsequent maize cash crop.

During the respective seasons, cover crop and maize shoot biomass and bulk and rhizosphere soil were sampled. Soil samples were analyzed for soil extracellular enzymes L-leucine aminopeptidase (LAP), β-1,4-N-acetyl-glucosaminidase (NAG), β-glucosidase (BG), and acid phosphatase (PHOS); dissolved organic carbon concentrations (DOC); inorganic nitrogen concentrations; and soil aggregate stability. Only rhizosphere soil was used for soil microbiome analyses. Of all the cover crops, cereal rye and hairy vetch

accumulated the most shoot biomass but did not differ from each other, and maize shoot biomass did not differ across treatments. Prior to cover crop termination in the spring, only rape seed stimulated LAP enzyme production compared to the control. Rape seed and cereal rye treatments had greater DOC concentrations than sorghum, and bulk soil DOC concentrations were greater than the rhizosphere. Inorganic nitrogen concentrations were lowest in cover crops that accumulated the most shoot biomass, i.e. cereal rye and hairy vetch. Soil aggregate sizes increased under living cereal rye relative to the control. Cover crops had several legacy effects that persisted into the maize crop, but these were not consistent with the effects found prior to termination: cereal rye stimulated BG and PHOS activity compared to sorghum. BG enzyme activity and DOC concentrations were greater in bulk soil compared to rhizosphere under the maize crop. Despite finding differences in cover crop effects on soil properties, we found no differences in microbial diversity indices or structure. However, under living cereal rye, one organism classified as *genus Massilia* was found to be enriched compared to the control but did not correlate with any measured soil functions. We were able to match our 16S BLAST results with a database to match with 15 MAGs at >97%.

Our research confirms findings from studies performed in controlled settings that within a single season cover crops can modify the soil microbiome at an organismal level and influence soil functions, and our results suggest that frameworks built to describe soil microbiome and soil health dynamics are also applicable to the field. We also show that 16S taxonomic results may soon be useful in proposing potential microbial function, given that such MAG databases continue to be improved. Overall, this research contributes to

the linking the soil microbiome with cover cropping and soil health, with further implications likely being microbiome management for sustainable agriculture.

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INTRODUCTION

In the movement to improve sustainable agriculture, cover crops have emerged as a management practice that can benefit farming by providing ecosystem services (Schipanski et al. 2014). Cover crops are plants grown between cash crops with the purpose of improving the physical, chemical and/or biological properties of soil (Fageria et al. 2007). These crops are either tilled and incorporated into the soil as green manure or left on the soil surface as mulch (Benedict et al. 2014). Several examples of the benefits of adopting cover crops, which belong to various, unique plant species, range from suppressing weeds through allelopathy and surface cover (Wortman et al. 2014; Afzal et al. 2023), covering the soil surface to prevent wind and rain erosion (Sharma et al. 2018), improving soil structure and water infiltration (Blanco-Canqui et al 2015), and improving nutrient conservation and cycling (O'Reilly et al. 2012; Echer et al. 2023).

Many of these benefits overlap with or are mediated by soil microbes. For instance, soil microbes are known to stabilize soil structure through aggregates (Oades and Watters 1991), decompose organic material and enhance biogeochemical cycling (Krishna and Mohan 2017; Bhattacharyya and Jha 2012), and assist in mitigating certain plant pathogens through competition and suppression (Schlatter et al. 2017). Soil microbial populations and activity tend to be particularly concentrated in the area of soil immediately surrounding the surface of crop roots, termed the rhizosphere. The rhizosphere can harbour a unique community of microbes residing a few millimetres away from the roots (White et al. 2017).

Given the potential to leverage the rhizosphere microbiome for improved ecosystem services in an agricultural setting, an area of productive research currently underway focuses on elucidating the influence of cover cropping on the rhizosphere microbiome. Cover crops, and plants, for that matter, are known to influence soil microbial dynamics through two main mechanisms: crop residue decomposition and root exudates (Spedding et al. 2004). There is a plethora of studies exploring the effect of identity(ies) of decomposing cover crop residues, whether as monocultures or mixtures, on rhizosphere microbiome composition (García-Gonzalez et al. 2023; Nevins et al. 2018; Rutan et al. 2021). Meanwhile, root exudates are a wide array of high- and low-molecular weight organic compounds that include amino acids, organic acids, sugars, and secondary metabolites that can act as a carbon source for rhizosphere microbes (Preece and Peñuelas 2019). In cover crop studies, exudates have been shown to modify microbiome community structure at the species and even crop variety level (Buyer et al. 2010; Seitz et al. 2022; Seitz et al. 2023). Root exudates shape the soil microbial community under a living cover crop while litter quantity and quality influence cover crop decomposition-related impacts on the soil microbiome. Studies researching the impact of living cover crops on the rhizosphere microbiome are less common (Finney et al. 2017; Leite et al. 2021; Lehman et al. 2012).

Seitz et al. (2024, *preprint*) conducted a comprehensive study to test the concept of cover crops modifying the rhizosphere microbiome membership and function through root exudates. There, they found that cover crops of various species shaped the rhizosphere microbiome and function differently through different exudate profiles. However, the

researchers acknowledge that there are limitations in scaling these cover crop exudate findings from the laboratory to the field. For instance, controlled experiments often analyze crop effects on the microbiome from one individual plant, whereas field studies often sample a section of a plot with several plants. The presence of several plants can lead to differences in root growth and interactions (Hess and De Kroon, 2007), and discerning a plant effect on the soil microbiome from the bulk microbiome can be difficult due to the challenge of sampling the rhizosphere from the field. Likewise, research has shown that an increase in plant growing area even between pot sizes had significant effects on plant biomass production (Poorter et al. 2012), not to mention the implication of this area change moving from the pot to the field scale. Other research has shown that the circadian rhythm of plants could alter rhizosphere structure and function (Hubbard et al., 2018). These additional factors could very likely confound the analysis of cover crops' effect on shaping the rhizosphere microbiome.

On the other hand, it is also important to consider the duration of the field study and management practices when studying cover crop microbiome dynamics. Many long-term studies have been conducted with sometimes conflicting results on how cover crops influence microbial function. Brennan and Acosta-Martinez (2017) conducted a 6-year study comparing annual and occasional cover cropping and observed, surprisingly, that there was no difference in soil enzyme response to cover crop quality (C:N ratio), N content and shoot biomass input. Similarly, Rankoth et al (2019) conducted a 2-year farm study and did not observe differences in microbially produced betaglucosidase (BG) enzyme activity with a cover crop mixture treatment under no tillage. Hai-Ming et al (2014),

however, found that the same BG enzyme activity could increase with cover crop residue addition in a rice system with 7 years of different cover crop rotations. These varying results can be attributed to field experiments entailing more variability from factors such as climate and management practices at a longer temporal scale and soil ecology.

Furthermore, measuring and defining changes in rhizosphere microbiome membership and function within soil environments requires careful consideration of the right analytical methods. A prevalent tool is 16S rRNA sequencing and characterization of rhizosphere communities to identify microbial membership and abundancy changes. This common approach sequences both conserved and variable regions of the ubiquitous, prokaryotic 16S rRNA gene to determine the taxonomic diversity and structure of microbial communities (Garibay-Valdez et al. 2019). More recently, potential microbial gene activity can now also be assessed under cover crop treatment through metagenomics, offering valuable insights into potential microbial metabolic and nutrient cycling pathways (Zheng et al. 2019; Wang et al. 2022). However, despite the availability of techniques to describe soil microbiome composition and potential function, there are still challenges in applying ‘omics along with 16S rRNA sequencing. In processing biomolecules for any ‘omics analysis, obstacles from inefficient cell lysis, molecule extraction, PCR bias, and bioinformatic data wrangling may complicate result interpretation (Biswas and Sarkar 2018).

When analyzing cover crop effects on the rhizosphere microbes, there is a need to continue advancing research that connects soil microbiome membership with microbial functions, as well as links these results from the controlled, laboratory setting to the less

controlled field environment. Additionally, as mentioned previously, studies detailing *living* cover crop-soil rhizosphere microbial dynamics through root exudation and the persistence of these effects into the following cash crop after cover crop termination are relatively rare. Many studies that do investigate cover crop-soil microbiome field dynamics are often longer-term studies which include other management practices, meaning isolating cover crop effects is challenging. These knowledge gaps motivated this study, where we researched the effects of four living cover crop species (*Secale cereale*, *Vicia villosa, Brassica napus, Sorghum bicolor,* hereafter referred to as cereal rye, AU hairy vetch, rape seed, and sorghum) on the rhizosphere soil microbiome community composition along with select soil functions (soil extracellular enzyme activity, dissolved organic carbon concentrations, extractable inorganic nitrogen, and soil aggregate stability) in a short-term field trial. We established a field experiment with these cover crop species and sampled rhizosphere and bulk soil at two time points: Spring 2023 under living cover crop stands; and Summer 2023 under the following maize cash crop. We expected that sampling at these two time points would allow us to capture cover crop rhizosphere effects and potential legacy effects during the summer cash crop from both rhizosphere effects and cover crop residue decomposition. Given that each cover crop belonged to a different functional group featuring different traits, such as AU hairy vetch being a significant soil nitrogen supplier (Brust 2019), we hypothesized that each cover crop species would shape the rhizosphere microbiome community and affect the soil functions uniquely.

MATERIALS AND METHODS

Experimental design and field management

The experiment took place at Colorado State University's Agricultural Research, Development and Education Center (ARDEC) facility near Fort Collins, Colorado (N 40°39'18.086", W 104°59'53.971", altitude 1555m). The field site receives an annual precipitation of around 408mm and is considered a semi-arid environment with a mean annual temperature of 10.2C (1981-2010 average, US Climate Data, 2024). The soil is categorized as an Aridic Haplustalf (USDA, NRCS 2019).

