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Soil microbial and plant biomass carbon allocation within perennial and annual grain cropping systems

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ABSTRACT

Perennial crops may improve the environmental sustainability of agriculture through their continuous growth, low inputs, and high root biomass. Extensive root growth of perennial grass crops, especially, can confer benefits such as improved soil health and soil carbon (C) storage both directly through biomass production and indirectly through stimulating soil microbial communities. To test these ideas, we compared crop productivity (grain, vegetative, and root biomass), soil microbial abundance, and soil microbial activity across six cropping systems for three years (2017–2019). The six cropping systems included the perennial species intermediate wheatgrass *(Thinopyrum intermedium* (Host.) Barkw. & D.R. Dewey*;* IWG), alfalfa (*Medicago sativa* L.), and a biculture of both. Annual crop rotations included wheat, soybean, and corn (*Zea mays*). IWG monocultures produced an average of 7.4 Mg ha^{-1} of root biomass over three years, two to three times more than annual systems. Because of early spring and fall vegetative growth, IWG and alfalfa had higher canopy density for a greater duration of the growing season than annual crops. IWG also had higher soil respiration in 2017 and 2019. These growth attributes of IWG were translating to higher fungal and Gram-negative bacterial lipid biomass than alfalfa or annual crops in 2019, also the year of the highest general microbial growth. The abundant root growth, annual duration of growing period, and conducive environment for microbial growth under IWG systems indicates the potential for future C storage, which may be offset to a degree by increased soil respiration.

1. Introduction

Climate change in the Upper Midwestern U.S. includes a greater risk of flash floods, erosion, and pest proliferation that could lead to yield declines of up to 25 % for corn and soybean by mid-century (Wilson et al., 2023). There is therefore an urgent need to build resiliency through soil conservation while also reducing greenhouse gases (GHGs). Carbon stored as organic matter is the primary pathway for conserving soil and building climate adaptation in agricultural lands, but crop choice and agronomic management strongly influence the fate of soil C. Globally, 0.3–1.0 Pg C year^{-1} are lost from agricultural soils (Chappell et al., 2016) via direct transport of soil C from farm land through water erosion and runoff (Polyakov and Lal, 2004), through wind erosion (Van Pelt et al., 2013) and through respiratory CO_2 emissions (IPCC, 2019). Farming practices such as soil tillage, crop rotation, residue removal, and frequency of soil disturbance can significantly influence the rate of soil C loss (Gaiser et al., 2008; Lal, 2009; Whitbread et al., 2003). On a larger scale, it is estimated that 8 Pg, or 10 %, in total of SOC stocks in the US have been lost from maize, soybean, and wheat cropping systems (Drewniak et al., 2015). This trend can also be reversed, with a potentially high capability of agricultural soils to store C (Bossio et al., 2020).

Perennial crops that can produce significant quantities of both harvestable aboveground biomass and belowground biomass are also a strategy for enhancing soil structure and contributing to soil carbon (C) storage (Rakkar et al., 2023; Crews et al., 2014; Gelfand et al., 2013; Glover et al., 2010). C storage may be achieved in agricultural lands by maximizing plant growth, using strategic crop rotations, strategic residue management, and minimizing soil disturbance (West and Post 2002; Gan et al., 2011; Whitbread et al., 2003). The accounting of C export from agricultural fields via grain and biomass harvest, and C

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inputs from root and shoot biomass are all important in the determination of net C storage (Jans et al., 2010). Efforts to increase continuous living cover and biomass production with cover crops, perennial forages, and trees have all shown potential for C sequestration, while avoiding conversion of established forest and perennial grasslands remains the most powerful management tool for soil C sequestration (Bossio et al., 2020; Rui et al., 2022).

The storage and protection of soil C can be improved with the presence of living roots. Perennial bioenergy crops like switchgrass (*Panicum virgatum*) contain up to 7.6 Mg C ha^{-1} yr^{-1} in root biomass (0–90 cm) alone (Jungers et al., 2017). Soil C accumulation could be especially impactful with deeply rooted species, but the net benefit must be balanced with the likeliness that root biomass leads to increased soil respiration of CO_2 in the short term (Button et al., 2022; Woeltjen et al., 2024a; Kuzyakov, 2010). In addition to direct C storage in root biomass, roots and the rhizosphere can have a strong indirect influence on C storage and soil stabilization through the stimulation of soil microbial communities (Button et al., 2022; Dwivedi et al., 2019, Link, 2023). The rhizospheres of perennial grasses, for example, have greater total microbial biomass, and acid phosphatases than of annual grasses in the same ecosystem (Lambiénou et al., 2017), and can have higher microbial lipid abundances in their rhizospheres compared to bulk soil (Liang et al., 2016). The conversion of agricultural land to perennial grassland has also resulted in a 5-fold increase in microbial C and N (Rosenzweig et al., 2017). Furthermore, an increase in soil fungi specifically, which has been observed in no-till systems (Mbuthia et al., 2015), may improve soil C storage potential. This can happen directly from greater microbial biomass contribution to SOC (Malik et al., 2016) or indirectly through fungal hyphal mediation of soil aggregation (Beare et al., 1997) and slowly cycling soil C pools (See et al., 2021).

Intermediate wheatgrass (*Thinopyrum intermedium* (Host.) Barkworth. & D.R. Dewey; IWG) is a promising cool-season perennial crop that produces grain for human consumption marketed under the tradename Kernza®. IWG also produces forage and can be economically viable as a dual-use forage and grain crop in the US (Hunter et al., 2020a, 2020b, Law et al., 2022). IWG produces extensive roots that confer soil protection, improve nutrient availability, and stabilize soil C pools (Cox et al., 2006; Pugliese, Culman, and Sprunger, 2019; Ryan et al., 2018, DeHaan et al., 2018; Bajgain et al., 2020. van der Pol et al., 2022). Modeling studies suggest that IWG can increase SOC by 0.3 Mg ha^{-1} yr^{-1} compared to annual wheat (Tang et al., 2024). The extensive perennial and root growth of IWG can also improve fungal diversity and biomass (Taylor et al., 2023; Audu et al., 2022; Duchene et al., 2020; McKenna et al., 2020) and improve soil health parameters such as soil aggregation (Rakkar et al., 2023). Although some studies have explored the use of organic fertilizers and legume intercropping to meet IWG fertility (Reilly et al., 2022; Fernandez et al., 2020), how these management factors affect biomass allocation and C cycling are not well known compared to widely used cropping systems.

Our study had two main objectives: 1) to compare the aboveground and belowground biomass production and seasonal duration of canopy cover over a range of perennial, continuous cover, and annual crop rotations and 2) determine the belowground microbial community development over time in each of the respective cropping systems. The cropping systems included two IWG monocultures varying in N fertilizer source, an IWG-alfalfa biculture, an alfalfa monoculture, and two annual crop rotations of soybean-corn-soybean and wheat-soybean-wheat. We hypothesized that IWG cropping systems would allocate more biomass to roots than other crops at the expense of shoot and grain yields, while supporting higher abundance of soil microbes, soil microbial activity, and soil carbon storage.

2. Materials and methods

2.1. Study area

The experiment was conducted at the University of Minnesota Research and Outreach Center Rosemount, MN (44.684658, −93.069299). The soil type was a Tallula silt loam, a coarse-silty, mixed, superactive, mesic Typic Hapludoll with a 6 % slope and eroded topsoil with pH, P, and K levels of 6.5, ~7.6 ppm, and ~139 ppm, respectively. The 30-year annual average temperature and precipitation at this site are and 6.7 °C and 86.66 cm, respectively (See Table S1 for monthly averages). Our study years slightly deviated from these averages, where 2017 was slightly warmer, and 2018 and 2019 were slightly wetter (Table S2). The previous crop was corn removed for silage in early September 2016.

