IMPACTS OF CROP ROTATIONS AND NITROGEN FERTILIZER ON SOIL BIOLOGICAL FACTORS IN SEMI-ARID MONTANA

by

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DEDICATION

I would like to dedicate this paper to my family and my godmother for inspiring me to seek this degree, to my scientist peers and mentors, and to Montana farmers.

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ABSTRACT

Evaluating the effects of cropping and fertilizing techniques is key to informing agricultural best practices. We must continue monitoring how we manipulate soils in order to preserve and cultivate high-quality soil ecosystems that can support us in the face of climate change and widespread soil loss and deterioration. We assessed the effects of common agricultural practices in Montana by measuring biological indicators of soil quality in the 18th year of a field plot experiment with 100% and 50% the recommended rate of synthetic nitrogen (N) fertilizer and crop rotations incorporating wheat, fallow, and legumes. The biological indicators measured were four soil extracellular enzymes, potentially mineralizable N (PMN), and microbial biomass. We sampled once in spring 2020 and subsampled in the fall. We also tested whether enzymes and PMN were correlated to aboveground plant residue, which was represented by the sum of the dried plant mass from past two years left on the plots after harvest. Plant residue was positively correlated with the C, N, and S-cycling enzymes and to PMN. The positive correlation between PMN and residue reflects that increased biomass inputs could increase easily mineralizable N. Soil with the high N-rate had a slightly higher geometric mean enzyme activity. This could be from the resulting increase in plant residue. The high N-rate treatment slightly decreased soil PMN but was not affected by crop rotation treatments. Fallow systems had lower enzyme function overall, indicating a lessened fertility and decomposition rate compared to continuously cropped treatments, which keep the soil covered with a crop for more months out of the year. The positive correlations of plant residue, along with the general lower performance of the fallow systems, especially the tilled fallow rotation, support that aboveground biomass inputs are a driver in soil ecosystem function. Continuous no-till crop rotations have increased aboveground plant organic matter, which could increase nutrient cycling and decomposition, and thereby soil biological quality and fertility.

IMPACTS OF CROP ROTATIONS AND NITROGEN FERTILIZER ON SOIL BIOLOGICAL FACTORS IN SEMI-ARID MONTANA

Introduction

Evaluating the effects of agricultural practices is essential to informing land management. One method to assess the effects of agricultural practices is to measure soil quality, which is “the capacity of a soil to function . . ., to sustain biological productivity, maintain environmental quality, and promote plant and animal health” (Doran and Parkin, 1994). The research discussed herein measured soil biological quality in the 18th year of a field plot experiment focused on wheat (*Triticum aestivum*) and legume systems with treatments of 100% and 50% the recommended rate of synthetic nitrogen (N) fertilizer and seven crop rotations, or planned sequences of crops, in southwestern Montana (Table 1).

Table 1. Simplified descriptions of crop rotations, all incorporating spring and winter wheat. Pea refers to both spring and winter pea. All rotations were no-till, except Tilled Fallow.

1. Tilled Fallow	Fallow - Wheat with conventional tillage
2. No-till Fallow	Fallow – Wheat
3. Continuous Wheat	Spring Wheat - Winter wheat
4. Pea-seed	Pea harvested for seed – Wheat
5. Pea-hay	Pea harvested for hay- Wheat
6. Pea-cover	Pea chemically terminated as brown manure – Wheat
7. CRP/Pea-seed	Grass/alfalfa mixture for 10 years, starting in 2002 and then converted to Wheat - Pea-seed for 2013-2019

Crop rotations have implications for soil ecosystem function and fertility (Acosta-Martinez et al., 2007). For example, incorporating legumes into a crop rotation can provide a N benefit to the next crop (Zentner et al., 2001) and economic stability (Miller et al., 2015). Increased N availability and biomass should positively affect many soil biological factors (Kim et al., 2020), although inorganic N fertilization is also associated with certain negative consequences, such as N leaching into water (Sigler, Ewing, and Jones, 2018) and soil acidification (Niu and Tian, 2015). For this study, we measured multiple soil biological indicators related to soil ecosystem function and nutrient cycling to assess the impacts of N fertilization and crop rotations. To assess mechanisms affecting soil biological quality in another way, we also tested whether enzymes and potentially mineralizable N (PMN) were correlated to early season wheat root biomass and aboveground plant residue (residue), which was represented by the sum of the dried plant mass from past two years left on the plots after harvest. Biological indicators included the activity of five extracellular enzymes, PMN, and microbial biomass.

Extracellular enzymes are considered short-term biological indicators of nutrient cycling and decomposition (Raiesi and Salek-Gilani 2018, Veres et al., 2015) and represent the equilibria of substrate, metabolism, and energy use (Sinsabaugh, Hill, and Follstad Shah, 2009). Bacteria, plants, and fungi exude extracellular enzymes into the soil, but microbes are responsible for most extracellular enzymes (Burns et al., 2013). Extracellular enzymes are excreted into the soil matrix to catalyze chemical reactions that break down corresponding larger molecules. These reactions aid in the breakdown of organic matter in soil and create by-products available for microbial consumption. They can also stay longer in the soil depending on the environment and the recalcitrance of the molecules is organic substrate (Burns et al., 2013). The extracellular enzymes measured and the nutrient cycle they are associated with were beta-glucosidase (C), beta-glucosaminidase (C/N), arylsulfatase (S), acid phosphatase (P), and alkaline phosphatase (P). The phosphatases are the only enzymes included that tend to have a negative relationship between their concentrations and their respective nutrient availability (Bandick and Dick, 1999).

PMN is a laboratory measurement representing how much organic N could be mineralized and made available in a typical growing season. PMN shows how much soil organic matter can readily be transformed into inorganic N, encapsulating the difference between total mineralization and uptake by soil organisms (Gregorich et al., 1994). As N availability increases and aboveground plant residue increases, PMN could increase (Kuzyakov et al. 2000), or N additions could suppress mineralizing bacteria, resulting in less PMN (O'Dea et al., 2013). Early season wheat root biomass could be correlated to PMN and enzyme activity if the roots are affecting nutrient cycling and N mineralization. If larger roots are negatively correlated with extracellular enzyme activity, that could indicate that wheat roots have increased biomass with decreased nutrient cycling, perhaps to forage for more nutrients. Or, as roots in this experiment are fertilized, perhaps there would be no correlation. The indicator of microbial biomass was measured by comparing microbial respiration rates over a short period of time and provided a gauge of microbial productivity. The procedure used for measuring microbial biomass produced respiration rates for microbial life showing which soils have more microbes that are readily alive or revivable in our soils in the laboratory. Microbial biomass generally should increase with greater N availability and residue inputs (Haynes, 2005).