A complete randomized block design trial was established with five replicate blocks of four cover crop treatments (rape seed, cereal rye, hairy vetch, sorghum) and one control treatment (no cover crop) (see Appendix A). Each plot measured 10x 5.5m. Composite soil samples (0-10cm) were collected from each block in September 2022 and analyzed for baseline extractable nutrients and texture by hydrometer (Ward Laboratories, NE). The field had a consistent sandy clay loam texture classification (46-50% Sand, 18-22% Silt, 30-34% Clay), average pH of 7.8, soil organic matter concentration of 24 g/kg, soil nitrate of 5.8 mg/kg, and Mehlich 3 extractable phosphorus of 27 mg/kg.

The experiment was initiated in 2022 after the harvest of the prior barley crop. Disc tillage was performed to create a level seed bed for cover crop planting. The cover crops were planted on August 29, 2022 and were terminated on May 22, 2023, using Cornerstone 5 glyphosate. Cover crops were seed at approximately the following rates: 9.0 kg/ha sorghum, 28 kg/ha hairy vetch, 90 kg/ha cereal rye, 5.6 kg/ha rape seed. The maize (*Zea*

mays var. *indentata)*, Producers Hybrids 5218 SSTX variety, was planted at 79,074 seeds/ha on May 23,2023. There were two timepoints when soil and crop shoot biomass were collected: Spring 2023 (sampling May 8 and 10, 2023) during living cover crop growth and Summer 2023 (sampling July 24 and 26, 2023) at approximately V10 growth stage of the maize cash crop, after cover crop termination. On October 24, 2023, corn grain yield samples were collected. Importantly, the sorghum produced biomass in the fall after planting and then winter-killed. Thus, the cover crop in the sorghum plots was not living during the spring sampling. Figure 1 details the field trial

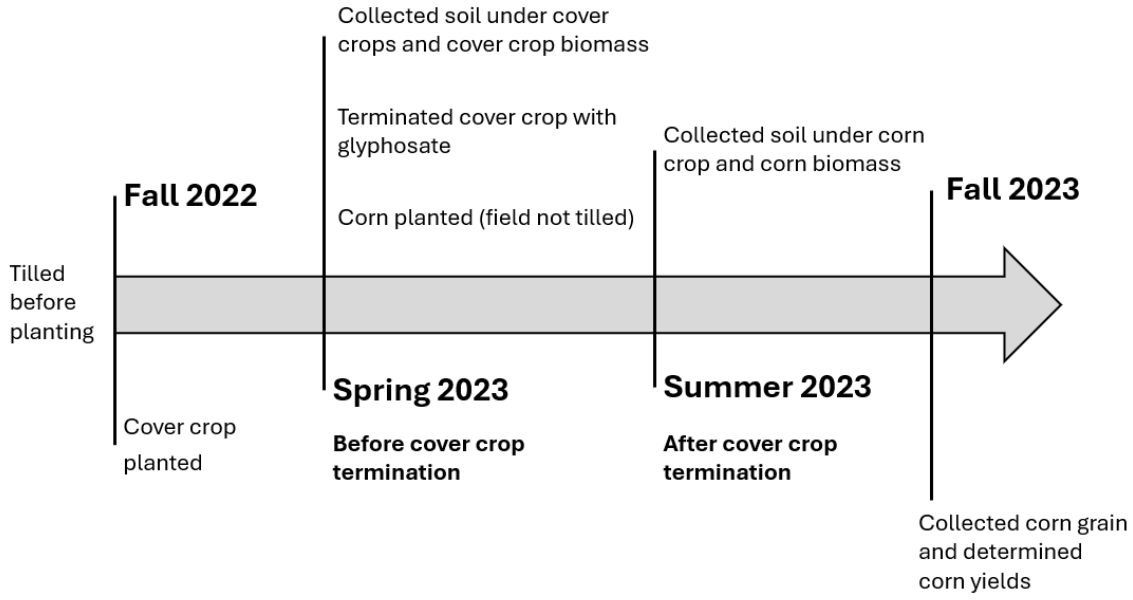

Figure 1. Timeline of the field experiment establishment and sampling time points. Before and after cover cropping are the two time points where we compare cover crop effects on the measured soil functions and the soil microbiome structure. We expected that sampling before cover crop termination would allow us to detect living cover crop effects on soil functions and soil microbes, which at this stage could potentially be mediated by root exudates. We also expected that sampling after cover crop termination under the corn would allow us to determine possible legacy effects of cover cropping that persist into the cash crop.

timeline starting from experiment establishment through the two sampling periods and corn grain collection.

Management inputs added to the plots included irrigation, herbicide and nitrogen and phosphorus fertilizers. Cover crops were terminated in May 2023 with Cornerstone 5 glyphosate applied at a 2.5 L/ha rate (including the control plot), and crop residue remained on the field without being incorporated into the soil. Glyphosate addition was chosen over tillage to minimize soil disturbance that likely could confound the analysis of cover crop effects on soil structure and soil microbial communities. Although the addition of glyphosate and its potential effect on the soil microbiome needed to be considered, the concentration of herbicide used in this field study was considerably smaller than a common field application rate of 10 mg/kg, where concentrations lower than this value seem to not have a significant effect on soil microbial biomass (Nguyen et al. 2016). Fall 2022 soil test results were used to guide spring 2023 maize fertilizer application rates based on area recommendations. We intentionally reduced nitrogen fertilizer below the recommended rate of about 200 kg/ha to 135kg N/ha such that any legume cover crop benefits would not be fully masked by the N fertilizer application. Nitrogen fertilizer was applied as urea ammonium nitrate (32-0-0) and phosphorus was applied as liquid ammonium polyphosphate (10-34-0) at a rate of 22kg P/ha within a week after planting. The field was sprinkle irrigated approximately 25mm once a week from May 2023 to September 2023 during the maize crop.

Sampling crop shoot and root biomass

We collected cover crop shoot and root biomass in Spring 2023 within one week of cover crop termination by clipping all vegetation at the soil surface within two 50cm x 50cm quadrats randomly placed within each plot. Relative root biomass was collected to 10cm deep by digging a 20x20x10cm cube in the center of each shoot biomass sampling quadrat with a shovel. The soil cube was placed in a labelled plastic bag and kept cool until root washing. Root samples were soaked in water and then passed through two sieves (4.0mm and 2.00mm), and the roots were collected with tweezers. Washed cover crop root biomass and shoot biomass were dried in an oven at 60C for at least 48 hours and weighed.

Maize shoot and root biomass were collected in Summer 2023 at V10 vegetative growth stage. The aboveground biomass was collected from a 1meter row in a 20cmx100cm rectangular section at 2 random locations within each plot. The number of plants were recorded for each sample and the biomass was dried at 60C and for at least a week then weighed. Maize root biomass was collected like Spring 2023 by carefully digging up a pair of corn plants from the center of the rectangular section and placing it in a labelled plastic bag. Maize root samples were processed using the same methods as in Spring 2023.

Sampling and partitioning soil

In order to analyze the direct effects of different cover crop species on soil functions and microbiomes, bulk and rhizosphere soil was collected in Spring 2023 and Summer 2023 at the same time as biomass samples were collected. The soil metrics analyzed for

bulk and rhizosphere levels included soil extracellular enzymes and dissolved organic carbon (DOC). Extractable soil nitrate and ammonium and soil aggregates were only sampled from bulk soil. Soil from which microbial DNA would be extracted for 16S rRNA analyses was collected only from the rhizosphere.

In Spring 2023, soil collection followed the same quadrat sampling procedure as with cover crop shoot sampling, but a separate, 20x20x10cm soil cube was dug up adjacent to the root sampling area. The soil cube was held over a clean bucket, and any soil that fell from this cube into the bucket was determined as bulk soil, homogenized, and 200g of this sample was placed in labelled plastic bag. The remaining soil in the cube that clung to the cover crop roots and less than about 2-3mm from the root was considered rhizosphere. For rhizosphere sampling, the sampler took care to wear clean gloves and disinfect with ethanol each time when moving to a new sampling area. Rhizosphere soil was gently pulled from roots and partitioned into subsamples in the field: 10g for DNA extractions, 10g for DOC and soil enzyme analyses. Upon collecting rhizosphere and bulk soil in one sampling area, buckets and gloves were rinsed with ethanol before moving to another. Soils were immediately placed in coolers with ice and kept refrigerated until processing.

In Summer 2023, soil collection followed the same rectangular section sampling procedure as with maize cash crop shoot sampling. Two maize plants adjacent to the plants collected for root biomass were carefully dug up for bulk and rhizosphere collection. The same procedure for partitioning bulk and rhizosphere soil in Spring 2023 was performed in Summer 2023.

Aggregate soil samples were collected separately. In Spring 2023 and again in Summer 2023, in an adjacent sampling area, a 10x10x10cm soil cube was dug up and gently placed into a brown paper bag, taking care to ensure the soil cube remained intact and no aggregates were crushed in the process. Aggregate samples were air-dried prior to processing.

Soil analyses

Soil samples were processed to analyze soil functions of interest and soil microbial community membership. One of these functions was soil extracellular enzyme activity. For both bulk and rhizosphere soil subsamples, soil enzyme activity was analyzed according to the protocol outlined by Saiya-Cork et al (2002). The enzymes were selected based on representative functions: degradation of protein (LAP, L-leucine aminopeptidase), chitin (NAG, β-1,4-N-acetyl-glucosaminidase), cellulose (BG, β-glucosidase), and mineralization of phosphorus (PHOS, acid phosphatase). For each sample, approximately 1 gram of soil was mixed with 60 mL of 50mM tris buffer (pH 8.1) and homogenized. Then, 200uL of homogenized soil slurry was pipetted into black, 96-well microplates; afterwards, 50uL of 200uM of enzyme substrate was added. These soil plates were then left to incubate at ~25C for 4 hours until fluorescence developed. A microplate reader (Cytation 5, BioTek, Vermont, USA) set to 365nm excitation and 450nm emission wavelength was used to read plates and approximate enzyme activity.

Another metric analyzed at the bulk and rhizosphere levels in both seasons was dissolved organic carbon (DOC), as a coarse proxy for net root exudation and microbially-available soil C. Approximately 8g of fresh soil was shaken in 45mL deionized water for an

hour, centrifuged at 3400 rpm for 15 minutes, filtered to 0.2um , and stored in -20C freezers until analysis. Quantification of DOC was completed by using a TOC-V-TN analyzer (Shimadzu Corp., Kyoto, Japan).