2.2. Experimental design and crop management

The experimental design was a randomized complete block with four replications. Plots were 7.5 m x 15 m. Treatments included six cropping systems: 1) IWG monoculture fertilized with 80 kg N ha^{-1} as urea (IWG+urea), 2) IWG monoculture fertilized with 80 kg N ha^{-1} as composted poultry manure (IWG+manure), 3) IWG intercropped with alfalfa (*Medicago sativa* L.; IWG+alfalfa), 4) alfalfa monoculture (Alfalfa), 5) spring wheat (*Triticum aestivum* L.) -soybean (*Glycine max* (L.) Merr.)-spring wheat rotation (wheat-soy), and 6) soybean-corn (*Zea mays* L.)-soybean rotation (soy-corn). Nitrogen (N) fertilizers and manure were applied to treatment 1, 2, and 5 in late April. Treatments 3,4, and the soybean phase of treatments 5 and 6 did not receive N amendments. See Table 1 for full management details for each cropping system. IWG monocultures were seeded in twin rows, with 41 cm between rows and 61 cm between each pair of twin rows. Planting was done using a no-till drill on September 29, 2016, after corn was harvested for silage. Alfalfa was no till seeded between 61 cm IWG rows on May 30, 2017. Annual crops and alfalfa monocultures were seeded in spring 2017 after rototilling to a depth of 20 cm. Alfalfa and wheat were seeded with 15 cm row spacing and corn and soybean were seeded with 76 cm row spacing. Spring wheat and corn received Chilean sodium nitrate (15−0−2) as a source of N. On 3 May 2018, and 9 April 2019, 67 kg P ha^{-1} bone meal (4−14−0) was added to all treatments to maintain P levels based on soil tests.

2.3. Plant biomass and grain yield sampling

Grain yield and aboveground vegetative plant biomass were collected and measured differently depending on crop type and row spacing. For all grain crops, sampling and measurement for yields occurred after physiological maturity (late July to mid-August for IWG and wheat, mid-October for corn and soybeans; Table 2). IWG grain and vegetative biomass was collected from hand sampled quadrats measuring 45.7 cm x 112 cm. Spring wheat grain and biomass was collected from quadrats measuring 61 cm× 61 cm. Seed heads for both IWG and spring wheat were dried and threshed with a laboratory thresher (Wintersteiger LD-50). For soybean, grain and stems were manually harvested from 3-m of the center two rows of each plot. For corn, grain and stover were manually harvested from 3-m of the center two rows of each plot, the ears were weighed, and then a 20-ear subsample was dried and kernels were separated from cobs. Crop residues of corn, wheat, and IWG were mowed and removed from plots after harvesting for yield. Alfalfa herbage yield was determined by harvesting on 12 June, 14 July, and 30 August 2018; and 24 June, 29 July, and 26 August 2019. Alfalfa yield was determined by harvesting a 1 m by 6 m area of the center of each plot using a small plot forage harvester (Carter Day International, Inc., Minneapolis, MN). For all crops, dry matter content of sampled and harvested plant materials was determined by drying a subsample to constant weight at approx. 35° C. Yields were then

Table 1
Agronomic management details of cropping systems from fall 2016- fall 2019.

Cropping system	Variety	Row spacing	Seed/planting rate	N Fertilizer (each spring)	Planting Dates
IWG+urea	TLI C5	Alternating 41/61 cm	15 kg ha^{-1}	80 kg N ha^{-1} urea	Sept. 29, 2016
IWG+manure	TLI C5	Alternating 41/61 cm	15 kg ha^{-1}	80 kg N ha^{-1} composted poultry manure	Sept. 29, 2016
IWG+alfalfa	IWG: TLI C5 Alfalfa - 54H91Pioneer	IWG - 41 cm Alfalfa - 20 cm	IWG – 15 kg ha^{-1} Alfalfa – 15 kg ha^{-1}	None	IWG: Sept. 29, 2016 Alfalfa: May 20, 2017
Alfalfa	54H91 Pioneer	20 cm	15 kg ha^{-1}	None	May 20, 2017
Wheat-soy	Wheat: Viking 211 Soy: MN0810CN	Wheat: 20 cm Soy: 76 cm	Wheat: 100 kg ha^{-1} Soy: 395369 plants ha^{-1}	Wheat: 80 kg ha^{-1} $NaNO_3$ Soy: None	Late May, annually
Soy-corn	Soy: MN0810CN Corn: O84–95-UP	Soy: 76 cm Corn: 76 cm	Soy: 395369 plants ha^{-1} Corn: 91429 plants ha^{-1}	Soybean: None Corn: 150 kg ha^{-1} $NaNO_3$	Late May, annually

Cropping systems abbreviations: IWG+urea = IWG monoculture fertilized with urea; IWG+manure = IWG monoculture fertilized with composted poultry manure; IWG+alfalfa = IWG intercropped with alfalfa; alfalfa = alfalfa monoculture; wheat-soy = spring wheat-soybean -spring wheat; and soy-corn = soybean-corn-soybean rotation.

Table 2
Measurement Timeline.

	Measurement	Property	2016	2017			2018			2019		
			Fall	Spring	Summer	Fall	Spring	Summer	Fall	Spring	Summer	Fall
Plants	Grain	Grain yields at maturity			X			X			X	
	Shoots	Vegetative biomass at maturity			X			X			X	
	Roots	Root biomass (0–60 cm)				X			X			X
	LAI	Plant canopy density		biweekly June-September			biweekly April-November			biweekly April-November		
Soil	Microbial biomass and activity	Microbial biomass C		X		X	X		X	X		X
		PLFA total and functional group biomass		X		X	X		X	X		X
		Enzyme activity		X		X			X	X		
	C & N content	Total carbon and nitrogen (0–60 cm)										X
	GHG flux	Respired CO_2		biweekly April-November			biweekly April-November			biweekly April-November		
Relevant Field Activity												
		Seedbed prep and planting	IWG	Alfalfa Annuals			Annuals			Annuals		
		Fertilization		X			X			X		

adjusted to a dry matter basis. We refer to stems, leaves, and dry stover as vegetative biomass in the results and discussion.

Soil cores were extracted using a hydraulic Gidding's soil probe to determine root biomass from the within-row and inter-row spaces each fall after crops were harvested (Table 2). One core with a 3.8 cm diameter by 60 cm deep was extracted from each within-row or inter-row space and separated into four 15-cm depth increments. Cores were washed of soil using a hydropneumatic elutriation system (Smucker et al., 1982), manually cleaned to remove sand and organic debris, then dried and weighed. Results presented are the sum dry weight across the four depth increments.

2.4. Plant canopy density

Leaf-area index (LAI; m^2 m^{-2}) was measured biweekly in each plot throughout the growing season, as a proxy for canopy density and plant productivity, using a Li-Cor 2200 plant canopy analyzer. Samples per plot were obtained from an average of five measurements taken in diffuse light conditions only, in an "X" pattern through the center of each plot. The "X" pattern included samples taken at points within and between planted crop rows, at angles parallel and perpendicular to the orientation of the rows. A 45° lens cover was also used to remove bias of the measurer's light attenuation. Sampling dates occurred 2017–2019, but data from 2017 was omitted from analysis because it did not encapsulate a full growing season. Sampling dates were: May 21, June 12, July 3, August 1, August 14, and October 6 in 2018; May 21, June 24, July 29, August 26, September 10, and October 18, in 2019.

