Soil microbial biomass and function are altered by 12 years of crop rotation

Marshall D. McDaniel* and A. Stuart Grandy

Department of Natural Resources and the Environment, University of New Hampshire, Durham, NH USA

*Corresponding Author:

Current address:

Department of Agronomy, Iowa State University

2517 Agronomy Hall

716 Farm House Lane

Ames, IA 50011

Phone: (515) 294-7947

Email: marsh@iastate.edu

Abstract

Declines in plant diversity will likely reduce soil microbial biomass, alter microbial functions, and threaten the provisioning of soil ecosystem services. We examined whether increasing temporal plant biodiversity in agroecosystems (by rotating crops) can partially reverse these trends and enhance soil microbial biomass and function. We quantified seasonal patterns in soil microbial biomass, respiration rates, extracellular enzyme activity, and catabolic potential three times over one growing season in a 12-year crop rotation study at the W.K. Kellogg Biological Station LTER. Rotation treatments varied from one to five crops in a three-year rotation cycle, but all soils were sampled under a corn year. We hypothesized that crop diversity would increase microbial biomass, activity, and catabolic evenness (a measure of functional diversity). Inorganic N, the stoichiometry of microbial biomass and dissolved organic C and N varied seasonally, likely reflecting fluctuations in soil resources during the growing season. Soils from biodiverse cropping systems increased microbial biomass C by 28-112 % and N by 18-58 % compared to low diversity systems. Rotations increased potential C mineralization by as much as 53 %, and potential N mineralization by 72 %, and both were related to substantially higher hydrolase and lower oxidase enzyme activities. The catabolic potential of the soil microbial community showed no, or slightly lower, catabolic evenness in more diverse rotations. However, the catabolic potential indicated that soil microbial communities were functionally distinct, and microbes from monoculture corn preferentially used simple substrates like carboxylic acids, relative to more diverse cropping systems. By isolating plant biodiversity from differences in fertilization and tillage, our study illustrates that crop biodiversity has overarching effects on soil microbial biomass and function that last throughout the growing season. In simplified agricultural systems, relatively small increases in crop diversity can have large

impacts on microbial community size and function, with cover crops appearing to facilitate the largest increases.

Keywords: crop rotation; agriculture biodiversity; soil carbon; soil nitrogen; nitrogen mining; community-level physiological profile; mineralization; extracellular enzymes; soil microbial biomass

Introduction

Research manipulating aboveground biodiversity in grasslands has shown a strong link between plant species richness and soil functions (Tilman et al. 1997, Zak et al. 2003, Eisenhauer et al. 2010, Mueller et al. 2013). While this research has contributed to our understanding of aboveground-belowground biodiversity in natural ecosystems, it fails to capture the biodiversity dynamics in agroecosystems, where crop rotations can be used to substitute temporal for spatial biodiversity. Given that species richness at any given time in a rotated cropping system is one (excluding any weeds), the aboveground-belowground relationships dependent on diversity in agroecosystems and spatially diverse ecosystems (e.g. grasslands) may not be the same.

Crop rotations have been shown to have large positive effects on soil C, N, and microbial biomass (McDaniel et al., 2014*a*), plant pathogen suppression (Krupinsky et al. 2002), and yields (Smith et al. 2008, Riedell et al. 2009). These positive effects on crop production have been colloquially referred to as the "rotation effect." However, the mechanistic processes that link aboveground crop rotational diversity and belowground soil processes and contribute to the "rotation effect" remain elusive. One hypothesis explaining the benefits of crop rotations is that greater diversity of plant inputs to soil organic matter (SOM) over time enhances belowground biodiversity and soil ecosystem functioning (Hooper et al. 2000, Waldrop et al. 2006, Grandy and Robertson 2007). Despite being low in spatial diversity, crop rotations have been shown to increase soil microbial and faunal biodiversity (Ryszkowski et al. 1998, Wu et al. 2008, Tiemann et al. 2015) and increase microbial carbon use efficiency (Kallenbach et al. 2015).

One essential function of soil microbial communities is the catabolism of newly added substrates from crops. The range and efficiency of microbial catabolism has great implications

for ecosystem services such as sequestering C and soil fertility (Carpenter-Boggs et al. 2000, Kallenbach et al. 2015), but also for ecosystem "dis-services" such as emission of soil-to-atmosphere greenhouse gases (McDaniel et al. 2014*b*). Furthermore, the partitioning of resources used in catabolism of residue and formation of SOM will affect long-term soil fertility (Lange et al. 2015; Kallenbach et al. 2015).

Soil microbial catabolism can be assessed using many different methods. The two most common measures are soil extracellular enzyme activities, microbe-produced catalysts for catabolism of soil substrates, and respiration response when supplying microbes with a source of C. The latter method, when multiple C compounds are added to the same soil, is commonly referred to as a community-level physiological profiles (CLPP), or as catabolic response profiles. The basic method for measuring soil CLPP involves adding a suite of C substrates to soils and measuring the catabolic response as CO_2 production or O_2 consumption with redox indicators (e.g. Biolog; Guckert et al. 1996). These C substrates are typically ecologically-relevant compounds found in soils, and are intended to represent root exudates, microbial or plant cell structures, or other more-processed soil organic molecules. Other studies have used CLPPs to establish a catabolic "fingerprint" to distinguish soil microbial communities from one another by how they utilize different C substrates (Lupwayi et al. 1998; McDaniel et al. 2014*b*). The CLPP data can also be used to derive measures of metabolic diversity including substrate-use richness or catabolic evenness.

What can catabolic potential, and even catabolic evenness, tell us about soil microbial functioning in agroecosystems? Previous studies have shown that these metabolic diversity measures are increased with agroecosystem management practices that also increase soil health,

71 e.g., reduced tillage or crop rotations (Lupwayi et al. 1998, Degens et al. 2000). In other words,
72 soil microbial catabolism may be a good proxy for long-term consequences of agroecosystem
73 management practices. Given that soil microorganisms, and the resources available to them in
74 the soil, regulate many critical processes in agroecosystems, CLPPs can provide an integrated
75 measure of how management practices alter microbes and substrates available to them. Modern
76 agriculture's use of monocultures could have unknown consequences for soil microbial
77 catabolism, and related processes such as SOM mineralization, but to date the effect of rotation
78 practices and crop diversity on soil microbial functioning remains poorly understood.

79 Considering a lack of understanding of how soil microbial functions are influenced by
80 crop rotations, we sought to examine the rotation effects on soil microbial biomass and function.
81 We measured soil microbial catabolic potential, C and N mineralization, extracellular enzyme
82 activities, and microbial biomass three times over one growing season in a long-term crop
83 rotation experiment at the W.K. Kellogg Biological Station (est. 2000). All soils were collected
84 during the same crop phase, allowing us to separate historical rotation from current crop effects.
85 We hypothesized that soils under more diverse crop rotations would show greater catabolic
86 diversity and have higher measures of soil function (enzyme activities, soil microbial biomass,
87 potentially mineralizable C and N). In addition, we hypothesized that crop rotation effects would
88 vary seasonally, being greatest in the spring and lessen over the growing season with the
89 emerging influence of the current crop. The rationale for this second hypothesis is that early in
90 the season all soils are coming out of different crops from the previous year, but over the
91 growing season under corn the soils will become more functionally similar as the immediate crop
92 has greater influence. Alternatively, significant Rotation by Season interactions on soil

93 microbial functioning that do not converge over the growing season point to historical effects of
94 rotations on differences in soil microbial communities and SOM.

95 **Materials and Methods**

96 This study was conducted in the Cropping Biodiversity Gradient Experiment (CBGE) at
97 the W.K. Kellogg Biological Station Long-term Ecological Research site (42° 24' N, 85° 24'
98 W). The CBGE was established in 2000 and consists of crop rotations ranging from
99 monocultures to a 5-species rotation (http://lter.kbs.msu.edu/research/long-term-
100 experiments/biodiversity-gradient/). The crop rotations were repeated but with different rotation
101 phases within all four blocks. For example, the corn-soy-wheat rotation is replicated three times
102 within each block, but these replicates are planted to a different crop each year. The plot
103 dimensions were 9.1 × 27.4 m and received the same chisel plow tillage to a depth of
104 approximately 15 cm, and received no inputs (e.g. pesticides or fertilizers) that would have
105 confounded the treatment effects of rotation diversity (Smith et al. 2008). Mean annual
106 temperature and precipitation at the site are 9.7°C and 890 mm. The two main soil series located
107 at the site are Kalamazoo, a fine-loamy, mixed, mesic Typic Hapludalf, and Oshtemo, a coarse-
108 loamy, mixed, mesic Typic Hapludalf (KBS, 2012). Soil pH in the top 10 cm ranges from 4.9 to
109 6.1 (1:1 w of 0.01 M $CaCl_2$).