Residue inputs varied among treatments with crop type and how much residue was returned to the plots. Residue inputs also changed depending on whether fallow was incorporated and whether the crop was terminated and left on-site, cut for hay, or cut for grain. Residue inputs should drive many of the biological variables patterns, as they are a primary substrate addition to the soil food web (Bandick and Dick 1999). For example, the amount of biomass removed was an integral component differentiating soil quality between cover crops, along with timing and tillage (Thapa et al., 2021). Soil with increased biomass additions should have increased C, N, and S enzymatic levels (Brennen and Acosta-Martinez, 2019); therefore, enzyme activity in fallow treatments should be lower than the other rotation treatments. Increased N availability also could also stimulate extracellular enzyme activity (Alster et al., 2013).

The objectives of the study were to: 1) measure effects of crop rotation and N fertilizer on soil biological quality with extracellular enzyme activity, PMN, and microbial biomass; 2) test whether residue and early season wheat root biomass are positively correlated to extracellular enzyme activity and PMN; and 3) assess whether a subset of enzymes measured in the fall after wheat harvest maintained similar patterns among crop rotations as in the spring.

Methods

Study design

The Greenhouse Gas Reduction Study contained seven distinct crop rotations with either high or low rates of N fertilizer (urea) for 18 years. It was a randomized 4-block design with split-plots in which each block (replicate) had seven crop rotation treatments, and each rotation treatment plot was split in half by N rates. The high rate was 100% of the recommended rate (52 kg N/Mg grain), and the low rate was 50% of the recommended rate (26 kg N/Mg grain). The treatment area at the split-split plot (the high or low N side of a crop rotation) was 1.8 m by 22.9 m. The seven crop rotations included: two wheat fallow systems (either tilled or no-till), continuous wheat, three systems with wheat alternated with a legume, and a mixed perennial/annual system that initially started with 10 years of grass and alfalfa and was followed by 8 years of pea-wheat rotations (see Tables A1 and A2 in appendix for crop rotation history).

The legume rotations had different manipulations on when and how much biomass was harvested. The Pea-hay rotation had most of its aboveground biomass removed as hay, whereas the Pea-cover rotation was terminated with herbicide and left on the plot for cover. The Pea-seed rotation refers to the peas being harvested for grain with stubble and plant residue remaining on the plot.

Site characteristics

The study took place at Montana State University's A. H. Post Agronomy Research Farm, near Bozeman, MT (45.6737° N, 111.1559° W). The soils are classified as Amsterdam silt loams, which are mollisols that originated from loess mixed with volcanic ash. The site is well-drained with slopes from 0-4%. Texture for the upper 10 cm is approximately 9% sand, 9% clay, and 83% silt (Dusenbury et al., 2008).

Sampling design

Soils were sampled May 28th, 2020 for soil and root cores on each rotation and subsampled on October 23rd, 2020 for soil cores in only three of the crop rotation treatments. The primary sampling was done in the spring as the wheat crop was beginning to grow. Seven cores (15 cm long x 2.5 cm diameter) per subplot were sampled with a soil push probe and composited to represent one sample per subplot, 56 samples total. Samples were collected systematically in a zig-zag pattern along the middle transects of plots to avoid edge effects, rodent holes, and bare patches. To reduce contamination, soil probes were wiped and sterilized with 95% ethyl alcohol between subplots. Samples were air dried 3 hours, sieved to 2 mm, and put into storage at 4 °C with a one-month limit to complete analyses. Wheat roots were sampled with a Eijkelkamp soil auger. Two soil cores (15 cm long by 7.5 cm diameter) were taken on either end of the plot, from a wheat row that was representative of the plot. The 112 cores were stored at 4 °C until processing.

Extracellular enzyme activity

To assess extracellular enzyme activity, soil was mixed with a para-nitrophenol (PNP) dye-labeled substrate, and the filtrate absorbency was measured with a spectrophotometer as per methods from Dick (2011) and Parham and Deng (2000). First, 1 gram of field-moist soil and the PNP labeled enzyme-specific substrate were added to a buffer solution at a specific pH and incubated at 37 °C for one hour. Samples were shaken gently by hand for 2 seconds before incubation to mix the solution slightly. Two solutions of bases (stopping agents) were added to the sample to terminate the reaction, which were 0.5 M $CaCl_2$ and either 0.5 M NaOH or 0.1 M THAM, depending on the enzyme. Afterwards, the sediments were filtered through Whatman 2 filters. The absorbency of the resulting filtrate was measured at a wavelength of 410 nm, except for arylsulfatase, which was run was at 420 nm. Two lab duplicates and one control were analyzed per sample for all enzymes. The control consisted of soil slurry with stopping agents in which the dye-labelled substrate is added after the stopping agents and immediately before filtering. Six 'blank' samples with no soil or dye were included for every 56 samples.

The filtrate absorbance was measured at a specified wavelength with a spectrophotometer, and absorbance was converted to micrograms (µg) PNP using the linear relationship of standard curves that plotted absorbency versus known concentrations of PNP (Lambert-Beer). Absorbance values were substituted into the equation for the best-fit line to solve for the concentration of µg PNP (Dick 2011; Parham and Deng, 2000). Finally, the field moist weight was adjusted to a dry weight equivalent, calculated with the gravimetric method (oven dried 105 °C for a minimum of 24 hours). Acid and alkaline phosphatase values were summed to create one variable, "phosphatase," for simplification and to achieve a more normal sample distribution. Final enzyme activity values are in µg PNP reacted per gram dry soil per hour (µg PNP/ g soil/ hour). The geometric mean for enzymes was calculated by taking the 4th root of the product of the 4 enzymes.

Potentially mineralizable N

Ammonium (NH_4) concentration was calculated by taking the difference between initial PMN and PMN after a 14-day incubation (Keeney and Nelson, 1982). For each sample, lab triplicates were made for the initial PMN reading, and then separate triplicates were made for the incubated PMN reading. Each lab sample received 5 g of field-moist soil and 12.5 mL of DDI water. For the initial reading, NH_4 was extracted after the water was added with 12.5 mL of a 2 M potassium chloride (KCl) extraction (making the solution 1 M KCl) and filtered through Whatman 5 filters. Then, the filtrate for the initial PMN samples was frozen immediately.

For the incubated lab samples, N_2 gas was added through a septum to create anaerobic conditions. The samples were placed in a water bath at 30 °C for 14-days. Upon removal, NH_4 was extracted with the same procedure as used for the initial triplicates. Lab blanks with only water and KCl were also included for both initial and incubated tubes and subtracted from respective samples in calculations (Keeney and Nelson 1982). NH_4 extractions were analyzed with a cadmium reduction analyzer (Lachat Instruments, Loveland, CO; Robertson et al., 1999).