2.5. Soil sampling

Soil samples were collected in late May from 2017 to 2019 by pooling 6 subsamples (3.1 cm diameter by 15 cm deep) taken in an "X" pattern with a hand probe from each experimental plot (Table 2). Soil samples were also collected each fall in late September-mid-October from 2017 to 2019 by pooling two subsamples that were taken with a hydraulic Gidding's probe (3.8 cm diameter by 60 cm deep) and divided into 15 cm depth intervals. One subsample was taken from the center of each crop row, and one from the center of the inter-row space. At each sampling, all samples were transported from the field in coolers and then partitioned the same day into fresh samples that were stored at 4° C, frozen samples that were stored at −20° C until analysis, or air dried at room temperature.

2.6. Total soil carbon and nitrogen

Combustion analysis was used to quantify total percent soil C and N on each soil sample in fall 2019 (Nelson and Sommers, 1996). Samples were air-dried and ground to pass a 2 mm sieve. A representative sample (~ 2 g) was then ground at 2500 rpm for 3 mins using a 2010 GenoGrinder (SPEX Sample Prep LLC, New Jersey, US), and 10 mg of soil was utilized to quantify total percent soil C and N using an elemental analyzer (varioPYRO cube, Elementer Inc, New Jersey, US).

2.7. Microbial biomass C

Microbial biomass C was determined using a modified chloroform fumigation extraction method (Gregorich et al., 1990). Field moist soil

from both spring and fall samplings each year as described above (Table 2) was passed through a 2 mm sieve and then duplicate 10 ± .05 g of soil were weighed for fumigated and nonfumigated analysis. Fifty µl of ethanol free chloroform was then added to each of the "fumigated" samples, followed by addition of 40 mL of 0.5 M K_2SO_4 to both fumigated and unfumigated samples. Tubes were then shaken for 4 hours, filtered frozen at −20° C. On day of analysis, fumigated samples were sparged to remove trace chloroform and analyzed on a Shimadzu TOC-L (Shimadzu, Scientific Instruments Inc., Kyoto, Japan) for total C and N analysis. Standards were diluted to 50 mg C L^{-1} using stock potassium hydrogen phthalate (1000 mg C L^{-1} KHP). Microbial biomass C was calculated by the difference in mg C kg dry $soil^{-1}$ between fumigated and unfumigated set of the same samples, using efficiency of extraction coefficients of 0.45 for carbon and 0.5 for nitrogen (Voroney et al., 2008).

2.8. Lipid analysis

Extraction of lipids from soil was performed as described in Oates et al., (2017) and Balser et al., 2019 for spring and fall collected soil samples collected at 0–15 cm depth. Briefly, 3 g of freeze-dried soil was weighed into labelled 40 mL centrifuge tubes followed by an extraction with chloroform, methanol, and citric acid buffer (0.9:1:2 ratio). The extraction was repeated 3 times followed by adjusting the reagents to a 0.9:1:1 ratio, respectively, followed by overnight phase separation. The bottom (chloroform) phase was transferred to 15 mL glass test tubes, followed by acid methylation to convert fatty-acids to fatty-acid methyl esters (FAME) before analysis on an Agilent 7890 gas chromatograph (Agilent technologies, Inc., Santa Clara, CA). Internal 13:0 lipid standards at a known concentration of 0.5 µg $µl^{-1}$ were used to convert peak areas of each fatty acid to nmol fatty acid g $soil^{-1}$. Individual lipids were used as biomarkers to indicate broad groups within the microbial community: 16: 1 ω5c for arbuscular mycorrhizal fungi (AMF; Balser et al., 2005; Gutknecht et al., 2012); 18: 1 ω9c and 18: 2 ω6,9c for other fungi excluding AMF (general fungi, GF; Balser et al., 2005; Gutknecht et al., 2012); 16: 1 ω7c and 18: 1 ω9 t for Gram-negative bacteria (Wilkinson et al., 2002); and 15: 0 iso and 17: 0 iso for Gram-positive bacteria (Wilkinson et al., 2002). Where a group had multiple indicators, we averaged them for the purpose of the manuscript. Preliminary analysis demonstrated that this approach accurately portrayed the lipid composition found in our soils. Lipid biomass (nmol g $soil^{-1}$) was calculated by summing all fatty acids 20 carbons or less in length. We took care in interpreting the AMF indicator (Ngosong et al., 2012), but the inclusion of both phospho and neutral lipids, where only fungi produce neutral lipids, added robustness to this indicator.

2.9. Extra-cellular enzyme activity (EEA)

Potential extracellular enzyme activity was measured as described by Sinsabaugh et al., (2003) and German et al., (2011). Assays were performed on frozen soil from spring and fall 2017, fall 2018, and spring of 2019 (Table 2), from the 0–15 cm depth increment. Samples were weighed to 0.5 g and mixed with 50 mL of 2.5 M Trizma base buffer at pH 7, followed by a 5 minute sonication. This soil slurry was then added to black 96-well plates that contained 4 different 4-Methylumbelliferyl linked substrates for hydrolytic enzymes (β-glucosidase and cellobiohydrolase for carbon degradation, N-acetylglucosaminidase for nitrogen acquisition, and phosphatase for phosphorous acquisition) or to clear 96-well plates containing a 3,3′,5,5′-Tetramethylbenzidine substrate for phenol oxidase and peroxidase. Hydrolytic enzyme plates were incubated in the dark for 1 hour before adding 10 µl of 1 M sodium hydroxide. Oxidative enzyme plates were incubated for 10 min before adding 30 µl of 1.8 M sulfurous acid. Fluorescence intensity or absorbance were measured on a BioTek® Synergy HT microplate reader (Agilent technologies, Inc., Santa Clara, CA). Internal standards on each plate included substrate controls. A standard curve of 0.16–2.5 µmol L^{-1} MUB with or without soil slurry added was included in order to calculate quench and emission coefficients, respectively, for converting fluorescence to µmol activity g dry $soil^{-1}$ hr −1 for hydrolytic enzymes. For oxidative enzymes, absorbance units were converted to µmol activity g dry $soil^{-1}$ hr^{-1} by correcting for substrate and plate blanks and dividing that net absorbance by a TMB extinction coefficient determined separately but under the same lab conditions.

2.10. Soil CO_2 fluxes

Soil CO_2 flux was measured biweekly during the growing season, as possible, using an Gasmet® DX4040 portable FTIR gas analyzer connected to a closed chamber system following the GRACEnet protocol for chamber measurements (Parkin et al., 2003; Parkin and Venterea, 2010). The chamber was a 16.2 × 52.7 × 10.2 cm tray constructed of 18-gauge stainless steel, wrapped in white reflective contact paper to avoid temperature increases within the chamber during sampling, and fitted with a removable 70 cm tall x 8 cm diameter PVC extension attached to the top of the chamber for accommodating vegetation (referred to as the "extension" below; Bergquist 2019). A small hole drilled into the side of the chamber and fitted with 8 cm tubing on was used to maintain atmospheric pressure but not influence gas fluxes (Parkin and Venterea, 2010). The chamber anchors were similar steel trays with the bottom removed. The anchors were inserted into the soil (~10 cm deep) so that the top rim protruded < 5 cm above the soil surface and soil was tamped around the anchor to ensure a good seal. Anchors were installed at least 24 h prior to sampling and left *in situ* during the growing season. A foam weather seal was attached to the rim of the chamber to ensure a tight seal prior to fastening the chamber to the anchor using alligator clips. The sample area volume was approximately 6250 cm^3 without the extension and 9550 cm^3 with the extension. Our chamber design was specifically intended to capture CO_2 flux from the whole root-soil system and thus our reported flux values represent the sum of both heterotrophic (microbial) and plant (primarily root) respiration at the time of sampling. Sampling was always performed between 10:00 and 16:00 hours to align with the time of greatest activity within diurnal gas flux patterns. At the time of sampling each plot, the chamber was clipped to each anchor as described, then measured for approximately 7 minutes where the data from the first two minutes were needed for equilibration between plots. Equilibration period data was then removed prior to calculations. We assumed, and validated with method testing prior to this experiment, that the temperature inside the chamber did not change during this short time of sampling. The flux of CO_2 was then calculated using the following equation (Collier et al., 2014) with the assumption that the change in concentration within the chamber increased linearly over time, which was validated based on data quality control procedures, and reported as reported as g CO_2 m^{-2} day^{-1}