110 Soils were collected from the following cropping systems: monoculture corn (*Zea mays*
111 *L.,* mC), corn-soy (*Glycine max,* CS), corn-soy-wheat (*Triticum aestivum,* CSW), corn-soy-
112 wheat with red clover cover crop (*Trifolium pratense,* CSW1), and corn-soy-wheat with red
113 clover + rye cover crops (*Secale cereale,* CSW2). Most of the year there was just one crop per
114 plot except when red clover cover crops were inter-seeded, and thus overlapped, with the cash
115 crop at the end of the growing season, *ca*. October (Fig. S1, Smith et al. 2008). Soil sampling

116 took place on April 27th, 2012; July 19th, 2012; and November 1st, 2012 – hereafter referred to as
117 spring, summer, and autumn. Corn was planted in all plots on June 11th, 2012. Three 5 cm
118 diameter soil cores (0-10 cm deep) were collected between rows from each plot, homogenized in
119 the field, and then put on ice and shipped to the University of New Hampshire. In the lab, field-
120 moist soils were immediately sieved using a 2 mm sieve. A sub-sample was taken from sieved
121 soil and dried at 105 °C to determine gravimetric water content. Water-holding capacity was
122 determined as the water content after soils were saturated and drained for 6 h.
123 *Soil carbon and nitrogen parameters*
124 Five g of field-moist soil were extracted for inorganic N with 40 ml of 0.5 M K_2SO_4. The
125 soil slurries were shaken for 1 h before the extracts were filtered on Whatman GF/C (5) filters
126 and filtrate frozen and stored until analysis. Soil nitrate (NO_3^-) and ammonium (NH_4^+) were
127 measured using the methods detailed in McDaniel et al. (2014c). We also used the same extracts
128 to measure dissolved organic C and N (DOC and DON). The extracts were run on a TOC-TN
129 analyzer (TOC-V-CPN; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). Total C
130 and N were analyzed by sieving soils through 2 mm sieve, grinding and analyzing on an ECS
131 4010 CHNSO Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA).
132 Potential mineralization rates of C (PMC) and net N (or PMN) estimate the quantity of
133 potentially-mineralizable SOM at an optimal temperature and soil moisture, and reflect both the
134 activity of the microbial community and availability of SOM (Paul et al. 1999, Robertson et al.
135 1999). These mineralization assays provide a good indicator of the potential for a soil to provide
136 plants with N (Stanford and Smith 1972, Robertson et al. 1999). Both PMC and PMN were
137 measured on 10 g of air-dried soils in Wheaton serum vials and brought to 50% water-holding
138 capacity, which is near optimal water content for respiration in these soils (Grandy and

Robertson 2007), and incubated for 4 months. During this 4-month period CO_2 efflux was measured on a LI-820 infrared gas analyzer (LI-COR, Lincoln, NE). Efflux was measured using the change in headspace CO_2 concentration measured between two time points. Each soil efflux measurement began by aerating jars, capping, and injecting a time-zero sample and then a second sample between 5 h to 2 d later. Efflux was calculated as the difference in CO_2 concentration between the two time points divided by time. Measurements of PMC occurred more frequently at the beginning of the experiment (daily), and became less frequent toward the end (once every other week), for a total of 19 sampling events over 120 d. High frequency measurements are required during the beginning of these incubations, when respiration rates are high, to prevent build-up of CO_2 (and lack of O_2). The PMN was assessed by extracting the inorganic N (NH_4^+ + NO_3^-) produced at the end of the incubation, measuring it with the methods described above, then subtracting this final value from the initial inorganic N extracted before the incubation began.

Soil microbial parameters

Soil microbial biomass C (MBC) and N (MBN) were determined using the modified chloroform fumigation and extraction method (Vance et al. 1987), but modified for extraction in individual test tubes (McDaniel et al. 2014c). Briefly, two sets of fresh, sieved soil (5 g) were placed in 50 ml test tubes, and 1 ml of chloroform was added to one set of tubes and capped. The tubes sat overnight (24 h) and were then uncapped and exposed to open air in a fume hood to allow chloroform to evaporate. Soils were then extracted in the tubes with 25 ml of 0.5 M K_2SO_4. The chloroform fumigated and non-fumigated extracts were run on a TOC-TN analyzer (TOC-V-CPN; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). We used 0.45 (Joergensen 1996) and 0.54 (Brookes et al. 1985) for the C and N extraction efficiencies.

Soils were analyzed for 8 extracellular enzyme activities (EEAs): β-1,4-glucosidase (BG), β-D-1,4-cellobiohydrolase (CBH), β-1,4-N-acetyl glucosaminidase (NAG), acid phosphatase (PHOS), Tyrosine aminopeptidase (TAP), Leucine aminopeptidase (LAP), polyphenol oxidase (PO), and peroxidase (PER). Given the large number of samples (60) and variety of measurements made at each of 3 sampling dates, soil EEAs were conducted on frozen samples within 4 weeks of sampling. While some studies show freezing has minor effects on EEAs (Peoples & Koide 2012), others show no effects (Lee et al. 2006, Deforest 2009), and we assume that any effects of freezing will be consistent among treatments. Extracellular enzyme activity assays were carried out following previously published protocols (Saiya-Cork et al. 2002, German et al. 2011), but with some modifications. Briefly, 1 g of soil was homogenenized with a blender in 80 ml of sodium acetate buffer at pH 5.6 (the average pH at the site). Soil slurries were pipetted into 96-well plates and then analyzed on a Synergy 2 plate reader (BioTek Instruments, Inc., Winooski, VT). For oxidoreductase enzymes, the supernatant from the slurry plates were pipetted into a clean plate to avoid interference with soil particles. Hydrolase assays were read at 360/40 and 460/40 fluorescence and oxidoreductases at 450 nm absorbance. For more details on the extracellular enzyme methods see McDaniel et al. (2014c).

Community-level physiological profiles (CLPP) were conducted using the MicroResp™ system (Chapman et al. 2007, Zhou et al. 2012, McDaniel et al. 2014*b*). The MicroResp™ system allows for high-throughput measurement of soil catabolic responses to multiple C substrates. Each soil was loaded into 96 deep-well plates using the MicroResp™ soil dispenser, and then brought to 50% water-holding capacity. Thirty-one substrates were used at concentrations ranging from 7.5 to 30 mg C per g of soil H_2O, as recommended by the MicroResp™ manual (Table S1). Soil and substrates were combined in analytical triplicates and

185 a CO_2 detection plate (agar containing creosol red) was immediately placed onto the deep-well
186 plate with an air tight seal provided by the MicroResp^{TM} kit. The soil and substrates were
187 incubated in the dark for 6 h at 25 °C. The detector plate absorbencies were read at times 0 and
188 6 h at 540 nm on a Synergy 2 plate reader (BioTek Instruments, Inc., Winooski, VT).
189 Absorbance data were normalized and converted to a CO_2 efflux rate (μg CO_2-C g soil^{-1} h^{-1}),
190 according to the MicroResp^{TM} procedure (Chapman et al. 2007).

191 *Data analyses*

192 Cumulative potentially mineralizable C and N were calculated in SigmaPlot v12.5 (Systat
193 Software, Inc., San Jose, CA) using the integration macro, *area below curves.* Data not
194 conforming to ANOVA assumptions of homogeneity of variances and normality were
195 transformed before analyses (Zuur et al. 2010). Catabolic evenness (CE), a measure of substrate
196 diversity, was calculated using the Simpson-Yule index, $CE = 1/\Sigma p_i^2$, where p_i is the proportion
197 of a substrate respiration response to the total response induced from all substrates (Degens et al.
198 2000, Magurran 2004). Metabolic quotient was calculated simply as the basal respiration over 6
199 h (determined in the MicroResp^{TM} method) divided by the MBC. Almost all the soil data were
200 non-normal, including: DOC, DON, PMC, PMN, microbial biomass, enzymes, and catabolic
201 evenness. All these data were log-normal transformed, except for catabolic evenness which was
202 square root transformed to meet normality requirements.
203 Response variables were analyzed using a 2-way analysis of variance (ANOVA), with
204 Season and Rotation as main effects. The ANOVAs were conducted in SAS 9.3 (SAS Institute,
205 Cary, NC) using the *proc mixed* function and post-hoc *t* tests were used to determine significant
206 differences among means using *ls means*. Block was assigned as a random effect variable within

207 the model. Correlations between variables were made using *proc corr*, and Pearson's correlation
208 coefficients are reported. Model effects were deemed significant if $\alpha < 0.05$.
209 All multi-variate data analyses were performed with R software v. 3.0.0 (The R
210 Foundation for Statistical Computing, Vienna, Austria). CLPP data were checked to ensure they
211 conformed to principal components analysis assumptions. The *prcomp* function in the *vegan*
212 package (Oksanen et al. 2016) was used for PCA of CLPP data. In order to correlate
213 environmental variables with the multi-variate CLPP data we used the *envirfit* function.