Microbial biomass

Substrate-induced microbial respiration (SIR) was used as a proxy for microbial biomass, and results reported as microbial respiration rate (Fierer, Schimel, and Holden, 2003). Duplicates of 5 g field moist soil with 10 mL of an autolyzed yeast and DDI water solution were shaken gently for 4 hours at room temperature. Carbon dioxide (CO_2) was measured in the headspace through an airtight rubber septum at time 0 (10-20 minutes after capping tubes), at two hours, and then at four hours. At each time (0, 2 hours, 4 hours), air was sampled from tubes through the septum with a syringe, and then the sampled air was injected into a vacuum sealed tube. Samples were analyzed the following day with gas chromatography (Varian CP 3800 gas chromatograph). Per each round of 50 samples, known concentrations of CO_2 were measured to create standard curves to calculate the ppm CO_2 in for each sample, which was divided by the dry mass of the soil sample (gravimetric method). Based on the three samples, the average linear slope of CO_2 concentrations over time (hr) and divided by dry soil mass (g) is the value used to represent microbial biomass (μL/g soil/hr) (Fierer, Schimel, and Holden, 2003).

Roots and aboveground residue biomass

Roots were washed of soil, dried at 60 °C for minimum 24 hours, and biomass (g) was recorded. Estimation of crop residues returned to the soil at harvest was determined using whole plot biomass samples to estimate harvest index, which then allowed calculation of crop residue based on plot grain yield measurement (Miller, unpublished data). The sum of the prior two years, 2018 and 2019, was used to capture the last biomass inputs to the fallow systems (Table A3).

Statistical Analysis

A linear mixed-effects model was used for each of the response variables (extracellular enzymes, PMN, and microbial biomass) with fixed explanatory variables of crop rotation and N rate and random effects of block and block by rotation using R package lme4 (Bates et al., 2015).

A Type III ANOVA was used on the model. Crop rotation treatment effects were compared with planned pairwise contrasts using the Tukey-Kramer honest significant difference with R package emmeans (Lenth, 2020). The levels of the explanatory Indicator variable for crop rotation treatments were the seven crop rotations, and the N-rate treatment Indicator variable had two levels: high and low.

Theoretical model: $\mu\{Response|Rotation, N\}$=$\beta_0 + \beta_1 I_{Rotation=2} + \beta_2 I_{Rotation=3} + \beta_3 I_{Rotation=4} + \beta_4 I_{Rotation=5} + \beta_5 I_{Rotation=6} + \beta_6 I_{Rotation=7} + \beta_7 I_{N=HIGH}$

H_0: Mean y response levels do not change with crop rotation or N rate.

H_A: Mean y response levels do change with at least one of crop rotation or N rate.

For fall enzyme subsampling of three rotations (Tilled Fallow, Pea-seed, and CRP/Pea-seed), the hypothesis was that rotation effects would still impact enzyme activity after the cash crop growing season. The model used was a linear mixed effects model with sampling times of spring and fall and rotation as fixed effects and block and block by rotation as random effects.

To assess the relationship between aboveground residue and the biological indicators, extracellular enzymes and PMN, a Kendall's rank coefficient was used as residue data was not normally distributed. The hypothesis was that greater residue input would be correlated with increased enzyme activity. A Pearson's correlation coefficient was used to test whether early season wheat root biomass was positively correlated with enzyme activity and PMN. All analyses used the R statistical package (R version 4.1.0 (2020)), and, along with the packages cited in text, ggplot2 from Kassambara, (2020) and mosaic from Pruim, Kaplan, and Horton (2017) were useful in data visualization and checking model assumptions.

Results

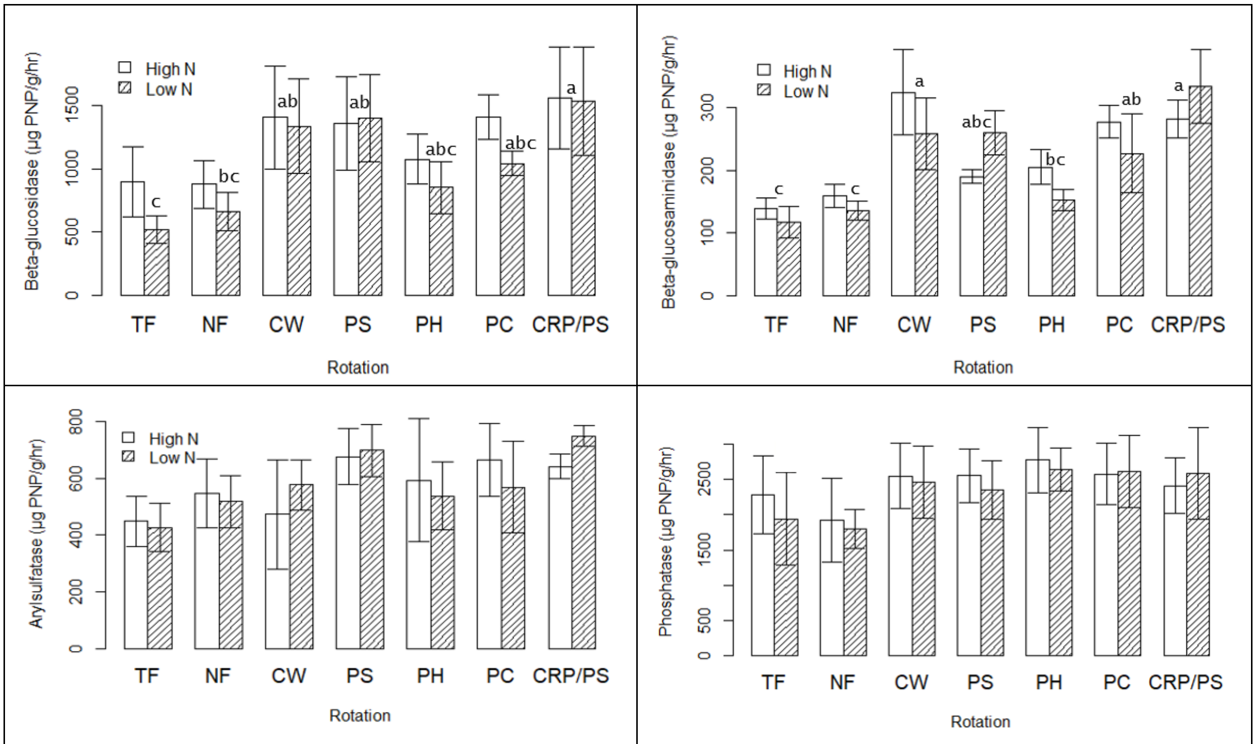
Objective 1) Measure effects of crop rotation and N fertilizer on soil biological parameters