$$F = S * V * A^{-1}$$

Where F = flux, S = slope of regression ($\frac{\Delta conc}{\Delta t}$), V = chamber volume, A = chamber area. Thus:

$$\frac{\left(\text{molL}^{-1} \quad \text{hr}^{-1}\right) \quad * \text{L}}{m^2}$$

2.11. Statistical analysis

All analyses were conducted with R version 3.4.4 (R Core Team 2018). For plant biomass production variables, each year was analyzed separately because different crops were present each year in the annual cropping systems and perennial crop stand age increased each year. A linear mixed-effects model (LME) (package, code: lme4, *lme*; Oates et al., 2015) was used to conduct one-way analysis of variance (ANOVA) tests with block as a random effect and cropping system as a fixed main effect. Means were compared by obtaining least-square means (package, code:

emmeans, *emmeans*; Lenth 2019) and adjusted for Tukey's HSD pairwise test of differences. Because leaf-area index (LAI) data was collected at multiple dates throughout 2018 and 2019, day was added as a main effect to the LME (including block as random effect) for that response variable. The final model for LAI included day as a factor instead of as a continuous variable due to a lower Aikake information criterion (AIC) estimate for the former model.

Due to the large significance of year from preliminary ANOVA results of LMEs of microbial data (including MBC, lipid, enzyme, and soil respiration data), years were analyzed separately with either a one-way ANOVA with cropping system as a main effect and block as a random effect, or a two-way ANOVAs using cropping system and sample date as main effects and block as a random effect for those data sampled on more than one date within a growing season. Soil respiration data for each year exhibited temporal autocorrelation and so these LME included 1st order autocorrelation structures with the appropriate time lag adjustments for each year. For 2018 soil respiration data, the initial LME analysis demonstrated an interaction between cropping system and date, and so we also performed a one-way ANOVA analysis of each date individually. Mean comparisons among cropping systems were made for each sample date using Tukey's HSD test, as described earlier. Relationships between soil moisture content and soil microbial biomass, lipids, and enzymes were tested using linear regression. Non-significant ($P > 0.05$) interaction terms were removed from LME models. Assumptions of normally distributed residuals, independence of error and constant variance were checked for each linear model using qqplots, histograms of residuals, and plots of the residuals against fitted values.

3. Results

3.1. Plant biomass

Total biomass production varied among cropping systems and from year to year, with corn in the soy-corn system in 2018 and third year IWG+manure or IWG+urea having the highest total biomass production (Tables 3 and 5). In 2017, the alfalfa and IWG+alfalfa cropping systems had approximately two times the total biomass production as soybean, which had the lowest total biomass (Tables 3 and 5, Figure S1). In 2018, the soy-corn system produced the highest biomass with 3 times that of the soybean in the wheat-soy, and IWG and alfalfa cropping systems had intermediate levels of biomass (Table 5). In 2019, IWG+manure and IWG+urea had the greatest total biomass, which was approximately 3 times higher than the total biomass of the soybean phase of the soy-corn (Table 5). In terms of the allocation of total biomass among plant tissue pools, IWG roots accounted for 61 % of the average three-year total biomass, alfalfa roots accounted on average for 36 % of the total biomass production, and roots of annual crops accounted for 27 % and 33 % of their total biomass production for soy-corn-soy and wheat-soy-wheat, respectively (Table 5). In contrast, grain only accounted for 0.04 % of total biomass in IWG systems, whereas grain accounted for 26 % soybean, 41 % wheat, and 56 % corn when present in rotations (Table 5).

Table 3
Analysis of variance (ANOVA) results to test the main effect of cropping system for grain, shoot biomass, root biomass (0–60 cm), and leaf-area index (LAI). ANOVA was run both for each entire univariate variable and separately by year because of strong effects of year on each variable.

		Plant biomass				Canopy density
		Grain	Shoot biomass	Root biomass	Total biomass	LAI
Cropping system		***	***	***	.	***
Year		***	***	.	*	***
Cropping system X Year		***	***	**	**	***
2017	Cropping system	***	***	*	NS	–
2018	Cropping system	***	***	**	*	***
	Date	–	–	–	–	***
	Cropping system X Date	–	–	–	–	***
2019	Cropping system	***	***	***	*	***
	Date	–	–	–	–	***
	Cropping system X Date	–	–	–	–	***

Abbreviations of statistical significance: - = not applicable; NS = $P > 0.05$;. = $0.05 < P < 0.10$; * = $0.01 < P < 0.05$; ** = $0.001 < P < 0.01$; *** = $P < 0.001$; Cropping systems were such that in wheat-soy = the spring wheat phase was present in 2017, soybean in 2018, and spring wheat in 2019; and for soy-corn = the soybean phase was present in 2017, corn in 2018, and soybean in 2019.

3.1.1. Grain yield

Grain yields were higher in annual systems than those from IWG (Tables 3 and 5, Figure S1). Spring wheat and soybean had approximately 10 times and 5 times the grain yields as IWG, respectively in the 2017 first year stand (Table 5). Across years and cropping systems, corn of the soy-corn-soy system produced the highest grain yield in 2018 whereas IWG consistently had the lowest grain yields. Among IWG systems, the highest grain yield was recorded in 2018 and the type of N source (fertilizer or alfalfa) did not affect IWG yield (Table 5).

3.1.2. Vegetative biomass

Vegetative biomass also varied among crops and years (Tables 3 and 5, Figure S1). Biomass production in monoculture alfalfa (the sum of three harvests per year) was 2–10 times higher than all other crops in 2017 and ~1.5 times higher in 2018. Soybean had the lowest vegetative biomass in 2017 in the soy-corn rotation and in 2018 in the wheat-soy rotation. Corn in 2018 had similar vegetative biomass as IWG treatments (Table 5, Figure S1). IWG shoot biomass increased in general from 2017 to 2019 with no consistent differences among IWG treatments.

3.1.3. Roots

Root production was generally higher in perennial cropping systems than in annuals, (Table 5, Fig. 1). In 2017, IWG+alfalfa produced more root biomass than all other cropping systems except IWG+manure (Table 5). In 2018, IWG+manure produced more root biomass (3 times and 2.5 times, respectively) than corn in the soy-corn and soybean in the wheat-soy systems, but there were no differences among IWG systems (Table 5). In 2019, IWG systems produced 2–3 times more root biomass than wheat, soybean, and alfalfa on average, but again there were no differences among the IWG treatments (Table 5, Fig. 1).