214 **Results**

215 It was a relatively dry year at the KBS-LTER in 2012, which had an annual precipitation
216 of 742 mm, compared to the historical mean of 870 mm (Hamilton et al. 2015). There was also
217 an anomalous warm spell in mid- to late-March (Fig. S2). After harvest, the corn yield (kg ha^{-1} ±
218 SE) in each treatment was as follows: mC = 2846 ± 152, CS = 4208 ± 575, CSW = 4107± 220,
219 CSW1 = 4015 ± 187, CSW2 = 5219 ± 1180 (KBS-LTER 2015).

220 *Soil C and N biogeochemistry*

221 There were few significant Rotation or Season effects on total soil C and N, except for
222 CSW1 had greater N than CSW ($P = 0.040$), although both soil C and N tended to increase with
223 the number of crops in rotation (Table1). Seasonal soil NO_3^--N concentrations were highest in
224 summer (10.33 ± 2.71) followed by spring (2.98 ± 0.69), and autumn (1.28 ± 0.20 mg kg^{-1}). Soil
225 NH_4^+-N was generally low, but summer had more than twice the concentrations of spring and
226 autumn. Dissolved organic C (DOC) and N (DON) were very dynamic over the year. The DOC
227 was highest in the autumn, while DON was over six times greater in the summer than the other
228 seasons ($P < 0.001$). The mean DOC:DON in autumn was 17.4 ± 5.9, five times higher than

spring and 13 times higher than summer. Soil NO_3^--N was the only variable that showed a significant Season × Rotation interaction ($P < 0.001$). There were significant main effects of crop rotation on DOC and DON (Table 1). During the summer the two cover crop treatments had the highest NO_3^--N concentrations (16.68 ± 0.87 and 12.14 ± 4.03 mg kg^{-1}), which was 67 % greater than CSW and CS treatments, and 158 % greater than mC. The CSW1 treatment had 112 % greater DOC concentrations than mC ($P < 0.001$), and two cover crop treatments had 107 % greater DON than non-cover crop treatments and 211 % more than the mC treatment.

The potentially mineralizable pools of C and N showed significant main effects of both Season and Rotation ($P < 0.03$), but no interactions. The PMC was highest during the autumn (636 ± 105 µg CO_2-C g $soil^{-1}$), while PMN was highest during the summer (89 ± 105 µg NH_4^++NO_3^- g $soil^{-1}$). Generally, both PMC and PMN increased with increasing number of crops in rotation (Fig. 1), and the incorporation of cover crops appeared important in regulating both PMC and PMN. For example, the PMC average of both cover crop treatments (CSW1 and CSW2) were 53 % and 41 % greater than mC and CS treatments ($P < 0.042$), respectively. The PMN average from the cover crop treatments was 36 %, 48 %, and 72% greater than the mC, CS, and CSW treatments, respectively ($P < 0.015$). The potentially mineralizable C-to-N ratio (PMC:PMN), considered an index of the quality of accessible SOM (Schimel et al. 1985; Clein & Schimel 1995), showed a significant Season × Rotation interaction ($P = 0.045$, Fig. S3). The PMC:PMN was markedly higher in the autumn than in summer and spring, indicating a greater demand for N in autumn. For summer and spring more diverse rotations had less CO_2 produced per unit of net inorganic N mineralized. However in the autumn, after harvest, the crop rotation effects on the PMC:PMN were reversed; meaning the more diverse crop rotations had greater CO_2 mineralized per unit of available N (Fig. S3).

252 *Soil microbial dynamics*

253 The range in soil MBC was 60 − 1661 μg C g soil^{-1} across all seasons and crop rotations,
254 but both Season ($P < 0.001$) and Rotation ($P = 0.008$) had significant effects on MBC (Fig. 2).
255 Soils collected in autumn had more than twice the MBC than those collected in spring and
256 summer. Generally, microbial biomass C was increased by increasing crop diversity across all
257 seasons (Fig. 2), but only CSW1 was 112% and 28% significantly greater than mC and CS,
258 respectively ($P = 0.023$). Microbial biomass N ranged from 6 to 61 μg N g soil^{-1} and also
259 showed both Season ($P < 0.001$) and Rotation ($P = 0.005$) effects, but no interaction. Once
260 again, MBN generally increased with crop diversity, with the CSW (57 %), CSW1 (54 %), and
261 CSW2 (50 %) significantly greater than the mC treatment ($P < 0.037$). Microbial biomass C:N
262 showed a significant interaction ($P = 0.013$), with more diverse cropping systems having greater
263 MBC:MBN in summer, but not in the spring or autumn. The metabolic quotient (qCO_2), is often
264 used as a proxy for microbial respiration efficiency (Anderson & Domsch 1990, 2010; Wardle &
265 Ghani 1995). Season ($P < 0.001$) and Rotation ($P = 0.024$) both influenced qCO_2, with summer
266 showing the greatest qCO_2 (0.11 ± 0.3) and autumn the lowest (0.04 ± 0.1) qCO_2. Crop
267 diversity significantly decreased the qCO_2 in the CSW1 by 40 % and 48% compared to mC and
268 CS.

269 Soil extracellular enzymes were very dynamic over the three seasons, as evidenced by
270 radar plots in which the area and shape for each treatment changes drastically over the growing
271 season (Fig. 3). A MANOVA with all eight EEAs showed significant Season ($P < 0.001$) and
272 Rotation ($P < 0.001$) main effects, but no interaction. Most individual enzymes showed only
273 significant Rotation effects except for PO, which also showed a significant Season effect with
274 autumn greater than the other seasons (Table 2). The soil enzyme responsible for cleaving a

glucosamine from chitin (NAG) and the lignin-reducing enzyme that uses peroxide (PER) were the only enzymes that showed a significant Season × Rotation interaction ($P < 0.001$). Spring had the greatest activities of LAP, 175% greater than the average of the other seasons (Fig. 3, Table 2). In summer, we see a shift to the highest PHOS activity – 25% greater than autumn and 99% greater than spring. There were no main effects of Season on BG or CBH, but Rotation main effects were significant, with the CSW1 treatment having an average of 42 and 50 % higher BG and CBH activity than CS and mC soils, respectively. The majority of the hydrolase enzymes were higher in the cover crop treatments compared to that of the non-cover crop treatments, especially mC (Table 2, Fig. 3). The two oxidoreductase enzymes (PO and PER) decreased with crop diversity. There were no significant main effects on the enzyme ratio used to assess C-versus-N demand (BG to NAG+LAP).

The community-level physiological profile (CLPP), a catabolic profile of the soil microbial communities, showed both significant Season ($P < 0.001$) and Rotation ($P = 0.003$) main effects (Figs. 4, S4; Table 3). A principal components analysis of the CLPP data showed that the summer soils corresponded with highest carboxylic acid utilization (Fig. 4), as Season was the strongest discriminating factor along principal component 1 (PC1, Table 3). However, when rotating and examining PC2 and PC3, there was a strong treatment gradient from the bottom-right to upper-left quadrants of the graph (Fig. 4, right panel). The lower-diversity treatments corresponded with greater use of carboxylic acid substrates. Across seasons, summer exhibited the lowest catabolic evenness (12.9 ± 1.4), but there was no crop rotation effect on catabolic evenness using all substrates (i.e. Full, Table 4).

Due to the overwhelming influence of carboxylic acids in the PCA variation, and their possible role in abiotic reactions leading to CO_2 emissions (Maire et al. 2012, Pietravalle and

298 Aspray 2013), we split the 31 substrates into two sets to analyze separately: 1) Non-carboxylic
299 acid substrates – a total of 21 substrates, and 2) carboxylic acids by themselves – 10 substrates.
300 Season, again, was a dominant significant effect on the MANOVAs in both groups of substrates
301 (P values < 0.001, Fig. S5, Table S2 and S3). The non-carboxylic acid CLPP showed a
302 significant treatment effect with PC1 and PC2, and clear separation between low and high
303 diversity cropping systems (P = 0.012, Fig. S4). The monoculture corn, and lower diversity
304 treatments, associated with more complex substrates. In the carboxylic acid CLPP there was also
305 a significant treatment effect, but with PC2 and PC3, and clear separation between low and high
306 diversity cropping systems along PC3 (P = 0.035, Fig. S5). The low diversity treatments
307 (especially monoculture corn) were more associated with simple (lower molecular weight)
308 carboxylic acids (Cit, Mlo, and Mli) on the positive half of PC3. When carboxylic acids were
309 split from the substrates, crop rotation had a significant effect on catabolic evenness – decreasing
310 the catabolic evenness both within non-carboxylic acids and carboxylic acids by as much as 4
311 and 13% respectively (Table 4).