To determine inorganic nitrogen concentrations in the bulk samples, 12g of fresh soil was shaken in 100mL of 2M KCL solution for one hour and filtered through Whatman Grade 1 paper and stored in -20C freezer until processing. The extracts were analyzed on a microplate reader (Cytation 5, BioTek Instruments, Winooski, VT) using colorimetric methods outlined in Sims et al (1995) and Doane and Horwáth (2003) to detect ammonium (NH_4-N) and nitrate (NO_3-N) concentrations.

Aggregate samples were processed using a wet sieving method described in Elliott (1986) to separate aggregates into large macroaggregate (>2000um), small macroaggregate (250-2000um), free micro aggregate (53-250um), and free silt and clay (<52um) fraction sizes. Briefly, around 30g of air-dried soil was submerged in water to slake for 5 minutes before sieving. The sieve was then dipped up and down over a shallow pan filled with deionized water for 2 minutes. Each fraction size was dried at 60°C, and aggregate mean weight diameter was calculated according to Van Bavel (1950).

Soil microbiome analyses with 16S rRNA sequencing

In parallel to the soil agronomic metrics outlined above, soil microbiome analyses were performed on only rhizosphere soils for both Spring and Summer 2023. DNA was extracted from 0.5g soil using the Zymo Research Quick-DNA™ Fecal/Soil Microbe kit. The DNA concentration present in the extracts was quantified with a Qubit® 2.0 fluorometer (Invitrogen) and then stored in -80C until further processing. Sample preparation was

performed at Colorado State University. Briefly, DNA samples were prepared for 16S rRNA gene V3 - V4 region using the updated primers 515F (Parada; GTGYCAGCMGCCGCGGTAA) and 806R (Apprill; GGACTACNVGGGTWTCTAAT). PCR mixes included 12.5 µL PCR-grade water, 10.5 uL 2X PCR master mix, 0.5 µL, each forward and reverse primers, and 1 µL template DNA. To selectively amplify the 16S rRNA V4 region, samples were incubated at 94 °C for 2 min followed by 35 cycles with the following protocol: denaturing at 94°C for 15 s, annealing at 60°C for 15 s, and elongating at 68°C for 1 minute, followed by 10 min final elongation at 68 °C.

DNA libraries were sequenced on Illumina Miseq 500cycle at the University of Colorado following the protocol outlined by Walsh et al. (2024). Bacterial community composition was analyzed from reads processed in Quality Insights into Microbial Ecology (QIIME2 v. 2023.9) (Bolyen et al., 2019). Reads were trimmed to 240bp and demultiplexed with the demux plugin. DADA2 (Callahan et al., 2016) was used to filter, denoise, and assign sequences to amplicon sequence variants (ASVs). Reads were rarefied to 10,000 reads/sample for a total number of 2732230 reads and 18385 ASVs. ASVs were taxonomically classified via Silva nr 138 (Quast et al., 2013) and GTDB v2 07 (Chaumeil et al., 2019).

Statistical analyses

Analysis of data and statistics on soil functions and soil microbial community was performed in R version 4.4.0 (R core Team 2024). Packages used to explore soil function data were *lme4* (Bates et al 2015), *lmerTest* (Kuznetsova et al 2017), and *emmeans* (Lenth et al 2018). Alpha diversity data was analyzed using *plyr* (Wickham 2011) and *lme4*, and

beta diversity was analyzed using *devtools* (Wickham et al. 2022), *stringr* (Wickham et al. 2023), *grid* (R core Team 2024), *corrplot* (Wei & Simko 2021), *corrgram* (Wright 2021), *ggrepel* (Slowikowski 2024), *pairwiseAdonis* (Martinez Arbizu 2020), and *vegan* (Oksanen et al. 2020) packages. Enrichment data was explored through *MaAslin2* (Mallick et al. 2021), *devtools,* and *gridExtra* (Augie and Antonov 2017) packages. For this field-based study, we used an alpha of 0.10 to define statistical significance and report specific p-values to communicate levels of confidence for our findings.

To perform statistical analyses on cover crop and maize shoot biomass along with soil functions (soil extracellular enzymes, DOC, inorganic nitrogen, and aggregates), *lmer* mixed models were used with block as a random effect, and soil type (Type), cover crop treatment (Treatment), and their interaction as fixed effects. For the aggregates and the inorganic nitrogen models, only Treatment as a fixed variable and Block as a random variable were included since there was no rhizosphere soil collected for either soil function. Estimated means (*emmeans*) contrasts were used to test for differences by cover crop treatment. Data were assessed for normality and homogeneity of variance. Log transformation was applied to spring cover crop shoot biomass, all spring enzyme data, summer NAG and BG, spring and summer inorganic N, spring and summer DOC, and summer aggregates to meet model assumptions Using the Interquartile Range (IQR) method, values below Q1- (1.5 x IQR) and above Q3 + (1.5 X IQR) were defined as outliers (Q1- 25^{th} percentile and Q3- 75^{th} percentile). No more than 6% of data points were removed as outliers for any one variable. In the spring soil enzyme dataset, however, many values that met our definition of an outlier were retained due to large differences attributed to

unexplained variation between lab processing days, which were done by block and thus the inclusion of block in our model addresses this variability.

When describing soil microbiome community composition in R, alpha diversity was determined by fitting Shannon diversity, evenness, and richness indices each into a separate, simple linear model with block, treatment and their interaction. Two alpha diversity models were run for Spring 2023 and Summer 2023 separately. For beta diversity analyses, 2% of samples was removed in order to fit field metadata with taxonomy data, since those samples were removed during the rarefaction step. ASV's were fitted into a PERMANOVA model with Treatment as an effect stratified by Block and then plotted in an NMDs cluster plot. Similar to alpha diversity, two separate models for the spring and summers seasons were run for beta diversity analyses. The clustered communities were then correlated with soil functions to test whether certain microbial clusters are associated with any soil processes. Furthermore, when exploring taxon enrichment in both seasons, the top and bottom 20% of enriched taxa (cover crop compared to control) were selected for further analyses.

RESULTS

Cover crop and maize shoot biomass

In Spring 2023 before cover crop termination, cover crops accumulated varying amounts of aboveground shoot biomass (Figure 2). The average shoot biomass produced across all cover crop treatments was 1.3 Mg/ha. Hairy vetch and cereal rye produced similar amounts of biomass, and both produced more biomass than any of the other treatments. Rape seed produced about 3 to 4 times less biomass than rye or vetch, followed by sorghum and control, where sorghum and control did not produce different amounts of biomass. Sorghum producing the least biomass was expected,

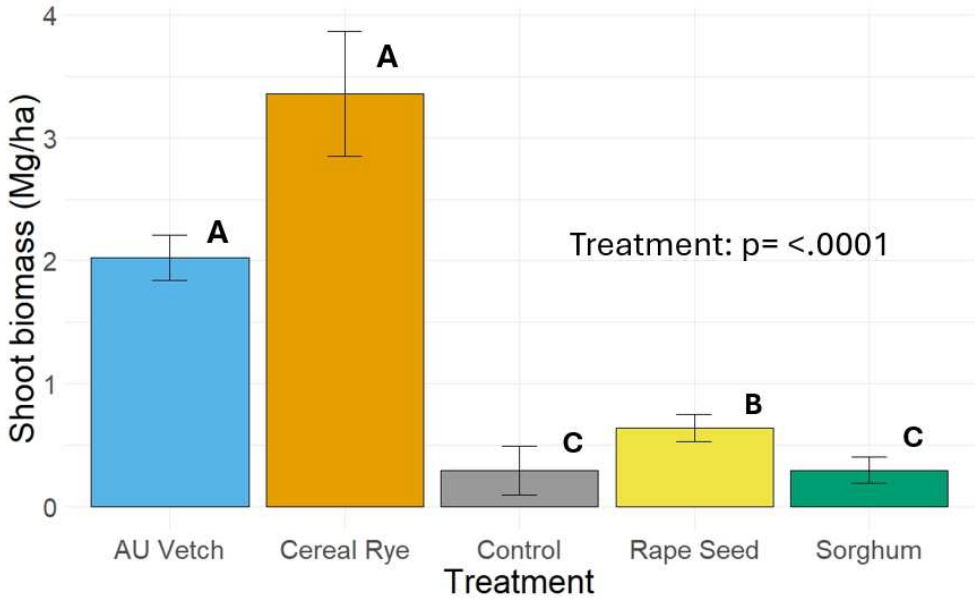

Figure 2. Mean cover crop shoot biomass prior to termination in Spring 2023. Error bars represent standard errors (n=5) and different letters indicate significant differences between treatment means ($p<0.1$).

given that the crop accumulated a small amount of biomass before being winter killed, whereas other crops survived and continued to grow into the spring.

After cover crop termination, the following corn crop biomass sampled in July along with the corn grain yields did not vary by cover crop treatment (see Appendix B, $p>0.1$). The lack of differences in maize shoot biomass across treatments suggests that there was no cover crop legacy effect on corn productivity. On average, the maize plants produced 24 Mg/ha of shoot biomass by July and 17 Mg/ha of grain at maturity.

Changes in soil functions during and after cover cropping

The responses of all soil functions during and after cover cropping are summarized in Table 1. Looking at the different enzyme responses, only the LAP enzyme had a treatment effect in Spring 2023 with living rape seed seemingly stimulating more enzyme production compared to the control (Figure 3). However, of all enzymes measured, LAP was the only enzyme in living cover crops to have a significant interaction between cover crop treatment and soil type, where the rhizosphere of spring rape seed stimulated more LAP enzyme activity compared to the rhizosphere of spring sorghum (Table 1, $p= 0.084$). Among the enzymes during cover cropping, only BG had a Type effect where BG enzyme activity was greater in the bulk soil compared to the rhizosphere across cover crop species.