3.2. Plant canopy density

Cropping system, year, date, and the interaction among these variables all affected plant canopy density as measured by LAI, with the most notable differences being that perennial systems had higher canopy density in the shoulder seasons (Table 3, Fig. 1). LAI differed among cropping systems on every day sampled in 2018 and 2019 (Table 3, S3, Fig. 1). In 2018, all perennial cropping systems had greater LAI than annual systems until July, when the mature corn canopy was denser than other cropping systems. Within perennial systems, alfalfa had the highest canopy density in mid-June. IWG+urea tended to have lower canopy densities compared to the other two IWG systems (Fig. 1). In 2019, IWG cropping systems had higher LAI in mid-June than both alfalfa and annual cropping systems (Fig. 1). From June 2019 until IWG grain harvest in August 2019, alfalfa had lower LAI than all other cropping systems except soy in the soy-corn system (Table 3, Fig. 2). After August 2019, the only major trend was that the regrowth of all three IWG cropping systems resulted in higher LAI than for the other

Table 4
Analysis of variance (ANOVA) results to test the main effect of cropping system for soil respiration (Rs), microbial biomass carbon (0–15 cm soil depth), activity of 4 soil hydrolytic and 2 oxidative enzymes (0–15 cm), and lipid biomass of 4 major soil microbial functional groups (0–15 cm). ANOVA analysis was run both for each entire univariate variable and separately by year because of strong effects of year on each variable.

		Microbial abundance						Enzyme activity					Soil respiration	Soil C and N
		MBC	Lipid Biomass	Actino	Fungi	Gram-neg	Gram-pos	B	C	N	P	O	Rs	CN
	Cropping system	NS	NS	NS	.	NS	NS	NS	.	NS	NS	.	.	NS
	Year	*	***	**	***	***	***	**	NS	***	***	*	***	NS
	Cropping system X Year	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	NS
2017	Cropping system	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	NS
	Date	**	***	***	**	.	***	**	***	**	***	**	NS	NS
	Cropping system X Date	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2018	Cropping system	.	NS	NS	.	NS	NS	.	NS	.	NS	NS	*	NS
	Date	**	–	–	–	–	–	–	–	–	–	–	***	NS
	Cropping system X Date	NS	–	–	–	–	–	–	–	–	–	–	*	NS
2019	Cropping system	NS	NS	NS	*	.	NS	.	*	NS	*	NS	*	NS
	Date	*	NS	**	NS	NS	NS	–	–	–	–	–	NS	NS
	Cropping system X Date	NS	NS	NS	NS	NS	NS	–	–	–	–	–	NS	NS

Abbreviations of statistical significance: NS = P > 0.05;. = 0.05 < P < 0.10; * = 0.01 <P < 0.05; ** = 0.001 <P < 0.01; *** = P < 0.001; MBC = Microbial biomass C based on fumigation extraction; Lipid Biomass = cumulative biomass of all lipids less than 20 carbons in length;
Actino = Actinomycetes; Gram-neg/pos = Gram-negative/positive bacteria; Enzymes: B = β-glucosidase; C = cellobiohydrolase; N = N-acetylglucosaminidase; P = phosphatase; O = oxidative (peroxidase + phenol oxidase)

Table 5
Average dry weight biomass (Mg ha^{-1}) of grain, vegetative biomass, root biomass, or in total biomass (± SE, n = 4) for each experimental year from 2017 to 2019. Different lower-case letters denote statistical significance at P < 0.05 based on Tukey's pairwise comparison tests.

Treatment/Crop	2017	2018	2019
Grain			
IWG+urea	0.41 (0.09)^{a}	0.53 (0.03)^{a}	0.34 (0.08)^{a}
IWG+manure	0.24 (0.08)^{a}	0.57 (0.07)^{a}	0.26 (0.07)^{a}
IWG+alfalfa	0.41 (0.06)^{a}	0.53 (0.04)^{a}	0.19 (0.08)^{a}
Alfalfa*	–	–	–
Wheat-soy	3.81 (0.79)^{c}	1.14 (0.25)^{b}	1.90 (0.33)^{b}
Soy-corn	1.73 (0.12)^{b}	9.86 (0.36)^{c}	2.06 (0.11)^{b}
Vegetative Biomass			
IWG+urea	3.28 (0.26)^{b}	4.23 (0.30)^{b}	5.42 (0.34)^{bc}
IWG+manure	2.31 (0.29)^{b}	4.92 (0.54)^{b}	6.71 (0.46)^{c}
IWG+alfalfa	2.27 (0.28)^{b}	4.23 (0.46)^{b}	3.94 (0.87)^{b}
Alfalfa*	7.23 (0.50)^{c}	6.69 (0.42)^{c}	7.03 (0.41)^{c}
Wheat-soy	2.22 (0.08)^{b}	0.57 (0.09)^{a}	3.46 (0.57)^{b}
Soy-corn	0.63 (0.12)^{a}	4.74 (0.13)^{b}	1.23 (0.08)^{a}
Root Biomass (0–60 cm depth)			
IWG+urea	4.10 (0.95)^{ab}	7.32 (0.63)^{ab}	9.51 (2.37)^{b}
IWG+manure	5.66 (1.14)^{bc}	8.80 (1.72)^{b}	9.38 (0.60)^{b}
IWG+alfalfa	6.08 (0.43)^{c}	5.73 (1.01)^{ab}	7.98 (0.47)^{b}
Alfalfa*	3.93 (0.18)^{a}	5.85 (1.50)^{ab}	1.91 (0.51)^{a}
Wheat-soy	3.25 (0.79)^{a}	2.50 (0.20)^{a}	2.17 (0.68)^{a}
Soy-corn	2.22 (0.31)^{a}	2.93 (0.41)a	2.30 (0.13)^{a}
Total Biomass Production			
IWG+urea	7.79 (0.99)^{ab}	12.1 (0.50)^{bc}	15.3 (2.03)^{c}
IWG+manure	8.21 (0.97)^{ab}	14.3 (1.62)^{bc}	16.3 (0.97)^{c}
IWG+alfalfa	8.75 (0.46)^{b}	10.5 (0.76)^{b}	12.1 (0.81)^{bc}
Alfalfa*	11.2 (0.66)^{b}	12.5 (1.80)^{bc}	8.94 (0.83)^{ab}
Wheat-soy	8.47 (1.07)^{ab}	4.21 (0.50)^{a}	7.53 (0.31)^{ab}
Soy-corn	4.58 (0.48)^{a}	17.5 (0.69)^{c}	5.60 (0.24)^{a}

* *Alfalfa herbage biomass was determined from the sum of three separate harvests each year.* Cropping systems abbreviations: IWG+urea = IWG monoculture fertilized with urea; IWG+manure = IWG monoculture fertilized with composted poultry manure; IWG+alfalfa = IWG intercropped with alfalfa; alfalfa = alfalfa monoculture; wheat-soy = spring wheat in 2017-soybean in 2018 -spring wheat in 2019; and soy-corn = soybean in 2017-corn in 2018-soybean in 2019.

cropping systems ($p < 0.001$, Fig. 1).

3.3. Soil respiration (CO_2 flux)

As with LAI, cropping system, year, date, and the interaction among these variables all affected soil respiration (Table 4, S4). Because of significant changes within years and sample dates (Table 4), we focused mainly on statistical testing for cropping system effects separately for each date. The CO_2 flux from systems including IWG were higher than those of the soy-corn system on 13-June, 28-September in 2017, and 6-May and 22-May in 2018 ($p < 0.01$, Fig S2). Alfalfa monocultures also had higher CO_2 flux than annual systems on 6-May and 22-May in 2018 ($p < 0.01$, Fig S2). In 2019, fluxes of IWG+alfalfa were higher than the wheat-soy system on 16-September and higher than the soy-corn system on 25-September ($p < 0.01$, Fig S2). Fluxes from all three IWG systems were higher than wheat on 25-September (mean *P*-value = 0.02, Fig S2).