312 *Relationships between soil biogeochemical factors, microbial functioning and yield*

313 Over the three seasons many soil biogeochemical factors correlated with microbial
314 catabolic potential, both with individual C substrate guilds and catabolic evenness (Table 5).
315 Abiotic factors such as pH and sand content correlated with the use of particular guilds of
316 substrates. Soil pH positively correlated with N-containing and complex substrates, but
317 negatively with carboxylic acids. Sand content negatively correlated with amino acids and
318 carbohydrates, but positively with carboxylic acids. The microbial response to amino acids and
319 amines correlated best with NO_3^--N (Table 5) and many of the specific enzyme activities,
320 showing negative relationships which indicated a linkage between demand for N and usage of N-

bearing substrates. Soil NO_3^--N was also significantly negatively correlated with catabolic evenness.

We used the soil microbial responses of EEA and the CLPP because we assumed they would be complementary. For example, adding N-acetyl glucosamine in the CLPP should be related to ß-1,4-N-acetyl glucosamindase (NAG) enzyme activity. Indeed, this was the case. Measuring NAG enzyme and adding the Nag amine to the soils showed a somewhat tight relationship, but this changed during autumn (Fig. S6). Additionally, when the CLPP substrates were grouped by guild they were significantly correlated with EEAs (Fig. S7). For instance, total amino acid catabolic response positively correlated well with LAP+TAP enzymes (r^2 = 0.35, $P < 0.001$) meaning that high activity of these enzymes in soils corresponded with high relative use of these substrates when added to soils, compared to other substrates added to the soil. This suggests that the LAP and TAP enzymes strongly reflect demand for N-bearing amino acids in soils. However, the catabolic response of the 'Complex' guild was negatively correlated with PO ($r^2 = 0.29$, $P < 0.001$). Soil PMN was better correlated with crop yields ($r^2 = 0.61$, $P <$ 0.001) than NO_3^- in early spring (Fig. S8), highlighting the importance of PMN-like measurements being used as soil fertility tests.

Discussion

Increasing biodiversity in this long-term crop rotation experiment has altered the soil microbial dynamics across an entire growing season. This occurred even though the soils in our study were all in the same crop phase (corn) for the season, indicating that observed differences among soils reflect long-term rotation effects. Microbial biomass C, N, potential mineralization, and catabolic potential were all altered by crop rotations, although the rotation effect for some of

these indicators of microbial functioning also depends upon the season. Soil microbial biomass and activity are now widely recognized as pillars of soil health (Doran and Zeiss 2000). Our results clearly indicate that diversifying agroecosystems (through crop rotations) enhances this aspect of soil health, and is also likely linked to changes in SOM dynamics (Tiemann et al. 2015) as well as the observed differences in yield among crop rotations (Smith et al. 2008, Fig. S8).

Crop biodiversity and soil microbial functioning

Both soil microbial biomass and functioning were strongly affected by increased crop diversity through rotation. This rotation effect was largely independent of the season, as indicated by the limited number of observed Season × Rotation interactions. The exception to this was microbial biomass C/N ratio (Fig. 2), potentially mineralizable C-to-N (Fig. 1 and S3), and two extracellular enzyme activities (NAG and PER, Table 2), which together are likely indicative of the enhanced ability of soil microbes under diverse rotations to process, provision, and retain soil N. The stoichiometric shifts in microbial biomass and potentially mineralizable SOM suggest seasonal changes in microbial communities and/or how microbes shift between C and N resources among crop rotations. For instance, the MBC:MBN ratio is only significantly wider in the two cover crop treatments than those without during the summer when inorganic N was plentiful, and labile C might have been limiting. On the other hand, during the autumn when the soils were most N-limited, the potentially mineralizable C-to-N ratio widened in all treatments but was widest among diverse crop rotations (Figs. 1 and S3). Together these findings suggest that labile C might be a major regulating factor of soil N cycling, and that crop rotations change these dynamics.

With regards to provisioning of N, the PMN, MBN, and NAG enzyme activity were greater in soils under more diverse crop rotations during the spring (Fig. 1, 2 and Table 2). NAG has been shown to be strongly related to net N mineralization (Ekenler & Tabatabai 2002), therefore the alignment between these two measures of microbial function were not surprising. Taken together, though, these data indicate that soil microbes from diverse rotations might be able to better supply crops with N via mineralization, at this critical stage when corn crop N demand is high (Blackmer et al. 1989). Thus, in this severely N-limited cropping system, it makes sense that spring PMN was better related to yield than soil inorganic N concentrations because these crops are relying almost exclusively on SOM-derived N. Most importantly, it also suggests that the greater provisioning of N from SOM to plants in more diverse cropping systems is a likely factor for the higher yields in our study (Fig. S8). These findings are consistent with plant biodiversity studies that find increased aboveground diversity enhances soil microbial biomass and functioning in natural (Stephan et al. 2000, Zak et al. 2003, Lange 2015) and agricultural ecosystems (Lupwayi et al. 1998, Xuan et al. 2012, McDaniel et al. 2014*c*).

While there were some significant differences in soil microbial dynamics between the non-cover-crop rotations (CS and CSW) and monoculture corn (Table 1, Fig. 1 and 2), the largest differences were between the two cover crop treatments and monoculture. This was particularly the case for the red-clover-only cover crop treatment (CSW1). A growing number of other studies show the large positive impact cover crops have on soil microbes and their activity (Mendes et al. 1999; Kabir & Koide 2000; McDaniel et al. 2014*c*; Mbuthia et al. 2015). The reason cover crops consistently increase soil microbial biomass and activity is likely due to the increased quantity and quality of crop residue inputs, but cover crops also have been shown to improve soil physical properties that enhance biological activity (Williams & Weil 2004;

387 Schipanski et al. 2014). Another contributing feature of crop diversity via rotation is a greater
388 likelihood of including 'keystone' species, such as legumes like soy and red clover used in this
389 study, which may have disproportionally large effects on soils (Wardle 1999). While total soil N
390 differences are largely undetectable, these legumes in diverse rotations are adding labile residues
391 (including more N) to these N-limited soils, which could also be reflected in the enhanced soil
392 microbial biomass and activity.

393 We hypothesized that increasing crop diversity through rotation would result in soil
394 microbial communities that are more diverse, and thus would more evenly use added C
395 substrates (i.e. increase catabolic evenness, or decrease the variation in use among substrates).
396 This hypothesis stems from arguments that soil community and functional biodiversity is linked
397 to plant biodiversity, mostly through the diversity of plant inputs to SOM (Lodge 1997, Hooper
398 et al. 2000, Waldrop et al. 2006, Korboulewsky et al. 2016). However, in our study, we found no
399 evidence that crop rotational diversity increased overall soil catabolic evenness (Table 4). There
400 is some evidence that crop rotations can alter soil bacterial catabolic diversity, or the ability to
401 use different C substrates (Lupwayi et al. 1998, Larkin 2003, Govaerts et al. 2007), however all
402 of these studies used Biolog, which has several limitations (Preston-Mafham et al. 2002). The
403 MicroResp™ system's main benefit is that it adds C substrates directly to the soil instead of
404 tranferring an inocullum from a soil slurry. The discrepancy between our study and these other
405 studies may be due to methodological differences between Biolog and MicroResp™. Our lack
406 of evidence for an aboveground-belowground link to catabolic potential aligns with findings
407 from other studies that have found functional diversity measures of soil microbes are not related
408 to plant diversity (Bartelt-Ryser et al. 2005, Jiang et al. 2012), nor plant species in general
409 (McIntosh et al. 2013).

410 In our study, when a subset of the C substrates were analyzed (all non-carboxylic acids,
411 or carboxylic acids only), we found that increased crop diversity decreased catabolic evenness
412 (Table 4). This is unusual considering soils from this same study, but collected a year prior,
413 showed increases of soil biodiversity (Shannon-Weiner index or H') with increased crop
414 diversity when measuring phospholipid fatty acids (Tiemann et al. 2015); and diversity has been
415 found to be strongly, positively related to species evenness in plants and animals (Stirling &
416 Wilsey 2001). In this study, our findings of a lack of an effect (or even a negative effect) of crop
417 biodiversity on catabolic evenness is also contradictory to the findings of Degens et al. (2000),
418 who showed that management practices that decreased soil C are associated with low catabolic
419 evenness. Yet, evidence from these same soil samples showed that crop diversity significantly
420 decreased H' for bacterial 16S rRNA by as much as 5 % compared to monoculture corn (Peralta
421 et al. *in review*). Taken together, the decrease of functional and structural diversity of soil
422 bacteria with crop diversity indicates that crop diversity might decrease bacterial diversity in this
423 crop rotation experiment. Nevertheless, a recent meta-analysis showed that crop rotations tend
424 to increase soil biodiversity by 3 % and richness by 15 % (Venter et al. 2016), but there was
425 large variability around these estimates. Regardless of aboveground-belowground diversity
426 trends, crop rotations did create functionally distinct microbial communities in our study (Fig. 4).
427 We still do not have a good understanding of how crop rotations alter soil microbial dynamics,
428 nor (arguably more importantly) how these changes in belowground communities might provide
429 beneficial soil ecosystem services like increasing soil C or mineralizing more N to increase crop
430 yields.