In terms of summer enzymes under the maize cash crop, Treatment had a marginally significant effect on LAP enzyme ($p=0.093$), though further investigation of pairwise comparisons did not find specific differences between cover crops for summer LAP activity ($p>0.1$). However, cover crop Treatments differed for summer BG and PHOS enzyme activity, where cereal rye had more enzyme activity for both compared to sorghum. Additionally, during maize vegetative growth, NAG, BG, and PHOS enzyme activities were

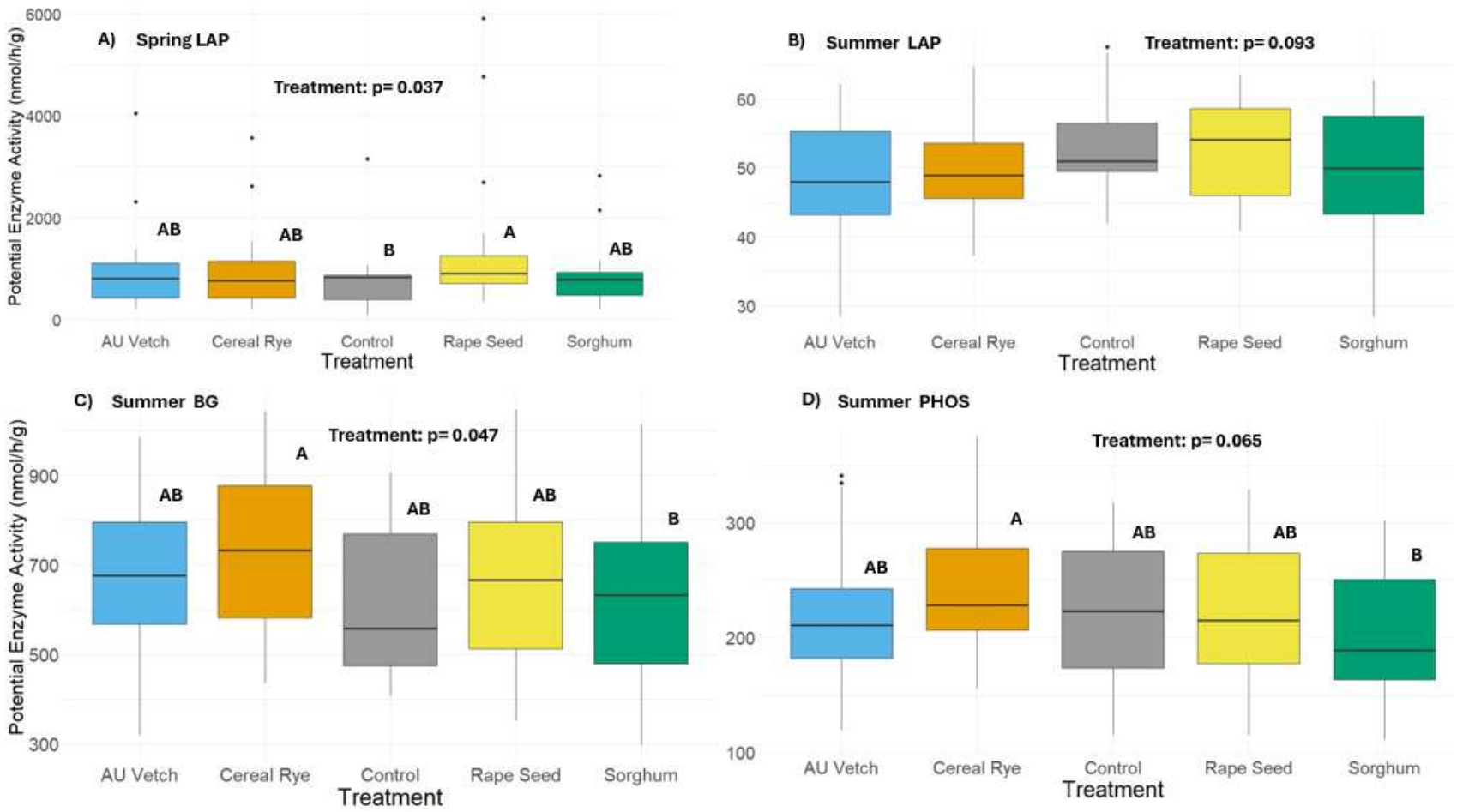

Figure 3. Measured enzyme activities cross cover crop treatments in nmol/h/g. Only one enzyme was directly affected by a living cover crop: A) LAP activity in Spring 2023 before cover crop termination. Under the maize crop, after cover crop termination, no cover crops effects were found for B) LAP, but cover crop legacies affected C) BG, and D) PHOS activities.

Table 1 Summary of select soil function means and standard errors (SE) during and after cover cropping by cover crop treatment and ANOVA p-values for soil type (B=bulk; R=rhizosphere), cover crop treatment, and their interaction. The Spring season shows values measured under living cover crops, and the Summer season shows values measured after cover crop termination and during the corn cash crop. The soil extracellular enzymes excreted by microbes are LAP, NAG, BG, and PHOS. INORGN stands for inorganic nitrogen and is the sum of soil inorganic nitrate and ammonium concentrations. DOC is the dissolved organic carbon concentration of the soil. AGG is aggregate mean weight diameter, and BD is the bulk density of soil.

		LAP (nmol/h/g)	NAG (nmol/h/g)	BG (nmol/h/g)	PHOS (nmol/h/g)	INORGN (ppm)	DOC (mg/ kg soil)	AGG (mm)	BD (g/cm3)
Season	**Treatment**	**Mean (SE)**	**Mean (SE)**	**Mean (SE)**	**Mean (SE)**	**Mean (SE)**	**Mean (SE)**	**Mean (SE)**	**Mean (SE)**
Spring	Control	784±141 B	18.9±5.0 A	902±214 A	1385±411 A	1.0±0.19 A	15.1 ±1.4 AB	323±34 B	
	Sorghum	878±139 AB	27.7±7.3 A	872±150 A	2274±589 A	0.5±0.14 B	13.1±2.3 B	474±65 AB	
	Rape Seed	1420±323 A	25.7±0.6 A	1384±284 A	1747±544 A	0.3±0.12 BC	19.6±2.2 A	409±39 AB	
	Cereal Rye	993±184 AB	23.5±4.6 A	1006±231 A	2118±582 A	-0.1±0.07 D	17.4±1.2 A	617±81 A	
	AU Hairy Vetch	967±195 AB	22.9±5.7 A	882±226 A	1913±474 A	-0.1±0.04 D	17.5±2.0 AB	493±58AB	
	Type	0.21	0.16	<.0001 (B>R)	0.78	NA	0.0098 (B>R)	NA	
	Treatment	0.037	0.72	0.42	0.18	<.0001	0.00081	0.0086	
	Type*Treatment	0.084	0.59	0.79	0.21	NA	0.78	NA	
Summer	Control	53.4±1.7 A	95.5±5.0 A	624±38 AB	221±13 AB	`-0.6±0.02 A	13±3.5 A	370±42 A	1.5±0.01 A
	Sorghum	49.3±2.0 A	93.1±5.6 A	633±43 B	203±13 B	-0.6±0.03 A	23±6.5 A	366±33 A	1.4±0.04 A
	Rape Seed	52.5±1.6 A	106±5.6 A	661±44 AB	228±14 AB	-0.7±0.01 A	13±4.3 A	454±45 A	1.5±0.02 A
	Cereal Rye	49.9±1.7 A	107±5.7 A	738±42 A	243±13 A	-0.6±0.03A	14±3.4 A	349±28 A	1.4±0.02 A
	AU Hairy Vetch	47.8±2.0 A	98.7±6.9 A	676±42 AB	221±14 AB	-0.6±0.02 A	23±6.0 A	421±38 A	1.4±0.02 A
	Type	0.12	<.0001 (B<R)	0.0042 (B<R)	0.00176 (B<R)	NA	0.015 (B<R)	NA	NA
	Treatment	0.093	0.19	0.047	0.065	0.42	0.11	0.21	0.097
	Type*Treatment	0.14	0.37	0.167	0.46	NA	0.16	NA	NA

greater in the maize rhizosphere than the bulk soil.

For dissolved organic carbon (DOC), cover crops and soil type had an effect on concentrations in Spring 2023 under living cover crops. When comparing the cover crop treatments, concentrations were higher in rape seed and cereal rye compared to sorghum with no differences between rape seed and cereal rye (Figure 4).

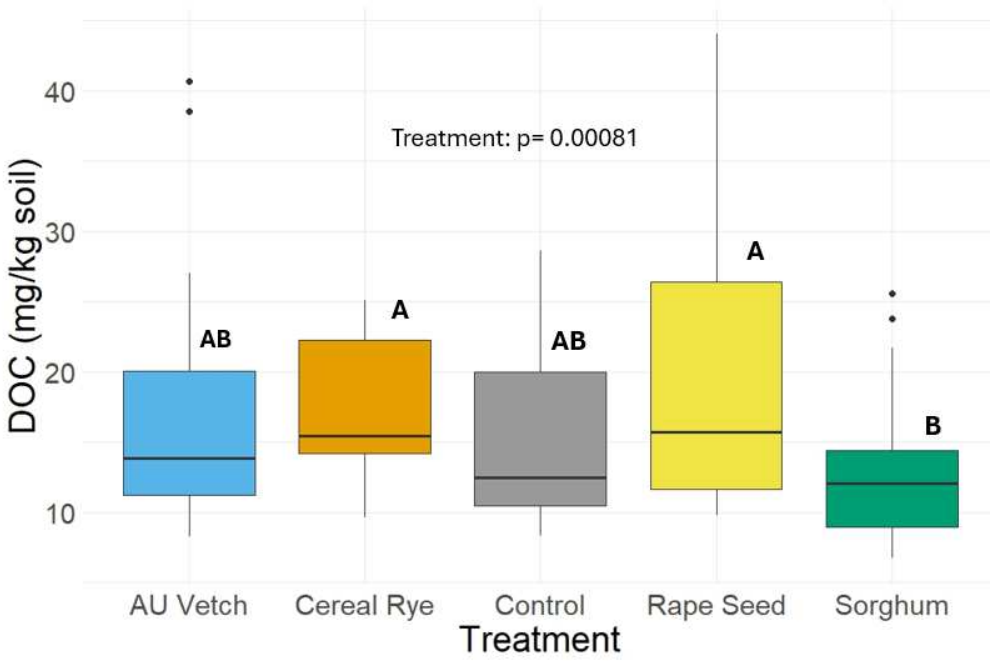

Figure 4. Average dissolved organic non-purgeable carbon concentrations (DOC) prior to cover crop termination. Concentrations were averaged across bulk and rhizosphere soils. The living rape seed and cereal rye had more DOC compared to the sorghum.