3.4. Soil C and N and extracellular enzyme activity

There were no effects of cropping system on total soil C or N (Table 4). There were few effects of cropping systems on extracellular enzyme activity (EEA), observed only at the May 2019 sampling date (Tables 4 and 6). Activity of cellobiohydrolase was higher ($p = 0.03$) under annual wheat compared to IWG+urea in 2019 and activity of β-glucosidase was marginally higher for this same cropping system comparison in 2019 ($p = 0.11$). Phosphatase activity was higher under annual wheat compared to IWG+urea and soybean in 2019 ($p = 0.04$ and $p = 0.02$, respectively). We saw no differences in the other C decomposition enzymes (peroxidase, phenol oxidase, or β-glucosidase), or N acquisition (N acetyl glucosaminidase) enzymes among the cropping systems in this study.

3.5. Microbial total biomass C and lipid biomarker abundance

Soil microbial biomass C (MBC) and total lipid biomass varied in magnitude through time but no differences were found among cropping systems (Table 4). One exception of marginal significance was that in 2018 IWG+manure had slightly higher MBC (mean = 197 mg C kg^{-1} soil) than corn (mean = 140 mg C kg^{-1} soil) ($p = 0.06$). Mean total MBC

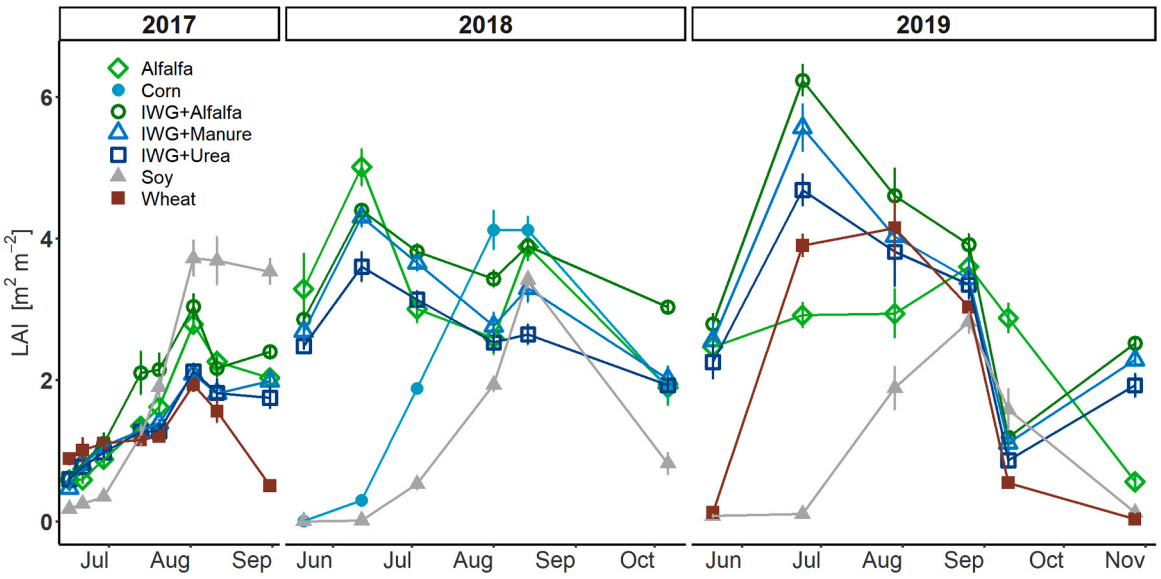

Fig. 1. Leaf area index (LAI) from 2018 and 2019. Error bars represent standard error from the mean (n = 4). LAI is reported in dimensionless units (m^2 m^{-2}). Alfalfa vegetative biomass was harvested on 12 June, 14 July, and 30 August of 2018, and 24 June, 29 July, and 26 August of 2019. Cropping systems abbreviations: IWG+urea = IWG monoculture fertilized with urea; IWG+manure = IWG monoculture fertilized with composted poultry manure; IWG+alfalfa = IWG intercropped with alfalfa; alfalfa = alfalfa monoculture; wheat-soy = spring wheat-soybean -spring wheat (in 2017,2018, and 2019, respectively); and soy-corn = soybean-corn-soybean (in 2017,2018, and 2019, respectively).

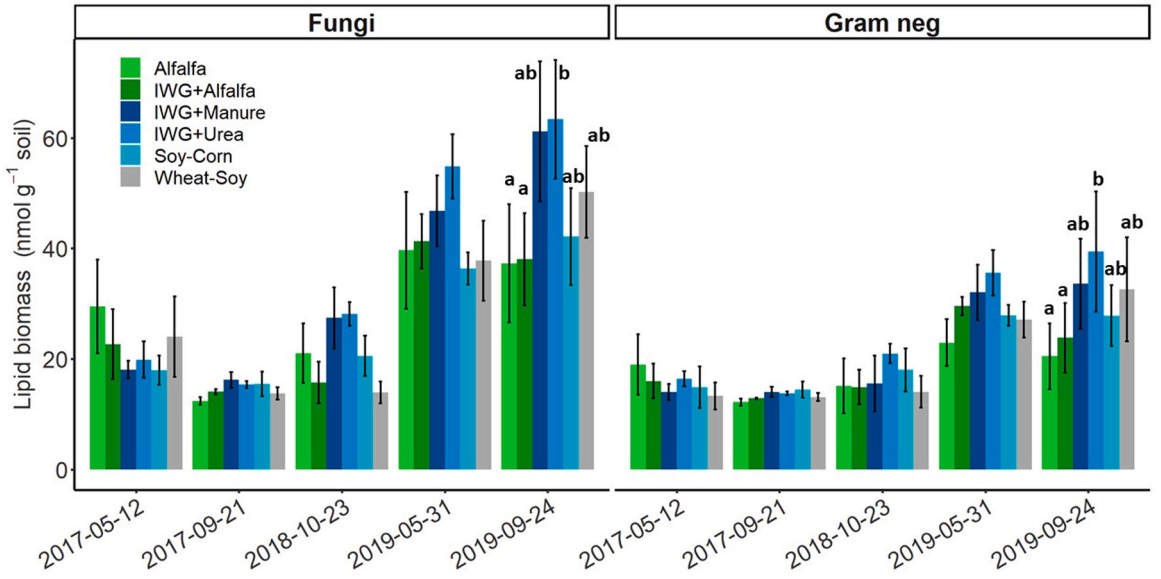

Fig. 2. Lipid biomass (0–15 cm; ± SE, n = 4) of two functional groups A) fungi, and B) Gram-negative bacteria from three years of fall samples and two years of spring samples. Dates in order from left to right; 2017 May 12, 2017 Sep 21, 2018 Oct 23, 2019 May 31, 2019 Sep 24. Letters denote significant difference based on posthoc pairwise comparison using Tukey's HD tests, α = 0.05. Cropping systems abbreviations: IWG+urea = IWG monoculture fertilized with urea; IWG+manure = IWG monoculture fertilized with composted poultry manure; IWG+alfalfa = IWG intercropped with alfalfa; alfalfa = alfalfa monoculture; wheat-soy = spring wheat-soybean -spring wheat; and soy-corn = soybean-corn-soybean rotation.

(including spring and fall samples from all cropping systems) was 113, 164, and 201 mg C kg^{-1} dry soil for 2017, 2018, and 2019, respectively.