431 One trend that emerges across the suite of 31 C substrates is that crop rotations altered the
432 preference for C substrates (i.e. complex versus simple C substrates). The soils from

433 monoculture corn corresponded to greater use of simple C substrates (especially carboxylic
434 acids), and showed less response to the suite of N-containing and complex substrates (Fig. 4).
435 This finding corroborates a previous study we conducted using whole-plant residues, in which
436 we showed diverse crop rotations resulted in greater decomposition of low quality crop residues
437 (e.g. corn and wheat, McDaniel et al. 2014*c*). Further, when looking only within the relatively
438 labile carboxylic acid substrates, microbial communities in the less diverse crop rotations (mC,
439 and to a lesser extent CS) responded to more labile, low-molecular weight carboxylic acids (e.g.
440 citric, malonic, and malic acid), while soil microbes from more diverse crop rotations responded
441 more to complex, higher-molecular weight carboxylic acids (e.g. caffeic, tartaric, and vanillic
442 acids - Fig. S5d). The strong effects of crop diversity on catabolism of carboxylic acids is not
443 surprising due to the small, yet dynamic, pool of these compounds in soil (Strobel 2001). Since
444 soil microbial function (as measured by CLPP) is an aggregate measure of both the community
445 composition and available resources, it is impossible to tease out which (or both) have changed
446 due to increased crop biodiversity. However, our overall findings indicate that increased
447 aboveground biodiversity through crop rotations and cover crops appears to facilitate soil
448 microbial communities' use of complex C substrates relative to simple ones.

449 *Seasonal dynamics and N limitation*

450 Season strongly influenced the measured pools of labile C and N (Table 1), as well as the
451 microbial biomass size and functioning within this agroecosystem (Figs. 1-4). We hypothesized
452 that soil microbial function would converge over the growing season, as the current crop exerted
453 greater influence over soil microbes. We did find some support for this hypothesis. Both
454 multivariate measures of extracellular enzyme activities and CLPP showed treatments becoming
455 more similar over the growing season (Figs. 3 and 4). This is based on three time points,

456 however, and we do not know for sure if this convergence was due to the influence of the corn
457 crop or other factors (like microclimatic). Some studies have shown that the current plant
458 species identity often trumps biodiversity legacy in controlling belowground microbial structure
459 and functioning (Stephan et al. 2000, Wardle et al. 2003, Bartelt-Ryser et al. 2005). Conversely,
460 several studies have pointed to weak or no influence of current plant species on soil microbial
461 structure and functioning (Costa et al. 2006, Kielak et al. 2008). The question of whether plant
462 species identity versus spatial and temporal diversity has a stronger control on soil biota remains
463 a critical question in terrestrial ecology.

464 The greatest microbial biomass and activity occurred in autumn, but potential N
465 mineralization peaked in summer. In perennial and annual cropping systems in Iowa, potentially
466 mineralizable N declined from spring to late summer; in addition, extracellular enzyme activities
467 peaked in July but there was little effect of the cropping system (Hargreaves and Hofmockel
468 2013). In another study, season was shown to affect microbial biomass and potentially
469 mineralizable C and N pools in a wheat-sorghum-soybean rotation in south-central Texas
470 (Franzluebbers et al. 1994, 1995, Franzluebbers 2002), but timing for peak values differed
471 depending on the study and cropping systems, likely reflecting different climates and soil types.
472 The frequently observed late-summer spike in microbial biomass and activity may be related to
473 higher temperatures during this time period; however, even within agroecosystems, the timing
474 for maximal microbial biomass varies substantially, although few microbial maxima are reported
475 in winter (Wardle, 1992). Our findings highlight the dynamic nature of soil microbial biomass
476 and activity, especially with regards to the supply and demand of N (e.g. microbial C:N,
477 substrate utilization, and extracellular enzyme activities), which is likely a limiting nutrient in
478 these agroecosystems that are receiving no exogenous N inputs.

479 The summer warrants discussion because the sample was collected after a prolonged
480 period of hot and dry days, but right after a large rainfall event. This rainfall event (> 18 mm d^{-1},
481 Fig. S2) increased the volumetric water content in the 0-10 cm of a nearby soil by over 54%
482 from the lowest value of the year (0.1 m m^{-3}, data shared from Hamilton et al. 2015); and we
483 know from previous research that drying-wetting cycles are important soil biogeochemical
484 drivers (Borken and Matzner, 2009) and can alter microbial structure and functioning (Fierer et
485 al. 2003, Schimel et al. 2007, Tiemann and Billings 2011, McDaniel et al. 2014*b*). Indeed, the
486 summer showed several signs of the soil microbial community being impacted by a rapid dry-
487 wet event: lower overall microbial biomass C, high NO_3^--N concentrations (Table 1), high
488 potential N mineralization (Fig. 1), high extracellular enzyme activities per unit of microbial
489 biomass (Fig. S9, presumably a result of lysed intracellular enzymes, Burns et al. 2013), and the
490 particularly strong response of the summer soils to carboxylic acids (a highly-labile class of
491 compounds used by fast-growing, opportunistic microbes, that would be found after a
492 disturbance such as a dry-wet event, Figs. 4 and S3). Dry-wet cycles may drive microbial C and
493 N to be reallocated to stress-response compounds instead of growth or reproduction, making C
494 and N more vulnerable to loss from soils (Schimel et al. 2007). We captured one of these dry-
495 wet events during one of the driest summers in the Kellogg Biological Station LTER's history
496 and we show high soil inorganic N concentrations and altered microbial dynamics relative to the
497 other dates. Climate change may increase the frequency and magnitude of these rapid dry-wet
498 cycles (Groffman et al. 2001, McDaniel et al. 2014*d*), and thus may have long-term impacts on
499 soil microbial functioning and biogeochemistry.

500 In the autumn we found several lines of evidence that indicate soil microbes are N, rather
501 than C, limited. These lines of evidence include: lowest soil inorganic N concentrations, low

potentially mineralizable N, high microbial biomass C:N and DOC:DON ratios, and high TAP and NAG enzymes relative to other enzymes (although interestingly not LAP), and finally strong respiration response to the addition of amines and amino acids (Fig. 4). The unusually wide microbial biomass C:N in autumn was very surprising (mean of 24 versus 10 and 8 in spring and summer, respectively), but microbial biomass C:N has been known to be as high as 30 in laboratory conditions (Schimel et al. 1989). Additionally, the few days before and after the collection of the autumn sample were unusually cold (Fig. S2), and cold temperatures and freezing can cause accumulation of carbohydrates in fungi (Tibbett et al. 2002), which could also widen microbial C:N ratio. While environmental conditions may be a factor in the microbial biomass C:N, it is likely that N limitation is a major factor in these long-term, unfertilized, agroecosystems .

Conclusions

As the growing population is increasingly reliant on soils for food, fiber, and fuel we will either need to consume less, put more land into production, or better use the land we already have in production. Putting more land in production will likely result in declines in local and global biodiversity. Thus, it is critical to incorporate biodiversity through any means possible into the existing managed ecosystems – even including biodiversity through time as with crop rotations. Here we show that both microbial biomass and function are strongly influenced by cropping diversity. In fact, the influence of crop rotations on soil microbes and functioning lasts over an entire growing season and even when all soils are under the same crop. Crop rotations clearly enhance soil microbial biomass and activity, which are now considered a pillar of soil health, and it appears from our study that rotations also facilitate microbes in supplying more soil N to crops (Fig. S8). Overall, our study highlights the importance of incorporating biodiversity

525 into agroecosystems by including more crops in rotation, especially cover crops, to enhance
526 beneficial soil processes controlled by soil microbes.