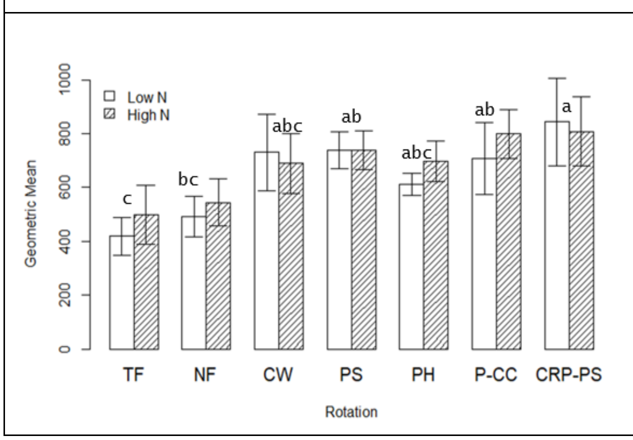
Extracellular enzyme activity: Crop rotation and N-rate treatments affected the enzymatic geometric mean in the spring (Table 2). Soil in both fallow systems had lower geometric mean activity than the CRP/Pea-seed treatment. The enzymatic geometric mean for soil in the Tilled Fallow treatment was also lower than Pea-cover and Pea-seed treatments. Soil from the Tilled Fallow treatment had a 94% lower mean enzyme activity than the CRP/Pea-seed treatment ($t_{15.4} = -3.00$, $p=0.10$). There was no evidence for rotation or N-rate treatments impacting arylsulfatase and phosphatase activity (Figure 1).

Table 2. Satterthwaite's ANOVA Type III for rotation and N-rate effects on extracellular enzyme geometric mean.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Rotation	192893	32149	6	17.11	3.73	0.01
N-rate	20492	20492	1	25.23	2.38	0.14

Crop rotation and N-rate treatments affected beta-glucosidase activity (Table 3). Beta-glucosidase activity was lower in Tilled Fallow and No-till Fallow than in the Pea-seed and CRP/Pea-seed treatments, both of which had legumes and higher residue returned to the plots than fallow treatments ($t_{18}= -3.53$, $p=0.03$ for Tilled Fallow and CRP/Pea-Seed; $t_{18}= -3.27$, $p= 0.05$ for No-till Fallow and CRP/Pea-seed).





Indicator	Pairwise comparisons
Beta-glucosidase	CRP-PS & PS>TF & NF
Beta-glucosaminidase	CRP/PS, CW, PS, PC>TF; CW & CRP/PS>NF; CRP/PS & CW>PH
Geometric Mean	CRP-PS > TF and NF; PC and PS > TF

Figure 1. Mean beta-glucosidase, beta-glucosaminidase, arylsulfatase, phosphatase (µg PNP/g/hr), and enzymatic geometric mean levels with 95% CIs plotted across N and rotation. Key to rotation acronyms: 1. Tilled-Fallow (TF); 2. No-till Fallow (NF); 3. Continuous Wheat (CW); 4. Pea-seed (PS); 5. Pea-hay (PH); 6. Pea-cover (PC); 7. CRP/Pea-seed (CRP/PS) (Sciplot graphic package in R from Morales, 2020).

Table 3. Satterthwaite's ANOVA Type III for beta-glucosidase fixed effects: rotation and N-rate.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Rotation	1404277	234046	6	18	3.77	0.01
N-rate	443220	443220	1	27	7.14	0.01

Crop rotation treatments, but not N-rate treatments, affected beta-glucosaminidase activity (Table 4). Tilled-Fallow soil had lower enzyme activity than Continuous Wheat, Pea-cover and CRP/Pea-seed treatments, while No-till Fallow soil only had strong evidence that it differed from Continuous Wheat and CRP/Pea-seed soils. In addition to the CRP/Pea-seed rotation, soil from the Continuous Wheat treatment had higher beta-glucosaminidase activity than the Pea-hay treatment (t_{18}=2.91, p=0.01). Soils from the CRP/Pea-seed treatment had approximately 72% greater beta-glucosidase activity than soils from the Pea-hay treatment (t_{18}=-3.34, p=0.05).

Table 4. Satterthwaite's ANOVA Type III for beta-glucosaminidase fixed effects: rotation and N-rate.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Rotation	189382	31564	6	18	6.55	<0.01
N-rate	2538	2538	1	27	0.53	0.47

Potentially mineralizable N: There was strong statistical evidence that the N-rate treatment impacted soil PMN ($F_{1,27}$=6.58, p=0.02). However, there was no evidence that rotation treatments affected PMN ($F_{6, 21}$=0.2926, P=0.93). Average PMN values for soil with low N treatments was 0.719 mg NH4-N/kg greater or approximately 30% higher than soils with the high N treatment (Figure 3) (t_{27}=2.65, p=0.01).

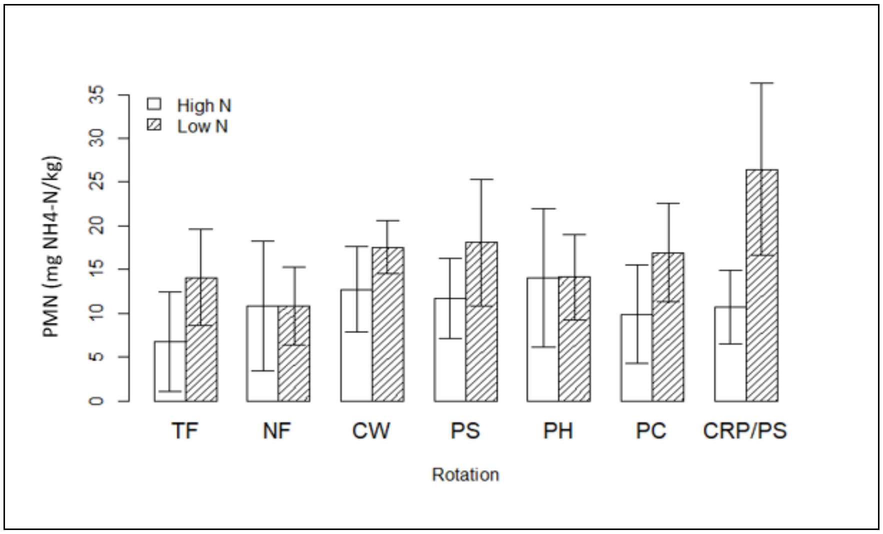


Figure 2. Mean PMN levels (mg NH_4-N/kg) with 95% CIs plotted across N-rate and rotation. Low N>High N (p=0.01). Key to rotation acronyms: 1. Tilled-Fallow (TF); 2. No-till Fallow (NF); 3. Continuous Wheat (CW); 4. Pea-seed (PS); 5. Pea-hay (PH); 6. Pea-cover (PC); 7. CRP/Pea-seed (CRP/PS) (Sciplot graphic package in R from Morales, 2020).