However, similar to the Type effect in Spring 2023 BG enzyme, DOC concentrations were greater in the bulk soil compared to rhizosphere soil across all cover crop treatments.

Soil inorganic N varied by cover crop treatment in Spring 2023 under living cover crops, but these differences did not persist into the following maize crop. In the spring, the control treatment had the highest inorganic nitrogen concentration followed by sorghum, rape seed, and cereal rye and hairy vetch, with the last two treatments not being different

from one another (Table 1). In Summer 2023 under the maize cash crop, no Treatment effect was detected.

Living cover crops influenced soil physical metrics prior to cover crop termination. In Spring 2023, the cereal rye had greater aggregate mean weight diameter (MWD) compared to the bare, fallow control (Figure 5). This result exemplifies living cover crop's ability to affect soil physical properties in the field in a matter of months. In the Summer under the maize crop, aggregate MWD did not differ by cover crop treatment. Results for bulk density, collected in Summer 2023 under the cash crop, interestingly, show a marginal treatment effect, Rapeseed plots had the highest bulk density and hairy vetch the lowest across all treatments, but there were no cover crop treatment significant differences when tested using pairwise comparisons.

Soil microbiome composition and enriched taxa

We analyzed the composition of the soil microbiome in the rhizosphere soil before cover crop termination and under the maize crop, because we were interested in learning about the response of soil microbes to cover crop root exudates. Several prominent microbial phyla were detected under living cover crops and the maize cash crop (Figure 6). The top 5 most abundant phyla in Spring 2023 were Acidobacteriota (21.8%), Proteobacteria (20.1%), Crenarchaeota (14.4%), Chloroflexi (11.9%), and Planctomycetota (11.4%), while the top 5 abundant phyla in Summer 2023 were similar (Acidobacteriota (23.4%), Proteobacteriota (20.1%), Planctomycetota (14.5%), other (13.6), and Crenarchaeota (12.3%)). Comparing across cover crop treatments and the two sampling points, there were no notable changes in the abundances of microbial phyla, and the phyla

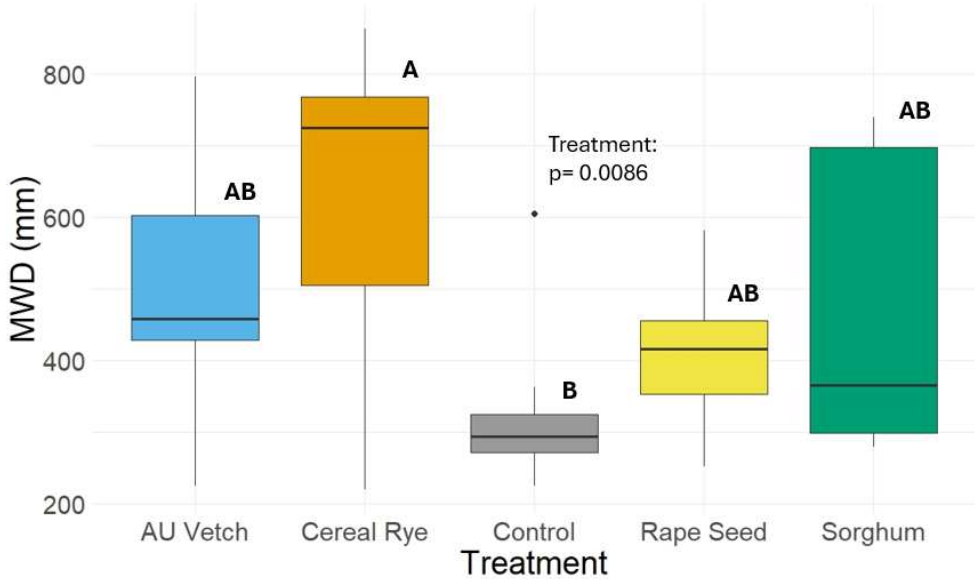

Figure 5. Spring 2023 soil aggregate mean weight diameter (MWD) in millimeters (mm). Aggregates size differed by cover crop treatment as living cereal rye increased MWD of soil compared to the fallow control, showing cover crops can affect physical structure in a period of several months.

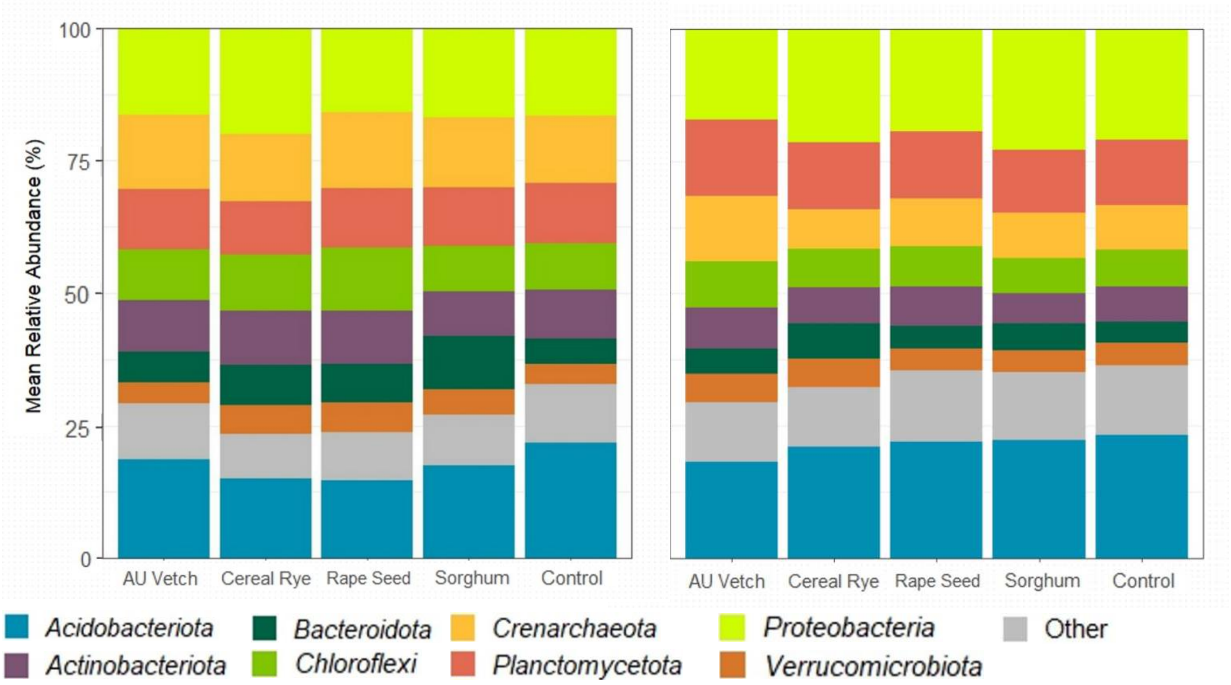

Figure 6. Distribution of bacterial and archaeal phyla present in a) Spring 2023 under living cover crops and b) Summer 2023 under the maize crop, determined from 16S rRNA sequencing of the rhizosphere portion of sampled soil. Phyla present in abundances <3.8% across the data set are represented as "other". Across cover crop treatments and the two sampling points, detected phyla and phyla abundances did not differ.

detected during and after cover cropping were consistent.

We hypothesized that cover crop treatments would differ in their rhizosphere microbiome prior to termination, and that some of these community differences may carry over to the maize crop as a legacy effect. However, alpha diversity did not differ by cover crop treatment during or after cover cropping, nor did indices such as evenness, richness, and Shannon diversity differ by treatment or sampling period (Figure 7). This indicates that