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Table 1. Soil carbon (C) and nitrogen (N) pools by season and crop rotation

Season	Crop Rotation	Total Organic C	Total N	NO_3^--N	NH_4^+-N	DOC	DON	C:N	DOC:DON
		---------- g kg^{-1} ---------		---------- mg kg^{-1} ----------					
Spring									
	mC	8.1 (0.8)	0.8 (0.1)ab	2.66 (0.79)	0.06 (0.01)B	14 (4)bB	5 (1)bB	9.8 (0.3)	2.8 (0.2)B
	CS	7.8 (1.2)	0.8 (0.1)ab	2.97 (1.13)	0.06 (0.01)B	11 (1)abB	5 (1)bB	10.3 (0.4)	2.1 (0.2)B
	CSW	7.0 (0.6)	0.7 (0.1)b	2.67 (0.39)	0.10 (0.02)B	21 (8)abB	6 (1)abB	10.4 (0.4)	4.2 (1.9)B
	CSW1	8.7 (0.4)	0.9 (0.1)a	3.10 (0.66)	0.10 (0.02)B	44 (18)aB	8 (1)aB	9.6 (0.2)	5.4 (2.6)B
	CSW2	8.2 (1.4)	0.8 (0.1)ab	3.49 (0.62)	0.12 (0.03)B	26 (7)abB	8 (2)aB	10.2 (0.2)	3.3 (0.4)B
Summer									
	mC	7.9 (0.8)	0.8 (0.1)ab	5.58 (0.67)c	0.08 (0.02)A	35 (4)bB	18 (1)bA	10.2 (0.4)	2.0 (0.1)C
	CS	7.6 (0.9)	0.8 (0.1)ab	9.47 (1.96)b	0.08 (0.01)A	32 (4)abB	33 (7)bA	9.8 (0.1)	1.0 (0.1)C
	CSW	7.6 (0.7)	0.8 (0.0)b	7.76 (0.75)b	0.08 (0.01)A	43 (7)abB	28 (4)abA	9.7 (0.3)	1.6 (0.3)C
	CSW1	8.1 (0.8)	0.9 (0.1)a	16.68 (0.87)a	0.37 (0.22)A	88 (32)aB	76 (8)aA	9.0 (0.2)	1.2 (0.4)C
	CSW2	8.7 (1.1)	0.9 (0.1)ab	12.14 (4.03)ab	0.34 (0.12)A	54 (7)abB	68 (13)aA	9.5 (0.1)	0.8 (0.1)C
Autumn									
	mC	8.1 (0.6)	0.7 (0.1)ab	1.31 (0.15)	0.07 (0.02)B	58 (21)bA	5 (1)bB	11.4 (0.3)	14.3 (7.3)A
	CS	7.7 (1.1)	0.7 (0.1)ab	1.44 (0.28)	0.06 (0.01)B	46 (15)abA	5 (1)bB	10.9 (1.0)	9.6 (3.2)A
	CSW	7.4 (0.8)	0.7 (0.1)b	1.28 (0.30)	0.08 (0.02)B	117(77)abA	6 (2)abB	10.6 (0.6)	15.6 (5.2)A
	CSW1	9.6 (0.6)	0.9 (0.0)a	1.41 (0.06)	0.05 (0.01)B	102 (27)aA	7 (1)aB	10.6 (0.5)	17.1 (7.2)A
	CSW2	8.9 (0.9)	0.9 (0.1)ab	0.96 (0.15)	0.05 (0.01)B	190 (42)abA	6 (1)aB	10.4 (0.4)	30.4 (4.0)A
ANOVA Factor					*P* values				
Season		0.756	0.769	**< 0.001**	**0.004**	**< 0.001**	**< 0.001**	0.213	**< 0.001**
Rotation		0.298	**0.040**	**< 0.001**	0.084	**0.038**	**< 0.001**	0.223	0.947
Season × Rotation		0.994	0.928	**< 0.001**	0.071	0.965	0.221	0.746	0.192

Note: Crop rotation abbreviations are: monoculture corn (mC), corn-soy (CS), corn-soy-wheat (CSW), corn-soy-wheat with red clover cover crop (CSW1), and corn-soy-wheat with red clover + rye cover crops (CSW2). Means (n = 4) are shown with standard errors in parentheses. Significant comparisons (*P* values in bold) are shown among Rotations (lowercase) and Season (capital) with letters.

Table 2. Soil extracellular enzyme activities (EEA) expressed as nano-moles of product per hour per gram of dry soil.

Season	Rotation	BGase	CBHase	LAPase	NAGase	PHOSase	TAPase	PPOase	PERase
		nmol hr^{-1} g^{-1} soil							
Spring									
	mC	94 (8)b	27 (2)b	24 (4)bA	27 (2)ab	133 (19)bC	10 (1)abA	140 (47)B	614 (12)a
	CS	107 (18)b	28 (5)b	28 (4)abA	20 (2)b	129 (20)bC	11 (0)abA	100 (30)B	634 (53)a
	CSW	118 (12)ab	31 (4)ab	26 (8)abA	33 (2)ab	152 (7)abC	12 (2)bA	92 (27)B	602 (59)ab
	CSW1	148 (5)a	50 (5)a	43 (5)abA	47 (3)a	188 (17)aC	16 (1)aA	87 (13)B	516 (24)b
	CSW2	153(13)ab	56 (12)ab	33 (5)aA	48 (5)a	208 (8)aC	16 (1)aA	137 (61)B	562 (24)b
Summer									
	mC	100 (5)b	37 (3)b	7 (2)bB	43 (4)	270 (42)bA	9 (2)abB	174 (67)B	676 (88)a
	CS	111 (17)b	43 (10)b	14 (3)abB	44 (7)	291 (25)bA	9 (1)abB	140 (50)B	580 (124)b
	CSW	102 (7)ab	47 (12)ab	14 (2)abB	47 (3)	280 (13)abA	7 (2)bB	96 (29)B	578 (68)b
	CSW1	146 (12)a	61 (10)a	20 (3)abB	69 (10)	370 (45)aA	14 (1)aB	236 (91)B	317 (144)bc
	CSW2	132 (17)ab	62 (14)ab	13 (4)aB	59 (9)	400 (56)aA	12 (1)aB	126 (73)B	392 (97)c
Autum									
	mC	111 (9)b	44 (6)b	5 (3)bB	67 (13)	238 (57)bB	14 (3)abA	330 (77)A	543 (113)a
	CS	110 (17)b	42 (8)b	8 (1)abB	55 (7)	209 (36)bB	11 (2)abA	234 (64)A	461 (103)bc
	CSW	115 (19)ab	49 (15)ab	9 (2)abB	54 (9)	245 (34)abB	14 (2)bA	176 (18)A	517 (150)b
	CSW1	138 (10)a	59 (6)a	8 (1)abB	63 (13)	277 (42)aB	18 (2)aA	300 (30)A	396 (76)c
	CSW2	117 (15)ab	46 (8)ab	17 (3)aB	63 (2)	308 (24)aB	18 (2)aA	202 (51)A	336 (49)c
ANOVA Factor					*P* values				
	Season	0.775	0.063	**<0.0001**	**<0.0001**	**<0.0001**	**0.003**	**<0.0001**	**<0.0001**
	Rotation	**0.017**	**0.006**	**0.007**	**<0.0001**	**0.0003**	**0.002**	0.224	**<0.0001**
Season × Rotation		0.852	0.839	0.314	**<0.0001**	0.967	0.647	0.837	**<0.0001**

Note: See Table 1 for crop rotation abbreviations. Means (n = 4) are shown with standard errors in parentheses. Significant comparisons (*P* values in bold) are shown among Rotations (lowercase) and Season (capital) with letters.

Table 3. Analysis of variance of results from the principal components analysis of community-level physiological profile (Fig. 4).

ANOVA§ Parameter	PC1		PC2		PC3		PC4		PC5		MANOVA (Total)	
Proportion of variance	38.7		17.7		14.5		9		3.8		83.7	
ANOVA Factor	F	*P* value	F	*P* value	F	*P* value	F	*P* value	F	*P* value	F	*P* value
Season	**64.02**	**< 0.001**	**22.57**	**< 0.001**	**5.4**	**0.008**	0.68	0.510	**10.33**	**< 0.001**	**33.28**	**< 0.001**
Crop Rotation	0.69	0.605	**3.03**	**0.028**	**12.82**	**< 0.001**	0.36	0.834	1.81	0.146	**2.19**	**0.003**
Season × Rotation	0.16	0.995	1.22	0.311	0.55	0.81	0.88	0.544	0.27	0.973	0.65	0.949
Significant comparisons¥	1=3≠2		1=2≠3, CS ≠ CSW1		1=2≠3, mC=CS≠CSW= CSW2				1≠2=3,			

§ Degrees of freedom: Season = 2, Crop Rotation = 4, Season*Rotation = 8.

¥ Significant comparison abbreviations: 1 = spring, 2 = summer, 3 = autumn

Note: See Table 1 for crop rotation abbreviations. Significant comparisons are in bold.