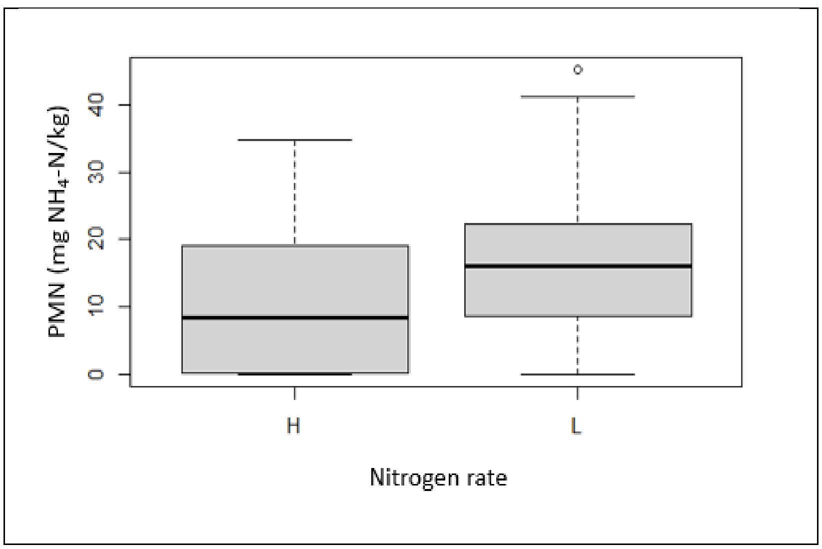


Figure 3. Box and whisker plots with median line and interquartile ranges for PMN (mg NH_4-N/kg) in soils with high and low N-rates Low N>High N (p=0.01).

Microbial biomass: Rotation treatments affected microbial biomass (Rotation: $F_{6,45}=1.91$, p=0.10), but N-rate treatments did not ($F_{1,45}=1.07$, p=0.31). With Tukey-Kramer HSD, there was weak evidence that microbial biomass was lower in soils from the Tilled-Fallow treatment than the CRP/Pea-seed treatment by an estimated 16.7 µL CO_2/g/hr (t_{18}=-2.78, p=0.14).

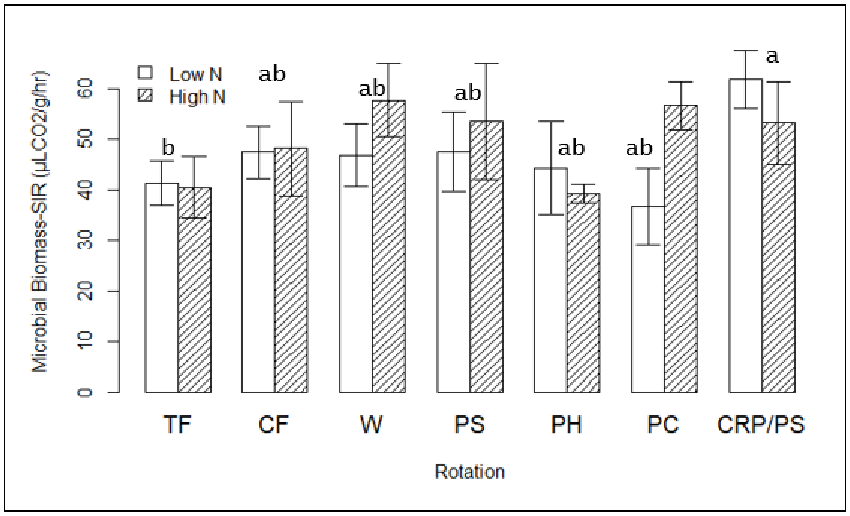


Figure 4. Mean microbial biomass (µL CO_2/g/hr) plotted across N-rate and rotation with 95% CIs. CRP/PS>TF (p=0.14). Key to rotation acronyms: 1. Tilled-Fallow (TF); 2. No-till Fallow (NF); 3. Continuous Wheat (CW); 4. Pea-seed (PS); 5. Pea-hay (PH); 6. Pea-cover (PC); 7. CRP/Pea-seed (CRP/PS) (Sciplot graphic package in R from Morales, 2020).

Objective 2) Test whether residue and early season wheat root biomass are positively correlated with extracellular enzyme activity and PMN

Beta-glucosidase, beta-glucosaminidase, and arylsulfatase were all correlated to plant residue using Kendall's rank correlations (Table 7). There was no evidence that phosphatase or the enzymatic geometric mean were correlated to residue. PMN was moderately correlated to residue. Using Pearson's correlations, early season wheat root biomass was not correlated to PMN, N-rate, or any of the enzymes, except for phosphatase activity, to which it had a positive correlation of 0.45 (Table 8). For early season wheat biomass and residue data, see Table 4 in Appendix.

Table 5. Extracellular enzymes, enzymatic geometric mean, and PMN with estimated Kendall's rank correlation (tau) to the sum of two years of crop residue left on the plot after harvest (Mg/Ha) across treatments and Pearson's correlations to early season wheat root biomass (g). See table A4 for residue and wheat root values.

Soil Biological Indicator	**Residue**		**Wheat Root Biomass**	
	p-value	**Correlation**	**p-value**	**Correlation**
Beta-glucosidase	<0.01	0.28	0.83	-0.03
Beta-glucosaminidase	<0.01	0.35	0.31	-0.14
Arylsulfatase	0.05	0.18	0.40	-0.12
Phosphatase	0.38	-0.08	<0.01	0.45
Geometric Mean	0.35	0.09	0.58	0.08
PMN	0.05	0.19	0.22	-0.16

Objective 3) Assess whether a subset of enzymes measured in the fall maintained similar patterns between crop rotations as in the spring

Beta-glucosidase activity in soils was not different from spring to fall and was not affected by rotation treatments in the fall. Rotation treatments did affect beta-glucosaminidase activity in the fall, and spring was different than fall sampling (time: $F_{1,11}$=15.77, p≤0.01 and rotation: $F_{2,6}$=6.91, p=0.03). Spring beta-glucosaminidase activity was approximately 55% higher than the fall measurement by an estimated 109 µg PNP/g/hr difference (Figure 6) (t_{11}=4.97, p≤0.01). Time and rotation treatments also impacted arylsulfatase activity (time: $F_{1,11}$=37.39, p≤0.01 and rotation: $F_{2,6}$=3.73, p=0.09). Spring arylsulfatase activity had a different pattern and was 19% lower than the fall measurement by 261 µg PNP/g/hr (Figure 7) (t_{11}= -6.12, p≤0.01). The geometric mean did not change from spring to fall. Rotation treatments affected geometric mean enzyme activity in soils ($F_{2,6}$=5.09, p=0.05). Geometric mean enzyme activity was 43% higher in the Pea-seed treatment soils compared to the Tilled Fallow treatment, and it was 65% higher in CRP/Pea-seed treatment soils than those from Tilled Fallow.