Within the soil microbial community, only fungal and Gram-negative bacterial lipids were responsive to cropping systems (Table 4, Fig. 2). In fall of 2019, Gram-negative bacterial lipid biomass and fungal lipid biomass were both highest in the IWG+urea system, but were only significantly higher than the alfalfa monoculture and the IWG+alfalfa biculture when compared to the other cropping systems ($p = 0.04$ for both biomarkers; Fig. 2). Mean total lipid biomass (including spring and fall samples from all cropping systems) of each year was 42, 46, and 93 nmol g^{-1} dry soil for 2017, 2018, and 2019, respectively.

4. Discussion

Our goals were to compare biomass production and grain yield of six perennial versus annual cropping systems, and to compare belowground soil microbial parameters between the cropping systems as well. The implications of this study reinforce the distinctions in phenology and biomass allocation for perennial versus annual cropping systems, and differences in soil microbial characteristics were observed among cropping systems only after three years despite the apparent distinctions in crop canopy density, root growth, and soil management. Perennial and annual cropping systems were uniquely but not unexpectedly

Table 6
Average soil enzyme activity (0–15 cm; ± SE, n = 4) in 2019, the year that cropping system significantly influenced these enzyme activities. Enzyme activity is represented only from the May 31 sample date when the assay was performed in 2019.

Crop	Enzyme activity (nmol h^{-1} g^{-1} soil)				
	B	C	N	O	P
IWG+urea	81 (4)^{a}	7 (1)^{a}	10 (1)^{a}	147 (50)^{a}	199 (8)^{a}
IWG+manure	87 (9)^{a}	10 (1)^{ab}	13 (2)^{a}	194 (83)^{a}	212 (22)^{ab}
IWG+alfalfa	115 (10)^{a}	12 (2)^{ab}	15 (2)^{a}	356 (160)^{a}	244 (17)^{ab}
Alfalfa	96 (9)^{a}	8 (1)^{ab}	15 (2)^{a}	415 (69)^{a}	217 (20)^{ab}
Wheat-soy	120 (16)^{a}	14 (1)^{b}	14 (2)^{a}	319 (149)^{a}	282 (46)^{b}
Soy-corn	86 (12)^{a}	9 (2)^{ab}	14 (3)^{a}	277 (55)^{a}	191 (37)^{a}

Enzyme abbreviations: B = β-glucosidase; C = cellobiohydrolase; N = N-acetylglucosaminidase; P = phosphatase; O = oxidative (peroxidase + phenol oxidase). Cropping systems abbreviations: IWG+urea = IWG monoculture fertilized with urea; IWG+manure = IWG monoculture fertilized with composted poultry manure; IWG+alfalfa = IWG intercropped with alfalfa; alfalfa = alfalfa monoculture; wheat-soy = spring wheat-soybean -spring wheat; and soy-corn = soybean-corn-soybean rotation.

different in their biomass C allocation as well. Accounting for agronomic function of the crop, the most vegetative biomass production was from alfalfa, the most grain production from corn, and the most root production from IWG. Results support our hypothesis that the increased allocation of biomass to roots in perennial systems influenced soil microbial communities and/or soil C dynamics. Early and late season plant canopy development, with increased soil respiration that paralleled this growth, was observed only in perennial systems. Theoretically this increased growth should translate to increased soil carbon but the duration of the experiment may have been too short to observed changes on the total percent carbon that was measured. Furthermore, lipid biomarker abundance suggested enhanced development of soil fungal and Gram-negative bacterial communities in association with the root systems of IWG after three years of crop development.

4.1. Annual duration of crop growth and plant biomass allocation is greater in perennial systems

The LAI and soil respiration data provide insight to seasonal patterns of crop growth for perennial and annual cropping systems. The plant canopy density of perennial crops established quickly in the spring, 5–8 weeks earlier than canopy establishment of annuals, and they also grew back after each alfalfa harvest and after IWG grain harvest in the fall. The longer growth season of perennials alters the soil environment by protecting topsoil from erosion and nutrient losses in the early spring and late fall. Higher soil respiration rates of IWG in the spring and fall, both from roots and heterotrophic respiration, imply some C loss where annual crops are neutral, which has also been suggested by ^{13}C labeling studies (Woeltjen et al., 2024a). These losses can be offset though by C assimilation during photosynthesis (Wiesner et al., 2022). The extended growing season of IWG is thus a critical factor in evaluating net ecosystem exchange and its capacity to retain C. De Oliveira et al., (2018) reported that IWG can act as a C sink in the US Central Great Plains region using a micrometeorological approach with eddy covariance, but more research is needed for other climates and using different approaches (De Oliveira et al., 2018).

Among all cropping systems in this study, IWG allocated the most biomass to roots and thus IWG monocultures or mixtures with alfalfa may be good candidates for increasing C sequestration in soil (Peixoto et al., 2022). Harvested shoot biomass and root biomass accounted for most of the annual C assimilation, however, it excluded IWG regrowth in the fall as well as fine root turnover between sampling times. Root biomass that is sampled once a year (the case for this study) does not capture the full extent of root growth dynamics, and productivity and mortality occurs at different rates throughout the growing season, and also between plant species (Vivanco and Austin, 2006). Vegetative shoot biomass of IWG was only harvested at peak (summer) biomass even though IWG fall biomass regrowth following grain and straw harvest in summer has been reported to range from 1.0 to 2.8 Mg ha^{-1} in the fall (Hunter et al., 2020b; Pinto et al., 2022). Thus our results may be an underestimation of total potential above ground biomass production from IWG.

4.2. Microbial enzyme activity and soil respiration responses varied over time

An effect of cropping system on soil microbial communities was present in the third year of production, at which point there were differences in EEA and lipid biomarker abundances. This was consistent with a similar study on IWG and soil food webs that did not observe changes in soil microbial communities until three years after initialization of the study (Sprunger et al., 2019), and in the same respect, we observed no changes in soil biological communities after 2 years in the same soil and research station in another study (Rakkar et al., 2023). However, in other recent studies of IWG, researchers found evidence that there can be significant increases of arbuscular mycorrhizal (AM) fungal lipid markers by the second year of growth (Duchene et al., 2020; Audu et al., 2022; Link, 2023). This change in the soil microbial community under IWG, regardless of the time needed to develop those changes, may be a result of perennial root systems' capacity for facilitating microbial growth in the rhizosphere (Liang et al., 2016; Jesus et al., 2016). The establishment of a dense root system in IWG could be provisional to the soil food web in the form of root exudation, rhizodeposition, and the transport of water and nutrients (Woeltjen et al., 2024b; Rakkar et al., 2023). All of these factors contribute to nutrient availability in the rhizosphere and, by also expanding the labile C pool from these inputs, influence the short-term soil C cycle (Puget and Drinkwater, 2001; Hawes et al., 2002). Compared to annual crops, the continuous living roots in the IWG cropping system are also likely to foster and conserve fungi, particularly AM fungi, because of their potential for mutualistic symbiosis (Bever et al., 2009; McKenna et al., 2020).

Soil EEA, involved in the decomposition of organic molecules like cellulose and proteins, was found to increase in both perennial and annual cropping systems at different times, suggesting complexities of this assay that warrant further testing. Two enzymes involved in the breakdown of cellulose, cellobiohydrolase and β-glucosidase, had greater activity in IWG+manure compared to IWG+urea which could be related to microbial responses to the organic matter added to the soil with the application of manure fertilizer. Similar studies have found that manure fertilization can lead to greater short-term C availability based on higher levels of mineralizable-C (Hurisso et al., 2016; Sprunger et al., 2019). However, the activity of these two cellulolytic enzymes, as well as the activity of phosphatase, was significantly greater in an annual wheat-soy-wheat crop rotation compared to IWG+urea in our third experimental year. This result unexpectedly contradicted the results of soil lipid indicators between cropping systems, but microbial assays are very prone to environmental influence, and it is possible the timing of these samples (late spring 2019) in annual wheat corresponded with a rapid priming of soil microbes in response to labile C in the previous years' litter and decaying roots. Another possibility is the growing evidence that annual wheat may be effective at stimulating microbial growth and activity (Woeltjen et al., 2024; Taylor et al., 2024).