Table 4. Catabolic evenness by season and crop rotation (showing full suite of C substrates, without carboxylic acids, and carboxylic acids only).

Season	Rotation	Catabolic Evenness		
		Full	No Carboxylic Acids	Carboxylic Acids Only
Spring				
	mC	24.37 (0.79)A	20.20 (0.05)aA	7.60 (0.23)aB
	CS	23.79 (0.91)A	19.80 (0.15)aA	7.21 (0.13)abB
	CSW	22.98 (0.63)A	19.65 (0.15)bA	6.56 (0.35)bB
	CSW1	24.28 (0.44)A	18.95 (0.19)abA	6.91 (0.12)abB
	CSW2	24.52 (0.72)A	19.75 (0.24)bA	6.90 (0.31)bB
Summer				
	mC	14.99 (1.61)B	18.95 (0.59)aA	4.91 (0.54)aC
	CS	12.86 (1.77)B	20.20 (0.18)aA	4.32 (0.38)abC
	CSW	12.10 (1.02)B	19.82 (0.54)bA	3.93 (0.20)bC
	CSW1	13.83 (1.65)B	18.59 (0.83)abA	4.34 (0.50)abC
	CSW2	12.78 (0.92)B	19.24 (0.51)bA	3.75 (0.11)bC
Autumn				
	mC	25.81 (0.79)A	19.62 (0.16)aB	8.47 (0.24)aA
	CS	25.82 (0.55)A	19.11 (0.22)aB	8.41 (0.22)abA
	CSW	25.71 (0.74)A	18.98 (0.28)bB	8.12 (0.61)bA
	CSW1	27.41 (0.63)A	18.63 (0.12)abB	8.90 (0.24)abA
	CSW2	26.08 (0.67)A	18.17 (0.28)bB	8.11 (0.08)bA

ANOVA Factor			
Season	**< 0.001**	**0.002**	**< 0.001**
Crop Rotation	0.357	**0.035**	**0.028**
Season × Rotation	0.928	0.058	0.807

Note: See Table 1 for crop rotation abbreviations. Means (n = 4) are shown with standard errors in parentheses. Significant comparisons (*P* values in bold) are shown among Rotations (lowercase) and Season (capital) with letters.

Table 5. Pearson correlation coefficients between soil properties and community-level physiological profile (CLPP) parameters.

Soil Variable	Substrate Guilds					Catabolic Evenness		
	Amino acids	Amine	Carboxylic Acids	Carbohydrates	Complex	Full	No Carboxylic Acids	Only Carboxylic Acids
Water content	ns	ns	ns	ns	ns	0.40	ns	**0.52**
pH	0.27	**0.43**	**-0.41**	ns	**0.53**	**0.68**	ns	**0.74**
Sand	**-0.36**	ns	0.28	-0.27	ns	ns	ns	ns
Silt	0.30	ns	ns	ns	ns	ns	ns	ns
Clay	ns	ns	ns	ns	ns	ns	-0.33	ns
Total C	ns	ns	ns	ns	ns	ns	**-0.40**	ns
Total N	ns	ns	ns	ns	ns	ns	**-0.40**	ns
C-to-N ratio	ns	0.27	ns	ns	0.30	**0.45**	ns	**0.53**
NH_4^+	ns	-0.31	0.33	ns	**-0.37**	**-0.40**	ns	**-0.38**
NO_3^-	**-0.58**	**-0.55**	**0.66**	-0.30	**-0.72**	**-0.74**	ns	**-0.70**
PMC	ns	0.29	ns	ns	ns	ns	**-0.63**	ns
PMN	ns	-0.27	0.32	ns	**-0.55**	**-0.49**	ns	**-0.52**
MBC	0.31	**0.49**	**-0.37**	ns	ns	**0.41**	**-0.38**	**0.47**
MBN	**0.36**	0.34	**-0.37**	**0.42**	ns	**0.36**	ns	0.31
MBC:MBN	ns	**0.40**	ns	ns	ns	0.31	-0.34	**0.40**
BGase	ns	**-0.43**	**0.30**	ns	ns	-0.29	0.32	-0.28
CBHase	-0.32	**-0.47**	**0.39**	-0.27	ns	-0.33	ns	-0.28
LAPase	ns	-0.29	ns	ns	ns	ns	**0.49**	ns
TAPase	ns	**-0.37**	ns	ns	ns	ns	ns	0.37
NAGase	**-0.35**	**-0.56**	**0.47**	**-0.39**	-0.29	**-0.46**	**0.29**	**-0.41**
PHOSase	**-0.45**	**-0.66**	**0.56**	**-0.46**	-0.34	**-0.63**	**0.34**	**-0.60**
PPOase	-0.38	-0.33	0.37	-.31	ns	ns	ns	ns
PERase	-0.40	-0.54	0.42	-0.37	ns	-0.30	0.43	ns

Note: Only significant correlations are shown (P values < 0.05), bold values are $P < 0.01$, ns = non-significant

FIGURES

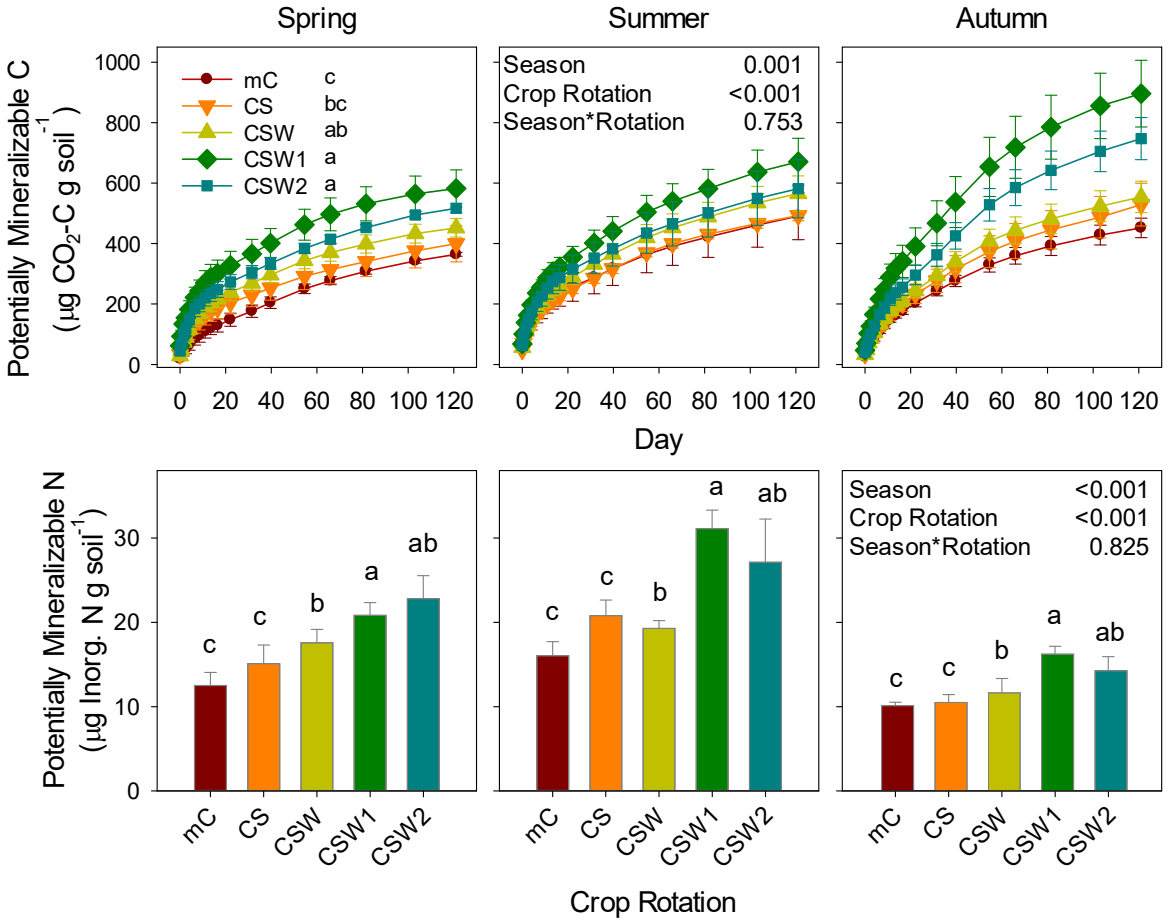

Figure 1. Potentially mineralizable carbon (top row) and potentially mineralizable nitrogen (bottom row). Crop rotation abbreviations are: monoculture corn (mC), corn-soy (CS), corn-soy-wheat (CSW), corn-soy-wheat with red clover cover crop (CSW1), and corn-soy-wheat with red clover + rye cover crops (CSW2). Means are shown and error bars are standard errors (n = 4). *P* values from ANOVA results are shown for each variable with the main effects (Season and Crop Rotation) and the interaction, as well as significant differences from post-hoc results shown as lowercase letters.