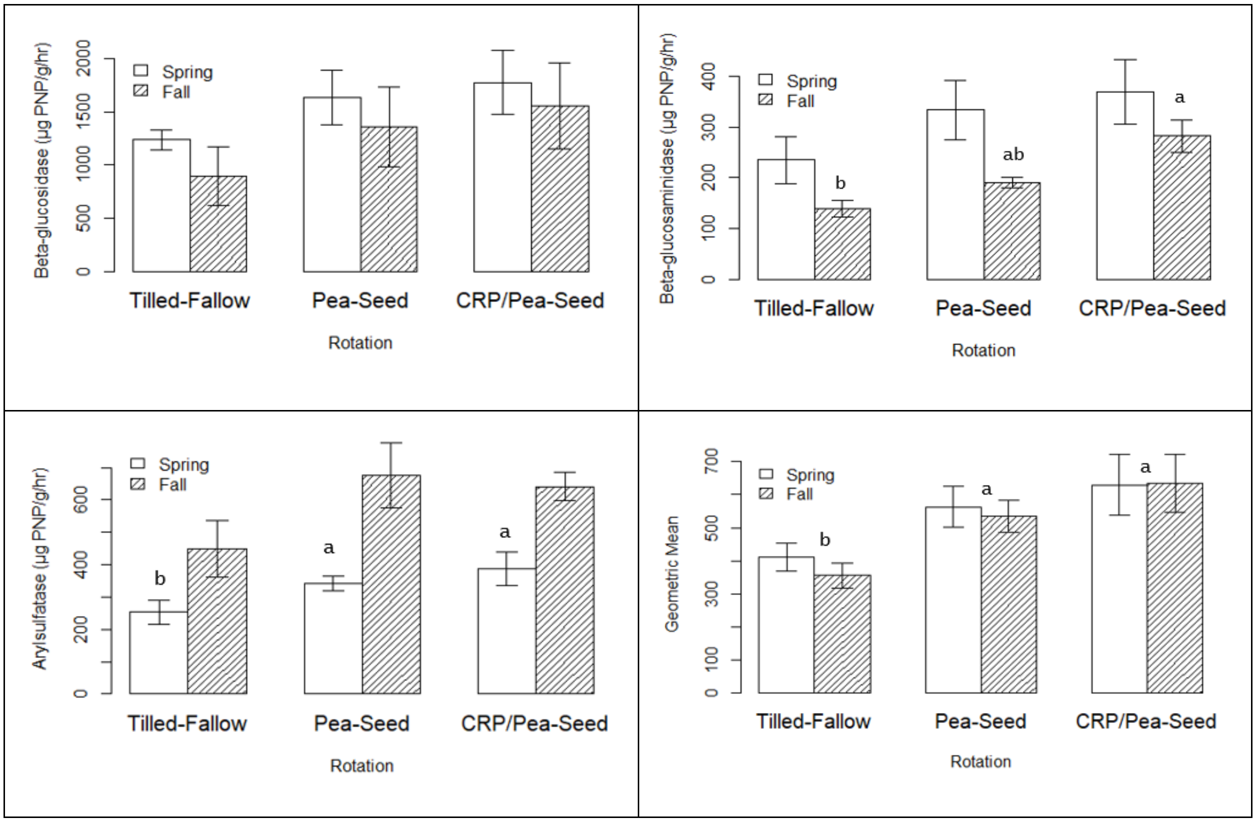


Figure 5. Mean enzyme activity of beta-glucosidase, beta-glucosaminidase, and arylsulfatase activity (µg PNP/g/hr), and their geometric mean with 95% CIs by the rotations: Tilled Fallow, Pea-seed, and CRP/Pea-seed. For beta-glucosaminidase, CRP/Pea-seed>Tilled-Fallow (p=0.02) and spring>fall ($p \leq 0.01$). For arylsulfatase, both CRP/Pea-seed (p=0.12) and Pea-Seed >Tilled-Fallow (p=0.13), and fall>spring ($p \leq 0.01$). For the geometric mean, both CRP/Pea-seed (p=0.09) and Pea-Seed >Tilled-Fallow ($p \leq 0.01$). (Sciplot package from Morales (2020)).

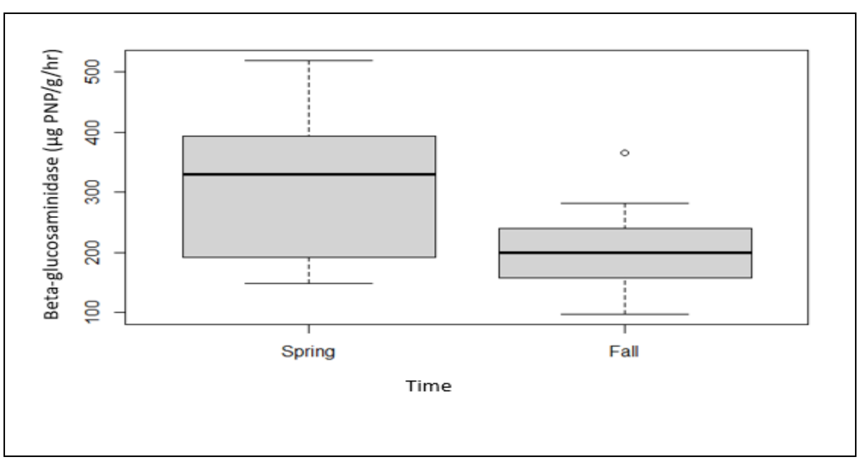


Figure 6. Box and whisker plots of spring and fall beta-glucosaminidase activity (µg PNP/g/hr). Spring > Fall ($p \leq 0.01$).

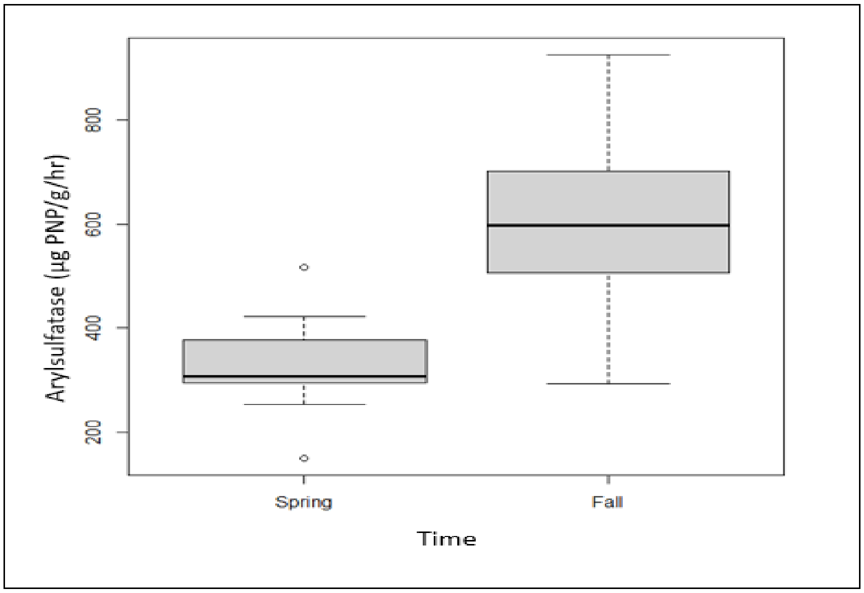


Figure 7. Box and whisker plots of spring and fall arylsulfatase activity (µg PNP/g/hr). Fall>Spring ($p \leq 0.01$).