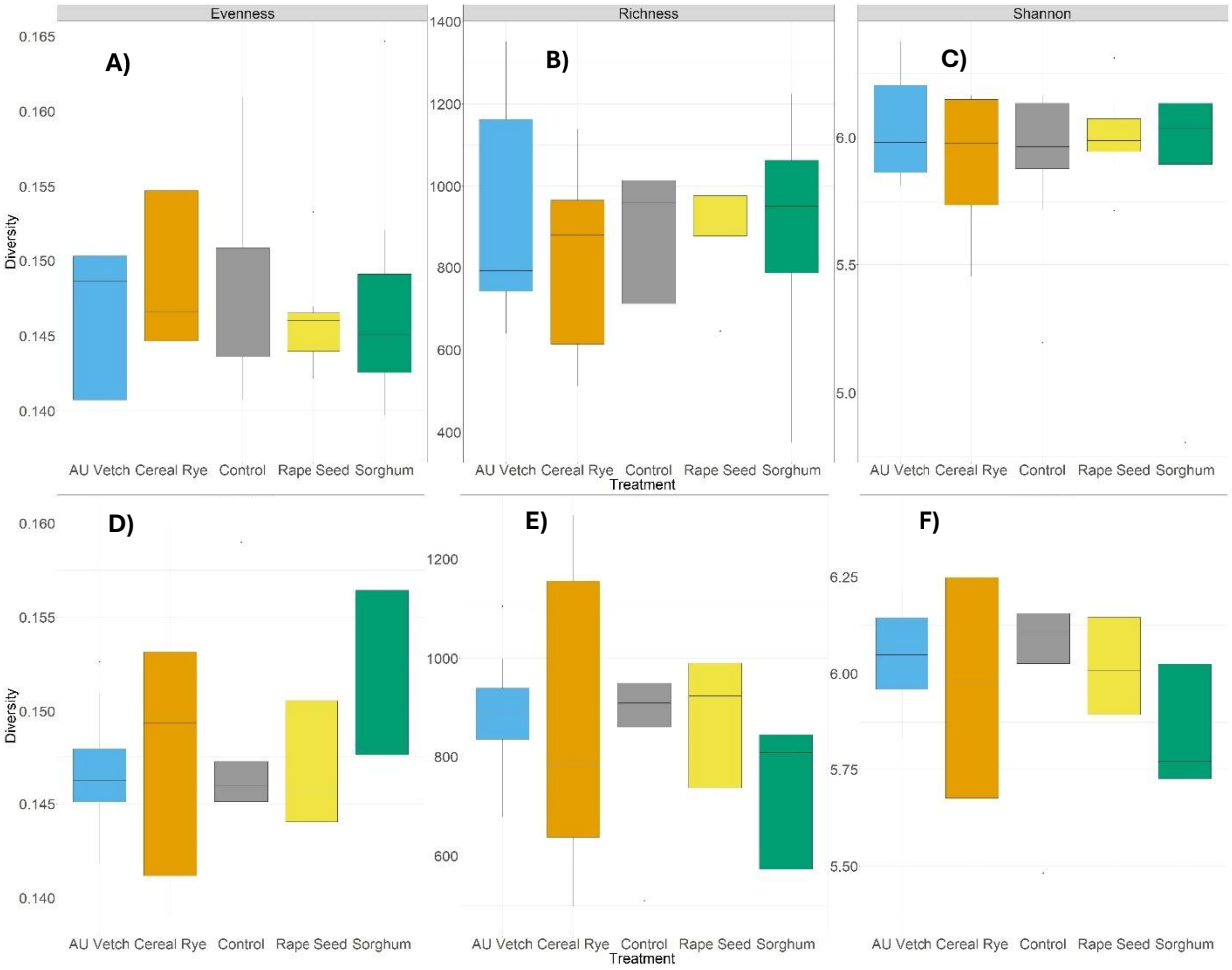

Figure 7. Alpha diversity evenness, richness, and Shannon indices in Spring 2023 before cover crop termination (A,B,C) and in Summer 2023 (D,E,F) after cover crop termination of the rhizosphere soil microbiome. No differences in microbial diversity were found across cover crop treatment treatments in both seasons. This indicates that soil microbiome communities across cover crops for both sampling points are similarly diverse and detected microbial species are evenly distributed.

the soil rhizosphere microbiomes of each cover crop during both sampling points are similarly diverse and detected soil microbes are evenly distributed. Likewise, beta diversity for both seasons did not show differences in rhizosphere microbiome community structure across cover crop treatments and the fallow control (Figure 8), signifying that microbiome structure across cover crop treatments during both sampling points is similar.

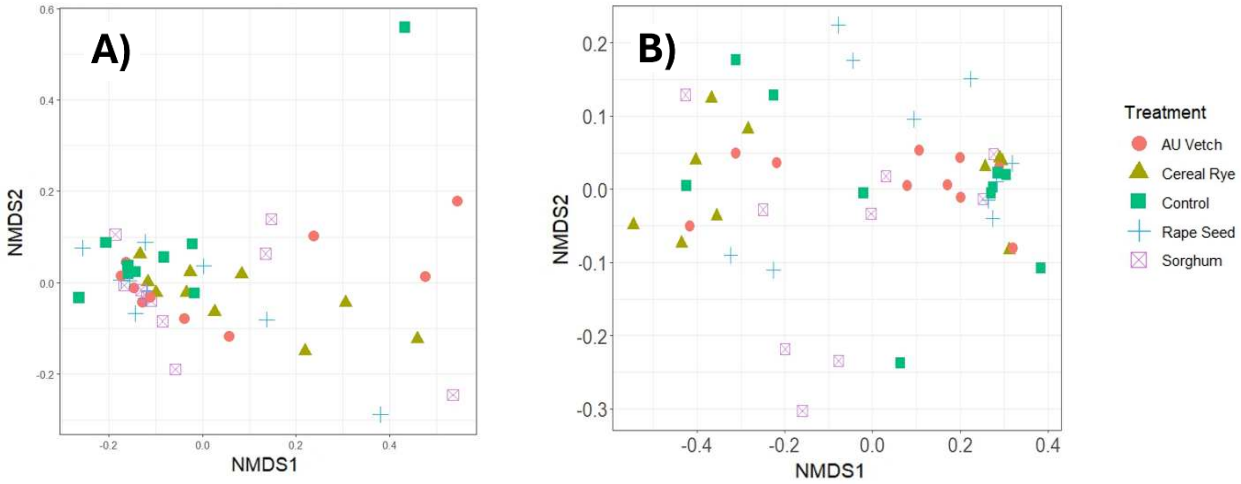

Figure 8. Beta diversity in Spring 2023 (A) and Summer 2023 B), before and after cover crop termination, respectively, of the rhizosphere soil microbiome. Treatment is indicated by color and shape. No statistical differences were determined in beta diversity for either season, indicating that microbial community structure across treatments for both sampling points is similar.

When we correlated the soil functions by these clustered communities, we also did not find any meaningful correlations with our quantitative measurements. However, while community composition did not differ by cover crop, we found specific taxa that were enriched under cover crop treatments for both seasons. The *genus Massilia* was the only taxa that was significantly enriched (q value 0.0009) under the cereal rye treatment relative to the control prior to cover crop termination. We did not find any correlations between

Massilia and any of our soil functions for both pre-cover crop termination and under the maize crop.

Furthermore, to assess compatibility with previously recovered exudate-responsive metagenomically assembled genomes (MAGs), (ARM database, Seitz et al 2022, 2024 preprint) ASVs were compared to MAGs two ways. First, ASV sequences were searched against MAGs. We considered matches that were at least 97% identity across the 253 base pairs. Second, we looked for taxonomic matches at the genus level. To do this, we compared ASV taxonomy classified using the naïve Bayes sklearn classifier trained with the GTDB-Tk species representative genomes (release 214) to MAG taxonomies classified using GTDB-tk (release 214). In total, these two methods identified linkages between 1,647 ASVs and 370 MAGs (full MAG set- ARM only: 1,400 ASVs and 270 MAGs), spanning 121 genera (103 if ARM only). We found 15 MAGs matched with the BLAST reads and were also found in the ARM database (Table 2). Several of these MAGs were found to contain genes involved in soil enzyme activities, but when we correlated the corresponding ASV's with the soil function measurements, we did not detect any correlations.

Table 2- ASV's were found to match metagenome assembled genomes (MAGs) in the ARM database at >97% across 253 bp by a BLAST search (Seitz et al., 2024, preprint). Functional profiles of these MAGs, generated in DRAM v1.14 (Schaffer and Borton et al, 2020), indicate that certain genomes have the potential to produce genes for the following enzymes: Leucine Aminopeptidase (LAP, EC:3.4.11.1), Beta-1,4-glucosiase (BGLU, EC:3.1.3.2), N-acetyl-glucosaminidase (NAG, EC:3.2.1.52), and acid phosphatase (PHOS, EC:3.2.1.21).

			Functional genes			
Genus	**% Match**	**#ASV**	**BGLU**	**LAP**	**NAG**	**PHOS**
g__CADEED01	100	7	--	3	--	1
g__CADEED01	100	6	3	2	1	1
g__JAJNBA01	100	7	--	1	--	--
g__UBA4722	100	1	--	1	--	--
g__WHTF01	100	5	--	1	--	--
g__CADEED01	99.605	6	4	2	--	1
g__JAJNBA01	99.605	8	--	1	--	--
g__WHTF01	99.605	5	--	1	1	--
g__Pseudobdellovibrio	99.209	1	--	3	--	--
g__Pseudobdellovibrio	99.209	1	--	3	--	--
g__VGBV01	99.209	3	--	1	--	1
g__VGCI01	99.209	14	--	1	--	--
g__JAJONM01	98.814	3	--	1	--	--
g__Vampirovibrio	98.814	2	--	3	--	--
g__Nitrososphaera	98.814	7	--	1	--	--

DISCUSSION

The study's objectives were to determine whether cover crops belonging to various species can modify the rhizosphere microbiome differentially and in a way that is characteristic to each crop's identity (e.g. hairy vetch recruits nitrogen-fixing bacteria). We hypothesized that we would find such effects before cover crop termination, and we expected that at least some of these microbiome compositional changes would carry over to the maize cash crop. Moreover, we expected that the microbiome communities of each cover crop would be correlated with some of the measured soil functions. Following a single over-winter growth period, cover crops influenced soil structure and enzyme activity. These effects were primarily limited to the cover crop with the greatest biomass production. Importantly, only the cover crop effect on soil enzymes persisted into the following cash crop. We found no evidence of cover crop influence on the soil microbial community composition, but some evidence that cover crops may enrich for specific taxa.

Cover crop shoot biomass and maize crop biomass and yield

As part of our broader analysis of cover crops and their connection with the soil microbiome, we measured shoot biomass along with shoot and grain mass of the succeeding maize crop. During cover crop growth and before corn planting, cereal rye accumulated more aboveground shoot biomass compared to rape seed and sorghum but produced similar biomass to hairy vetch. Other studies have shown that cereal rye grown as a monoculture can be more productive or similar to hairy vetch (Poffenbarger et al. (2015); Vaughan and Evanylo (1998)). However, what is interesting is the presence of cover

crop effects on enzymes and soil aggregate stability, discussed in the following sections, under cereal rye but no treatment effects from the hairy vetch on any soil functions. This difference in results when comparing the two cover crops may indicate that the cover crop effects are not a simple result of more biomass addition, but rather are dependent on cover crop identity.

To discuss the lack of differences in cash crop yield, a similar field-based, Rodriguez et al. (2023) conducted a meta-analysis of the effect of growing hairy vetch as a cover crop prior to maize and cash crop yield differences. The authors of this study found that across diverse experimental setups, meaningful maize yield differences after hairy vetch were dependent on certain management practices such as not fertilizing maize with nitrogen. Similar to the reasoning behind a lack of cover crop effects on inorganic nitrogen under the maize crop, our modest application of nitrogen fertilizer might explain the lack of yield differences.