The respiratory soil losses of CO_2 can be significant where there is high root biomass and active soil microbial communities (Drewniak et al., 2014; Button et al., 2022; Woeltjen et al., 2024b), so it would have been expected for us to also see higher soil respiration in IWG cropping systems. While we did observe this, it was only at a few select timepoints with no clear trend present.

4.3. Soil fungal and bacterial responses were strongest after three growing seasons

These results support the idea of the rhizosphere as a hot spot for soil microbial activity and suggests that in some systems such as ours, time and minimized disturbance of the root systems are needed for these relationships to develop (Guggenberger et al., 1999). Increased microbial biomass, especially fungi, and root biomass have been shown to increase SOC accumulation in other perennial grasses (McGowan et al., 2019). However, the increase of Gram-negative bacteria, which represent only a small fraction of soil bacteria (Millar and Casida, 1970), was unexpected. Another study of soil lipid biomarkers found an increase of Gram-positive bacteria under no-till cropping systems, but no change in Gram-negative markers (Mbuthia et al., 2015). Despite sharing the absence of soil disturbance, no-till cropping systems are not identical to perennial systems, and perhaps the constant of a living (and large) root system in a perennial crop protects Gram-negative bacteria that are more sensitive to environmental stress. A comparison of restored tallgrass prairie and agricultural cropland (including no-till systems) found that the restored prairie had increased fungal activity and total soil C (Bailey et al., 2002), indicating that perennial grasses more than tillage regime were responsible for the C gains. The connection between fungal activity and higher soil C storage has been tied to the capacity for fungal mycelia to improve soil aggregate stability and thus protect SOC from mineralization (Beare et al., 1997). Positive associations between fungal activity and increasing soil C may also be related to the ability of fungal mycelia to breakdown lignin and other large and more complex organic residues that, from their decomposition, subsequently produce SOC by-products which are retained for longer in the SOC pool (Malik et al., 2016). However, some fungi are also equipped to initiate further decomposition of these recalcitrant by-products if soil resources, especially C and N, are limited (Fontaine et al., 2011).

Importantly, the increase of soil fungal lipids we observed in IWG+urea was greater than in annual crops but only statistically greater than alfalfa and, surprisingly, IWG+alfalfa—the latter two cropping systems contained the smallest amount of fungal and Gram-negative bacterial lipids in 2019. This opposite response, where a perennial grass was apparently facilitating soil microbial growth while a perennial legume appeared to suppress microbial growth, was unexpected. This contradicts the results of a recent study investigating a similar comparison of IWG and alfalfa (Peixoto et al., 2022). Peixoto et al., 2022 attributed higher microbial biomass in alfalfa to an increased concentration of amino acids in the alfalfa rhizosphere compared to IWG. However, an important distinction between this study and Peixoto et al., 2022 is that Peixoto et al. sampled rhizosphere soil exclusively whereas our study homogenized bulk and rhizosphere soil together from each field plot. Peixoto et al., 2022 applied a higher N fertilizer rate to IWG as well, which may influence rhizodeposition (Sainju and Allen 2023).

The difference in soil microbial biomass between IWG and alfalfa from this study could be related to differences in plant root architecture, exudate chemistry, or a combination of both. For example, alfalfa typically invests more energy into tap roots that can account for more than 90 % of alfalfa's total root system (Louarn et al., 2015). Unlike alfalfa, IWG root systems have a higher proportion of small-diameter fine roots (Sprunger et al., 2019). This difference in root architecture might affect soil microbial communities in the rhizosphere, especially in the surface soil (0–15 cm) where IWG produces many fine lateral and crown roots. With a more fibrous root structure than alfalfa, IWG may have the capacity to retain greater microbial biomass in mycorrhizal associations and in proximity to the root system where exudates and rhizodeposition can provide additional energy and substrate.

Because fungal and Gram-negative bacterial lipid biomass of the IWG+alfalfa was significantly lower than IWG+urea in 2019, this may indicate a potential suppressive or selective recruitment effect the alfalfa roots are having on the soil microbiome even when grown together with IWG. Despite this seemingly negative result, alfalfa has been shown to increase SOC in other studies looking at inter-seeding in rangeland ecosystems (Mortenson et al., 2004) and in pure stands of a northern temperate climate (Angers, 1992), as well as support nitrogen needs of a companion grass such as IWG (Tautges et al., 2018).

The potential for long-term soil C storage may also be greater when soil fungi are abundant in the agroecosystem (Bailey et al., 2002; Six et al., 2006). However, the mechanisms behind SOC accumulation in fungal-rich soil are not completely understood (Six et al., 2006), as well as the effects of rhizodeposition on soil C cycling that are difficult to quantify but very important for future research (Pausch and Kuzyakov, 2018). Furthermore, the effects of perennial roots on soil microbial communities diverged in this experiment, indicating that other attributes of the plant, beyond the presence of continuous living roots, are responsible for influencing soil microbial communities and thus SOC.

5. Conclusion

This work underlies a question of growing importance in global agriculture which is, how do we balance the needs of food production (C outputs such as grain) with the soil integrity (C inputs such as roots) that can support both ecosystem resilience and longevity of food production? Annual and perennial cropping systems have distinct life cycles that lead to different biomass allocation and this in turn can influence C balance and microbial activity in the soil. Annual crops allocated more of their biomass to seed production, and perennial crops allocated most of their biomass to roots (IWG) and shoots (alfalfa). Perennial crops also remained productive and covered the soil for a longer duration of the growing season, potentially physically protecting the soil and continuing to develop an extensive root system. We observed an increase in fungal and Gram-negative bacterial biomass in IWG systems in the third growing season. However, further investigation is needed to explain the contradiction of soil microbial influence from alfalfa which did not have the same tendency to increase soil fungi and bacteria that was observed in IWG. Since alfalfa is both a perennial and a legume, these results were unexpected. Furthermore, the intercropping of IWG and alfalfa exhibited a reduced soil microbial response similar to alfalfa monoculture, suggesting that alfalfa may have a strong influence the soil microbiome in an intercropping system. Since responses were strongest after three growing seasons, and in the highest year of microbial growth, further research should consider the intersections between root systems, annual climate, and conditions for microbial growth from year to year. More synthetic research on the development of plant-soil systems with respect to different cropping systems, within the context of local edaphic factors, will better inform the adoption of practices which will foster societal crop production needs and improved environmental outcomes from those systems.

CRediT authorship contribution statement

Gutknecht Jessica LM: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Jungers Jacob:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Bergquist Galen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Wyse Don:** Funding acquisition. **Rakkar Manbir:** Writing – review & editing, Methodology, Investigation. **Sheaffer Craig:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Galen Bergquist, Craig Sheaffer, Jessica Gutknecht, and Jacob Jungers reports financial support was provided by General Mills Foundation. Galen Bergquist, Craig Sheaffer, Jessica Gutknecht, and Jacob Jungers reports financial support was provided by Minnesota Clean Water Fund via the UMN Forever Green Initiative. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2025.109535.

Data availability

Data will be made available on request.

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