Discussion

This research on soil biological quality in wheat and wheat-legume crop rotations with high and low N fertilizer rates, while small in scope, gives insight to important aspects of agricultural practices as it used robust biological soil quality indicators (Bünemann et al., 2018) and was implemented during the final year of an eighteen-year experiment. While the variables described here are but a portion of those measured in the GGRS experiment, we investigated an aspect of soil quality related to nutrient cycling and decomposition, both of which contribute to soil ecosystem function.

Nitrogen fertilizer rates impacted soil biological quality in different ways for the response variables. It did not affect microbial biomass. However, the soils with the high N-rate had a slightly higher enzymatic geometric mean. The soils with the high N-rate also had increased beta-glucosidase activity, presumably via increased biomass and thereby C cycling, similarly to

Bowles et al. (2014). However, in contrast to that study, we saw no effects in soils due to the N-rate on the C and N cycling enzyme, beta-glucosaminidase.

The high N-rate treatment decreased soil PMN, representing organic N mineralization. One mechanism for the decrease could be from N fertilizer inhibiting the bacteria responsible for mineralization (O'Dea et al., 2015). It was surprising that there was no statistical difference in PMN in soil from different rotations, as compared to a study that observed higher PMN in continuous cropping compared to fallow (Biederbeck et al., 1998). Nonetheless, it is clear the soils with high N fertilizer rate decreased PMN; although, it should be noted that PMN was only measured at one point in time and does not encompass total organic N mineralization. O'Dea et al. (2015) found that urea decreased total mineralizable N on these same soils, and hypothesized that urea decreases microbial need for mineralization and/or favors organisms that rely more on inorganic N than organic N. More sampling dates across the season would be needed to assess the full seasonal story of how N fertilizer treatment affected organic N mineralization.

Crop rotations also caused different responses for the soil quality indicators. Soil from both the Tilled Fallow and No-till Fallow treatments had lower enzymatic geometric mean activity than soil from the CRP/Pea-seed treatment, which suggests that fallow treatment diminishes soil quality. Soil in the Tilled Fallow treatment also had measurably lower enzymatic activity than soil from the Pea-seed and Pea-cover rotations, which could be a combination of the effects of fallow and those of tillage, which also negatively impacts soil quality (Mbuthia et al., 2015). The enzymatic geometric means for soils from the Continuous Wheat and Pea-hay treatments were not measurably different from any other rotations treatments. While soil from the Tilled Fallow treatment had lower microbial biomass than the CRP/Pea-seed treatment, the soil from the No-till Fallow treatment did have not lower microbial biomass compared to any of the other rotational treatments in this study. Reduced geometric mean enzyme levels in soil from both Tilled and No-till Fallow treatments provide further evidence of reduced enzyme cycling in both tilled and no-till fallow systems, as supported by Tanaka, Wienhold, and Liebig (2004), who saw evidence of improved soil quality with continuous cropping compared to fallow.

Compared to the Continuous Wheat treatment, crop rotations with legumes did not appear to change the enzymatic geometric mean, PMN, or microbial biomass. Increasing diversity did not affect indicators in this case, although substrate quality is often measured as an important factor in decomposition (Kuzyakov et al., 2017). The soil in the Pea-hay treatment, presumably due to its large biomass removal, resulted in a decrease in beta-glucosaminidase. Although the Pea-hay treatment incorporated legumes, its soil had lower enzyme levels than that of Continuous Wheat, emphasizing the importance of returning plant biomass to the field and perhaps that total biomass can be as or more influential on nutrient cycling and decomposition than the C:N ratio of inputs (Schmatz et al., 2017).

Rotation effects on enzymes persisted in the fall after a summer of growing wheat. This supports that extracellular enzymes are also somewhat stable over time (Burns et al., 2013) and that the rotations were affecting enzymatic activity in the soil. Differences between Tilled Fallow treatment and Pea-seed and CRP/Pea seed remained in the fall in beta-glucosaminidase (C and N cycling) and arylsulfatase (S cycling), but not for beta-glucosidase (C cycling). Beta-glucosaminidase and arylsulfatase had opposite trends between spring and fall sampling. Beta-glucosaminidase had much higher activity in the spring compared to the fall. Whereas arylsulfatase activity was slightly higher in the fall compared to the spring. There is an added confounding variable in the comparison between spring and fall that relates to samples being stored for slightly different amounts of time for the spring enzyme procedures compared to the fall procedures. As soil samples deteriorate over time for biological readings, the confounding variable of storage time (not more than a week of difference for when they were run. This adds another element of doubt to the seasonal patterns. Overall, the geometric mean of the fall subsampling shows no difference between spring and fall sampling.

While early season root biomass was not correlated with enzyme activity and PMN, that could be due to wheat having relatively small root systems (Table A4). Perhaps, both small size and recalcitrance make roots less important to the easily mineralizable portion of organic matter and its associated microbes of decomposition. Rather, roots could be more correlated with soil organic carbon (SOC) (Kuzyakov et al., 2017). The only indicator that root biomass was positively correlated to was phosphatase, which could indicate slight increases in early season root mass in plots where phosphorus was locally limiting for a root (Hermans et al., 2006).

Biomass residue, in this case, the past two years of organic matter left on the plots after harvest, was positively correlated to PMN, and to the C, N, and S-cycling enzymes, although not phosphatase or the enzymatic geometric mean. The positive correlation between PMN and residue reflects that increased biomass inputs could increase easily mineralizable N. The positive correlations of residue, along with the general lower performance of the fallow systems, especially the Tilled-Fallow rotation support that aboveground biomass inputs are a driver in soil ecosystem function. The positive correlation to biomass is known, and while the benefit of increased biomass is supported by this research, high N fertilizer rates are also associated with increased soil acidity and nitrate leaching (Liu et al., 2010). Long-term management plans and research should address how to mitigate negative effects of inorganic N fertilizer and develop alternative ways to supply N to the soil, like incorporating legumes into crop rotations (Zentner et al., 2001; Miller et al., 2015).

Overall, for the crop rotations, continuously cropped rotations generally had higher soil quality than the fallow treatments, especially the Tilled Fallow treatment. In the spring, soil in the CRP/Pea-seed treatment was the only rotation with higher enzymatic geometric mean activity than soil from the No-till Fallow rotation treatments, although, the CRP/Pea-seed treatment was not statistically different from the other continuously cropped rotations. In the fall, notably with fewer measurements, there was no difference in the enzymatic geometric mean between soil from the CRP/Pea-seed and Pea-seed treatments. However, soil in the CRP/Pea-seed treatment was the only rotation that had higher microbial biomass than the Tilled-Fallow treatment, although, again, the CRP/Pea-seed treatment microbial biomass was not statistically discernable from any of the other rotation treatments aside from Tilled Fallow.