Potential cover crop effects on soil enzyme activity and soil chemistry

In our one-year trial, we detected several cover crop effects on enzymes before cover crop termination and during the maize crop. This information gathered on soil enzyme activities is a step towards understanding the potential function of the soil microbes before and after cover crop termination, because we consider these enzymes to be proxies for microbial activity. When the cover crops were alive, rape seed stimulated LAP enzyme activity more than the control. Likewise, the greatest DOC concentrations were detected in rape seed along with cereal rye at this sampling. That only living rape seed had an enzyme effect of all the cover crop treatments may be explained by the litter quality

of rape seed residue. Compared to cover crop grasses like oats, the residue in canola is easily decomposable with a lower C:N ratio and less lignin and structural carbohydrates, and it is recognized to have quick turnover rates (Ghimire et al. 2017). We observed some winter senescence of aboveground rapeseed biomass following by spring regrowth, and this deposited litter may have stimulated microbial LAP production. Alternatively, Seitz et al. (2024, preprint) observed high microbial functional diversity in indole-3-acetic acid (IAA) production in rape seed exudates through two pathways: indole-3-acetonitrile (IAN) and indole-3-acetamide (IAM). IAA, the product of these two pathways, is an organic acid that is readily consumed by rhizobacteria (Oburger et al. 2018). The observation of microbial LAP stimulation under the living rape seed in our study may also be a result of its specific root exudate chemistry. The LAP enzyme breaks peptide bonds and accesses the nitrogen pool for soil microbes, which could complement other carbon nutrient cycling nutrient potential. Connecting this point with DOC concentrations, the potential diversity in metabolic pathways might indicate that there is more carbon exudate processing occurring in the rhizosphere. Apart from IAA metabolites, rape seed is known to produce fumigant compounds like 2-Phenylethylisothiocyanate (PEITC), which can degrade quickly and select for certain microbes in the rhizosphere microbiome (Rumberger and Marschner, 2003). In other words, the stimulated production of LAP enzyme under rape seed prior to cover crop termination may originate from rapid litter decomposition and/or the recruitment of microbes with diverse exudate processing functionalities. Likewise, the increase in DOC concentration could be attributed to the availability of a diverse array of metabolites and root carbon exudates from rape seed.

Across all cover crop treatments, we found contrasting rhizosphere effects of cover crops and maize on soil enzyme and DOC concentrations. Greater BG enzyme activity was observed in the bulk soil compared to rhizosphere soil prior to cover crop termination, but the opposite trend was observed with greater BG activity in rhizosphere under the maize crop. Similarly, DOC concentrations followed the same trend with higher concentrations in bulk soil under the cover crop and higher amounts in rhizosphere during maize crop growth. A parallel can be drawn between BG activity and DOC concentrations, since BG is recognized as an enzyme that accesses the carbon source for soil microbes to produce energy (Kim et al. 2022). Thus, greater BG activity and DOC concentrations in bulk soil under cover crop may be the microbial community drawing DOC concentrations down around the cover crop roots, since a depleted rhizosphere carbon source could represent less substrate for microbial BG to act on. Meanwhile, the opposite trend in the maize crop may be explained by simple microbial stimulation from the maize roots, where maize exudation rates were higher than those of the cover crops and microbes were less carbon limited. Kumar et al. (2017) demonstrated stimulation of BG, NAG, LAP, and PHOS enzymes in maize rooted soil and explained this as likely due to increased root exudated carbon. Similarly, we were able to detect stimulation of BG, NAG, and PHOS enzymes in the maize rhizosphere compared to the bulk soil. The maize roots, just like in the cover crops, exhibits root exudate properties that influence microbial activity.

We did detect a cover crop treatment effect on soil enzyme activity that persisted into the maize crop. Cereal rye stimulated BG and PHOS more than sorghum, which as noted in the results of cover crop biomass accumulation, had grown the least compared to

the rest of the cove crops. Because of the minimal, yet detectable growth of sorghum in the fall and its early winter kill, the sorghum treatment became a sort of nutrient depleted control that may explain these observed differences with cereal rye for the two enzymes. Since no cover crop was alive during the maize crop sampling, finding a cover treatment effect indicates a legacy effect of the prior cover crop on enzyme activity. This legacy effect could be due to varying cover crop litter decomposition and its influence on enzyme activities, as terminating a cover crop creates an immediate supply of carbon and other nutrients to the soil microbes (Lehman et al. 2012). However, the rates at which crop residues decompose and release nutrients into the soil are dependent on residue quality, including the C:N ratio. For example, cereal rye is known to have a high C:N ratio and decompose slower than hairy vetch (Sievers and Cook 2018). Thus, the combination of high biomass and high C:N of the rye cover crop could have potentially stimulated BG activity even after cover crop termination.

The cereal rye also increased PHOS activity in the maize crop compared to the same effect in sorghum. A decomposing cereal rye residue effect that simulates PHOS activity under the maize crop may also explain this result, since cereal rye has been shown to immobilize soil phosphorus (Gonzales et al. 2022). Even though the phosphorus content of the rye could be similar to legumes, cereal rye has a high carbon: phosphorus ratio that could lead to microbial immobilization or delayed mineralization (Maltais-Landry et al. 2014). Thus, after termination, the gradual release of phosphorus may carry over to the maize crop as a legacy effect. What is interesting in these results is the lack of legacy effects of the hairy vetch on any enzyme activity, since other studies reported increases in

PHOS activity with the incorporation of decomposing hairy vetch residue into soil (Stegarescu et al 2021; Kataoka et al. 2017; Mancinelli et al. 2013). However, as mentioned previously with cereal rye, hairy vetch can decompose quickly with a lower C:N ratio and thus release nutrients at a greater rate. The implications of this faster decomposition are, perhaps, greater nutrient mineralization and thus faster acquisition by the succeeding maize crop established not long after cover crop termination. Another point to consider is the fact that, after cover crop termination, some of the hairy vetch was not entirely killed and there was still shoot biomass present after establishing the maize crop. This could mean that due to delayed decomposition, a more subtle enzyme response to nutrient release from hairy vetch was detected, resulting in a lack of visible cover crop legacy effects.

This delay in hairy vetch residue decomposition may also explain the lack of cover crop legacy effects on inorganic nitrogen during the maize crop. In a similar vein, the partial hairy vetch residue that decomposed might have had a subtler effect on the inorganic nitrogen concentrations during our maize crop sampling. This result is unexpected and does not line up with the literature, because incubation studies like Hansen et al. (2021) have shown that within 80 days and comparing among legume and non-legume crops, one of the highest amounts of shoot biomass-nitrogen mineralized into soil originated from hairy vetch. Yet, since we fertilized the field with nitrogen in our study, this amendment may have muted any cover crop effects on inorganic nitrogen.

Potential cover crop effects on soil physical properties

Along with soil enzyme responses and soil chemistry, we also evaluated the effect of cover cropping on soil physical properties such as soil aggregate stability and bulk density. The only living cover crop that affected aggregates was cereal rye, which increased aggregate mean weight diameter relative to the control. This relatively fast change in aggregate stability under the living cereal rye crop has been demonstrated in other another study where this crop increased aggregate stability in 8 months (Liu et al. 2008). However, after cover crop termination, the cereal rye increase in aggregate MWD did not carry over to the maize crop as a legacy effect. There were no cover crop treatment effects on MWD under the maize crop (Table 1). Kumar et al. (2017) investigated the effects of maize roots on aggregate sizes and found that, with increase planting density, macroaggregates seemed to break down into smaller microaggregate sizes. A similar process might have happened in our field study, resulting in a loss in aggregate stability differences under the maize crop. Likewise, other studies have found that cover crop root biomass, in particular, can reduce bulk density, which is what we observed in our field experiment.

Challenges in studying cover and cash crop effects on the soil microbiome

A key component of this experiment was to connect cover crop effects to rhizosphere microbiome composition, and thus relate this change to shifts in soil microbial functions. However, before cover crop termination and under the maize crop, we did not detect any changes in microbial evenness and richness, nor find meaningful correlations between microbiome structure and our soil functions. The lack of differences in microbial diversity indices such as alpha and beta diversity in the rhizospheres of the

cover crops is surprising. There are many studies conducted in a short timescale that detected living and decomposing cover crop effects on microbial community and functions. For example, Finney et al. (2017) studied living cover crop effects on the bulk soil microbiome in one growing season and found that crops such as oats and cereal rye increased AM fungi, while crops like hairy vetch were related to higher abundancies of non-AM fungi. Cazzaniga et al. (2023) also studied living cover crop effects along with legacy effects post-cover crop termination and, over the course of 4.5 months, found differences in community structure with oilseed radish cultivars producing the most microbial footprints that included bacterial taxa. Meanwhile, Ogunleye et al. (2013) observed the changes in microbial community structure and function at different stages of cover crop decomposition in a short-term, one-year study. Although they found differences in bacterial and fungal abundance across cover crop monocultures and mixtures, similar to our study, they could not connect cover crop mixture diversity with microbial functionality mediated by soil enzymes. These contrasting results highlight the need to continue advancing knowledge in other factors that shape the soil microbiome composition and enhancing approaches to research soil microbial functions.

Given that the soil is a heterogenous environment, many other variables can confound the effects of cover and cash crops on soil microbial membership and function. Garland et al. (2021) conducted a study analyzing the differences in cover and intercropping practices in fields scattered across different European countries and considered the confounding impacts of such external factors. The researchers found that environmental variation and soil cover duration played a greater role in shaping microbial

diversity and even taxon-specific responses than cover crop identity. They also discuss the potential challenges in detecting cover crop legacy effects when sampling soil where only one crop (e.g. cash crop) is growing on the field compared to sampling under intercropping. Alahmad et al (2019) also point out that functional microbial differences from cover cropping can be detected below the usually sampled top 10 cm of soil, where even some microbial species at a lower depth had a greater response to cropping treatments.