Interestingly, the CRP grass and alfalfa treatment that appears to set it slightly apart from other rotations was from 8-18 years prior to soil sampling. This could, perhaps, indicate a legacy effect from the 10 years in a grass and alfalfa rotation prior to its conversion to Pea-seed rotation. Perhaps it was affected by increased macrofauna abundance that changed the physical structure of soil in ways that are still benefitting nutrient cycling (Schipanski et al., 2020). Perhaps, the legacy was finally ended by the fall sampling.

In conclusion, crop rotations that increased soil organic matter could improve soil biological quality, as indicated by increased enzymatic activity and microbial biomass. In this case, the highest soil quality parameters were generally found in the continuously cropped systems. Fallow systems had lower enzyme function and microbial biomass, indicating a decreased fertility and decomposition rate compared to continuous cropping. For N fertilizer, the high rate increased carbon cycling and diminished organic N mineralization and would need to be taken into consideration with other soil quality indicators to inform management decisions. Exploring where and how grazing could be incorporated could also be an interesting avenue in research following off Schipanski et al. (2021), who demonstrated grazing of cover crops can be done and soil quality sustained. Further research is needed to continue to quantify and understand the mechanisms that drive soil quality and to monitor the effects of crop rotations and N fertilizer impacts in different climates and soils in Montana.

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APPENDIX

APPENDIX

ADDITIONAL TABLES

Table A1. Table of crop rotations from 2013-2020: ‘S’ is spring, ‘W’ is winter, and ‘CCM’ refers to a cover crop mixture (2 fababean:3 proso millet:4 tillage radish plants/ft2).

<table>
<tr><th>System</th><th>2013</th><th>2014</th><th>2015</th><th>2016</th><th>2017</th><th>2018</th><th>2019</th><th>2020</th></tr>
<tr><td>Tilled Fallow</td><td>Fallow</td><td rowspan="7">Spring Wheat</td><td>Fallow</td><td rowspan="7">Winter Wheat</td><td>Fallow</td><td rowspan="7">Spring Wheat</td><td>Fallow</td><td rowspan="7">Winter Wheat</td></tr>
<tr><td>No-till Fallow</td><td>Fallow</td><td>Fallow</td><td>Fallow</td><td>Fallow</td></tr>
<tr><td>Continuous Wheat</td><td>Spring Wheat</td><td>W Wheat</td><td>S Wheat</td><td>W Wheat</td></tr>
<tr><td>Pea-seed/pulse</td><td>S Pea</td><td>W Pea</td><td>Lentil</td><td>W Pea</td></tr>
<tr><td>Pea - hay</td><td>S Pea</td><td>W Pea</td><td>CCM</td><td>W Pea</td></tr>
<tr><td>Pea-cover</td><td>S Pea</td><td>W Pea</td><td>CCM</td><td>W Pea</td></tr>
<tr><td>CRP/Pea-seed first 10 years grass/alfalfa)</td><td>S Pea</td><td>W Pea</td><td>Lentil</td><td>W Pea</td></tr>
</table>

Table A2. Crop history in 9 cropping systems since 2003 near Bozeman, MT. System 4 and 8 were excluded *a priori* due to management changes over the 18 years.

Year	1	2	3	4	5	6	7	8[Org]	9[CRP]
2003	Ftill	Fnt	SWht	SPea	WPea	WPhay	WPhay	WPgm	Alf/grs
2004	WWh	WWh	WWht	WWht	WWht	WWht	SWht	SWht	Alf/grs
2005	Ftill	Fnt	SWht	SPea	WPea	WPhay	WPhay	WPgm	Alf/grs
2006	WWh	WWh	WWht	WWht	WWht	WWht	SWht	SWht	Alf/grs
2007	Ftill	Fnt	SWht	SPea	WPea	WPhay	WPhay	WPgm	Alf/grs
2008	WWh	WWh	WWht	WWht	WWht	WWht	SWht	SWht	Alf/grs
2009	Ftill	Fnt	SWht	Flax	SPea	SPhay	SPbm	SPgm	Alf/grs
2010	SWht	SWht	SWht	SWht	SWht	SWht	SWht	SWht	Alf/grs
2011	Ftill	Fnt	WWht	WCan	WPea	WPhay	WPbm	WPgm	Alf/grs
2012	WWh	WWh	WWht	WWht	WWht	WWht	WWht	WWht	Alf/grs
2013	Ftill	Fnt	SWht	Flax	SPea	SPhay	SPbm	SPgm	SPea
2014	SWht	SWht	SWht	SWht	SWht	SWht	SWht	SWht	SWht
2015	Ftill	Fnt	WWht	WCan	WPea	WPhay	WPbm	WPgm	WPea
2016	WWh	WWh	WWht	WWht	WWht	WWht	WWht	WWht	WWht
2017	Ftill	Fnt	SWht	Lentil	Lentil	CCMha	CCMb	Lentil	Lentil
2018	SWht	SWht	SWht	Corn	SWht	SWht	SWht	SWht	SWht
2019	Ftill	Fnt	WWht	WCan	WPea	WPhay	WPbm	WPea	WPea
2020	WWh	WWh	WWht	WWht	WWht	WWht	WWht	WWht	WWht

Org – managed organically through 2016.
CRP – Alfalfa/grass mixture managed as CRP and single-cut hay (split plot) 2003-12.
Abbrevs.: bm=brown manure, Can=canola, CCM=cover crop mixture (2 fababean:3 proso millet:4 tillage radish plants/ft2), F=fallow, gm=green manure, grs=grass, nt=no-till, P=pea, S=spring, W=winter, Wht=wheat

Table A3. Residue for 2018 and 2019 (Miller, unpublished data) and early season wheat root biomass (mg).

Rotation	**N-rate**	**Average early season wheat root biomass (mg)**	**2018 Residue (Mg/ha)**	**2019 Residue (Mg/ha)**	**Residue (Mg/ha) summed from 2 years prior to sampling (2018, 2019)**
Tilled Fallow	L	82	8186	0	8186
Tilled Fallow	H	62	5383	0	5383
No-till Fallow	L	52	7629	0	7629
No-till Fallow	H	32	5254	0	5254
Continuous Wheat	L	53	6941	4178	11119
Continuous Wheat	H	44	5994	3038	9032
Pea-seed	L	64	7193	2913	10106
Pea-seed	H	9	5803	3524	9327
Pea-hay	L	107	8185	225	8410
Pea-hay	H	105	6716	258	6974
Pea-cover	L	73	8052	3214	11266
Pea-cover	H	4	4450	3679	8129
CRP/Pea-seed	L	75	7963	4077	12040
CRP/Pea-seed	H	63	6497	4848	11345