These environmental and sampling nuances also impact our ability to analyze cash crop effects on the soil microbiome. Fernandez et al. (2016) determined that maize can reshape microbiome composition during peak growth, but they also highlighted the need to consider soil type and research site latitude and location in making conclusions about these effects. Even though their study was conducted on long-term, organic management field sites, their observation of similar taxa across sites with similar rainfall and soil texture emphasizes the challenge of generalizing results. Likewise, Rüger et al. (2021) further illustrate the complexity of characterizing maize's effect on the rhizosphere microbiome with varying microbial diversity along the maize root, such as the root tip having a different microbial profile compared to other sections. The lack of differences in microbial community response to cover cropping in our study could be explained by several or a combination of these external factors.

Cover crop taxon-specific versus microbiome community effect and a new approach to connecting 16S taxa with potential genetic function

While we were not able to find microbiome structural or diversity changes in our study, we were able to observe a change in the abundance of one taxon under the cereal rye treatment prior to cover crop termination. This enriched taxon found in the living cereal rye was *genus Massilia*. *Massilia* are non-spore forming, aerobic bacteria with flagella (Kämpfer et al. 2011; La Scola et al. 2011) that have been discovered in various environments, ranging from air, freshwater, soil, and rhizosphere samples. *Massilia* are classified as r-strategists that proliferate and can take advantage of root carbon exudates before rhizosphere biotic competition intensifies (Ofek et al. 2012). In our study, this genus was detected during maximum, late vegetative growth of cereal rye before cover crop termination, meaning our findings may contribute to expanding knowledge of the lifestyle and habitat of *Massilia*.

The finding of a taxon-specific response to a living cover crop in the field is exciting and supports similar results from research conducted in more controlled settings. Seitz et al. (2024, preprint) demonstrated that cover crop root exudates can induce an organismal (MAG) response within the broader rhizosphere microbiome, and they propose that such results can inform the development precision agriculture for enhanced crop performance. This ability of a plant to recruit certain microbes has been demonstrated in other studies. Chaparro et al. (2014) studied the microbiome of *Arabidopsis* in a controlled environment at different stages of growth and found the plant seemingly selects for a subsect of microbes and microbial functions which the core microbiome does not exhibit. In other

words, our study results suggest that cover crops could also modify individual microbial membership of the rhizosphere microbiome at a more complex field scale.

Our study also contributes to the work of the scientific community to bridge the gap between soil microbiome membership and soil functions mediated by microbes in the field environment. Several cover crop studies have attempted to link these two important components by combining microbe screening methodologies with ‘omics techniques. For example, Dasgupta et al. (2023) attempted to reconcile 16S rRNA and metagenomics to describe both taxonomic and potential functional diversity of microbes at the field scale. Cazzaniga et al (2023) took a step further and employed a combination of metagenomics and metatranscriptomics to differentiate between active and potentially active microbially communities, whose signals differed depending on which cover crop was planted. In our research, we were able to find nearly perfect matches between the soil microbes identified with 16S rRNA surveying and the MAGs containing genes for certain soil enzymes. We were not able, however, to correlate the soil functions measured in the field (i.e. soil enzymes, DOC concentrations) with these identified microbes and their matching MAGs.

CONCLUSION

In our interdisciplinary cover crop study, we found that cover crop species can have varying effects on soil functions. Out of the four cover crops we studied, only rapeseed and cereal rye had stimulatory effects on specific soil functions and these effects differed if measured during cover crop growth or during the following crop. For example, rape seed was found to increase soil DOC concentrations and stimulate LAP enzyme production before termination relative to the control, but these effects did not persist into the maize crop. In contrast, cereal rye, which was one of the highest biomass producers, increased soil aggregation during cover crop growth and had a legacy effect of increased soil enzyme activity relative to sorghum, which produced no spring biomass. These differing findings on soil chemical and physical properties indicate that cover crop effects do not arise from the mere addition of shoot biomass, where greater biomass leads to, for example, greater enzyme activity. Rather, cover crop identity is an important factor determining the source of nutrient inputs from biomass residue and rate of release into the soil.

In contrast to soil functions, we did not see differences in rhizosphere soil microbiome structure and diversity across the cover crops before or after their termination. Instead, we saw that living cereal rye could shift the soil microbiome at a taxon-specific level by enriching *genus Massilia*. This field finding confirms cover crops' ability to alter abundances of individual microbes, which was observed in previous controlled experiments. However, we did not find meaningful correlations between enriched *Massilia* and other detected taxa with any of the measured soil functions.

Instead, we were able to match our 16S BLAST results at >97% with a database containing MAG's identified in soil from the same research site of this study. Many of these near-perfectly matched MAG's contained genes coding for our soil enzymes of interest such as LAP, NAG, BG, and PHOS. This suggests that, given that such MAG databases on soils continue to expand and be developed, information on potential microbial function could be extracted from 16S data, which traditionally was only used to assign taxonomy. Our results are encouraging because they show that research on cover crop-soil microbiome dynamics from a controlled setting can be upscaled to the field, and they highlight that cover crop effects are detectable in a field environment in shorter timeframes. Further research should focus on refining the connection between measured soil biogeochemical parameters and microbiome membership/functional data, as a solid connection between these components would present a strong case for describing microbiome dynamics under cover cropping. Down the line, an improved understanding of the nexus of the soil microbiome, cover cropping practices, and soil functions can lead to intentional microbiome management for improved, sustainable agriculture.

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Appendix A

A)

	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6
Block 5	4	1	5	3	2	*
Block 4	2	5	3	4	1	*
Block 3	*	5	3	2	4	1
Block 2	3	*	5	1	4	2
Block 1	*	5	4	1	2	3

1- Rape Seed

2- AU Hairy Vetch

3- Sorghum

4- Control

5- Cereal Rye

* Empty plot

B)

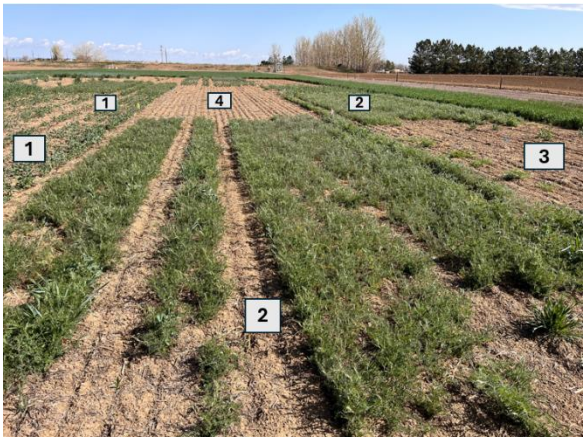

Figure S1. A) Diagram of randomized block experimental field setup including 4 different cover crop species. The field is organized into plots and blocks, which represent a unique cover crop planted as a monoculture and replicates of the cover crop, respectively. Each treatment including the control is designated a number and replicated across 5 blocks (n=5). One extra plot (*) in each block was left out of the study due to the lack of seed availability at the time of planting. B) Images of the field in May 2023 before cover crop termination and during the first sampling time point under living cover crops. Several plots of the first two blocks (blocks 1 and 2) are labelled with the same number scheme as in A).

Appendix B

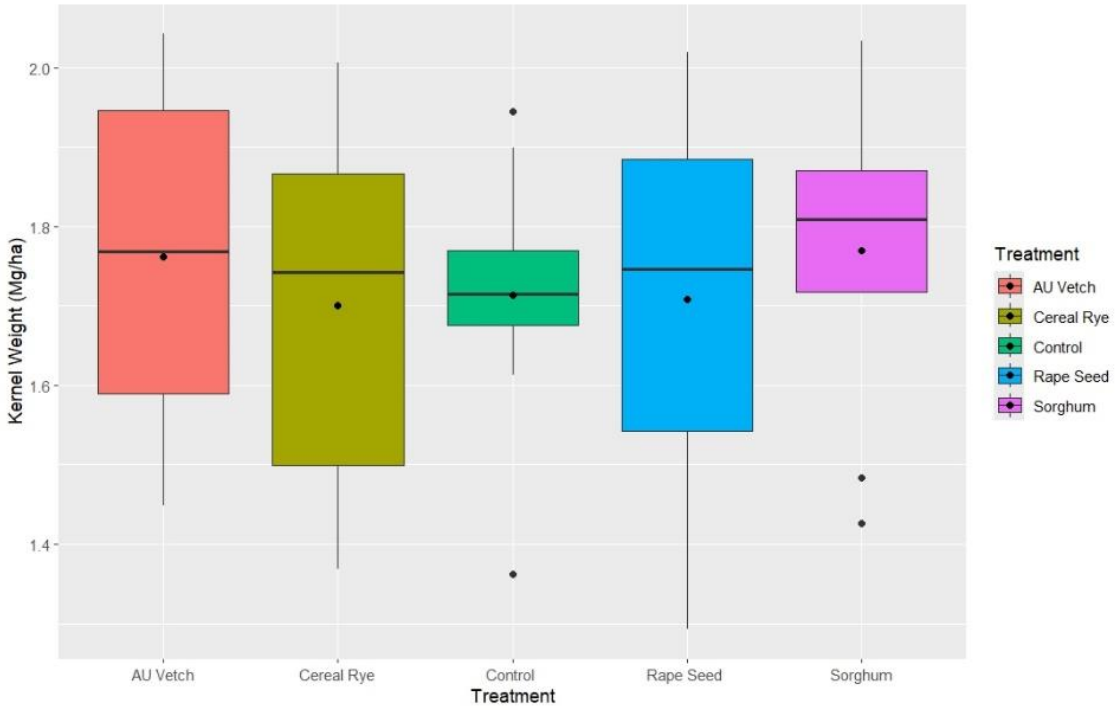

Figure S2. Boxplot of the kernel corn weight in (Mg/ha) collected in Fall 2023 across cover crop treatments after termination and under the maize crop. No statistical differences in corn yield were detected across the legacy plots, indicating that the earlier cover crops did not have a legacy effect on corn grain accumulation.