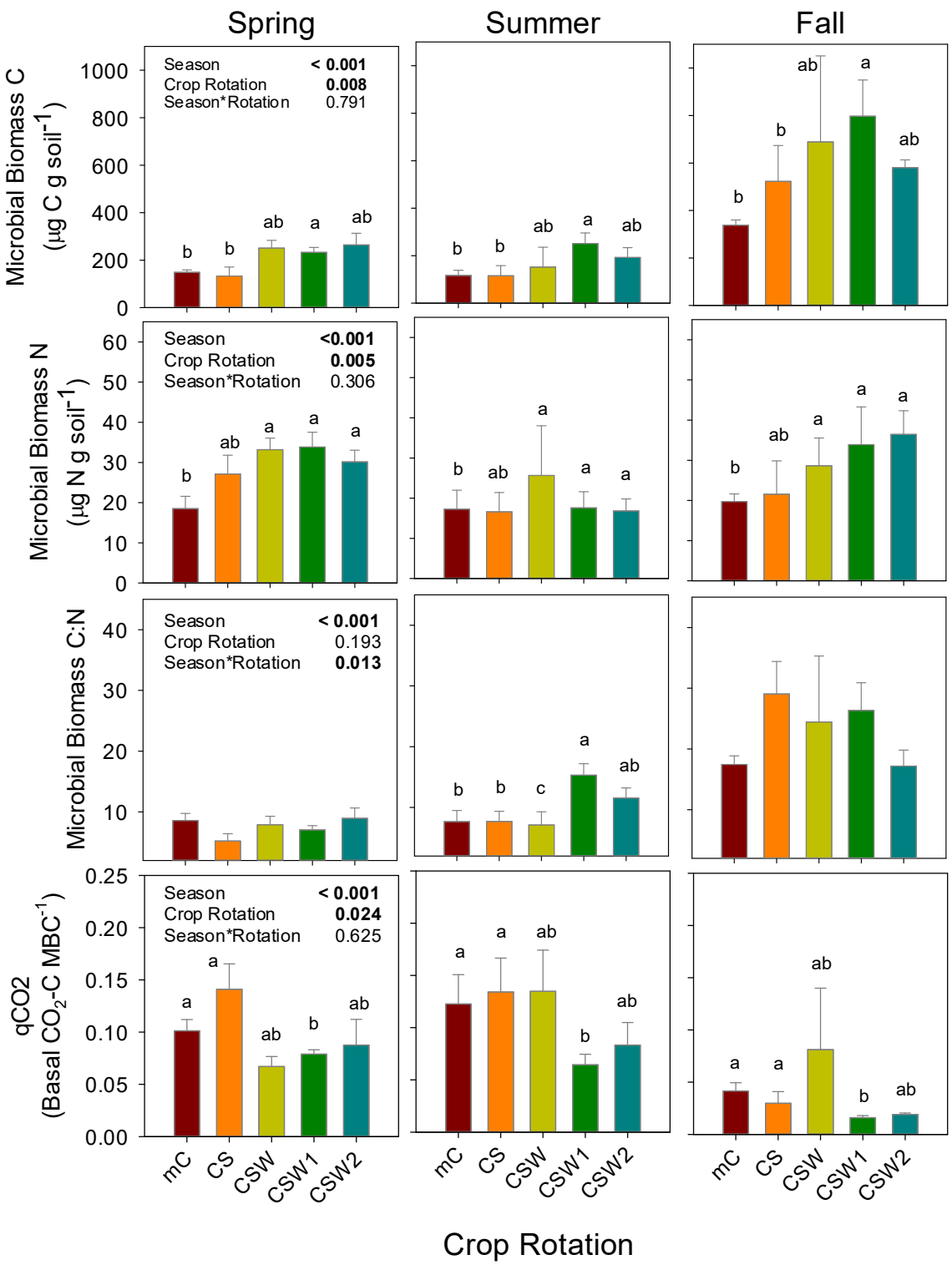

Figure 2. Soil microbial biomass parameters by season and crop rotation. See Fig.1 for crop rotation abbreviations. Means are shown and error bars are standard errors (n = 4). *P* values from ANOVA results are shown for each variable with the main effects (Season and Crop Rotation) and the interaction, as well as significant differences from post-hoc results shown as lowercase letters.

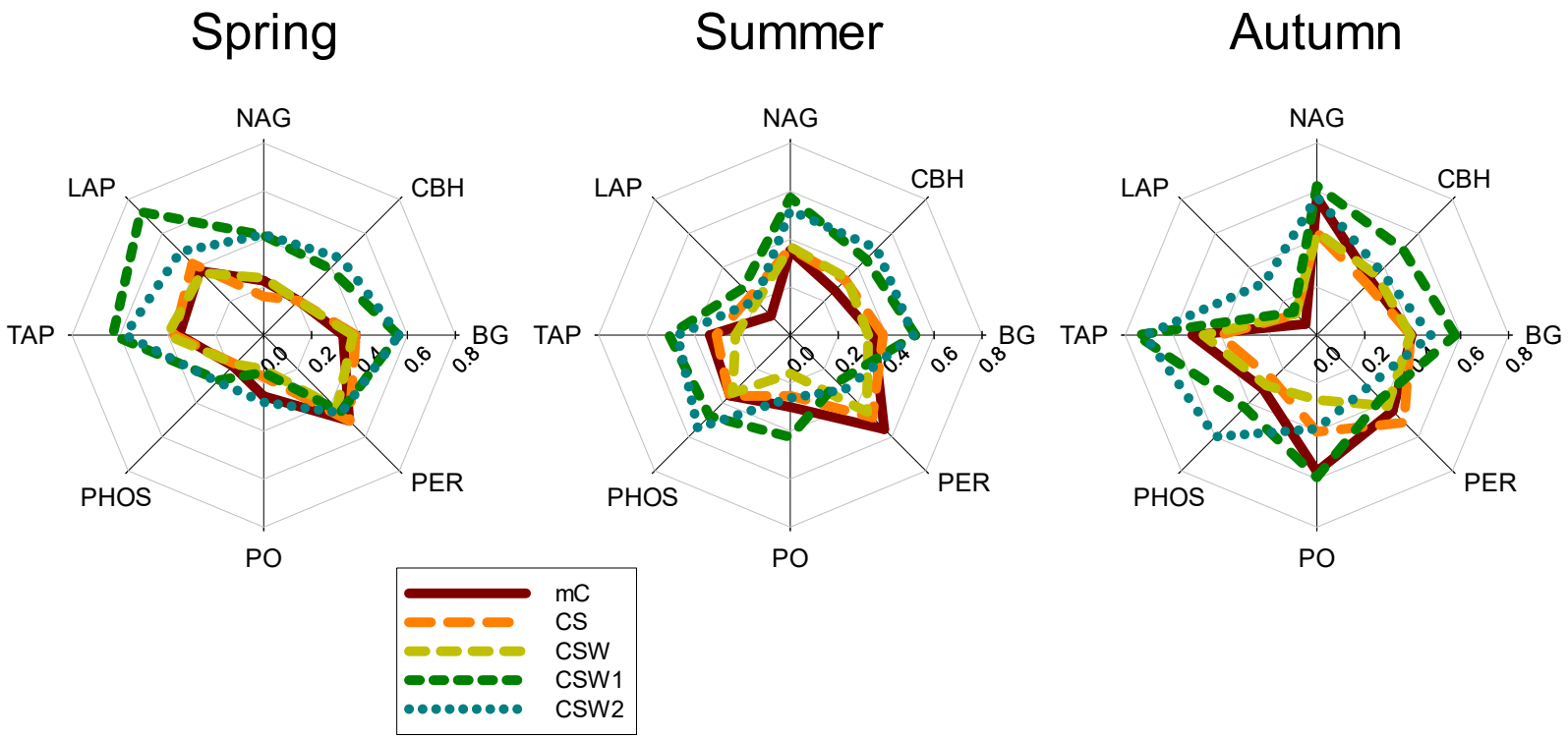

Figure 3. Extracellular enzyme activities (EEA) normalized for the maximum value during each season. EEA abbreviations are: β-1,4,-glucosidase (BG), β-D-1,4-cellobiohydrolase (CBH), β-1,4,-N-acetyl glucosaminidase (NAG), acid phosphatase (PHOS), Tyrosine aminopeptidase (TAP), Leucine aminopeptidase (LAP), phenol oxidase (PO), and peroxidase (PER). See Fig.1 for crop rotation abbreviations.

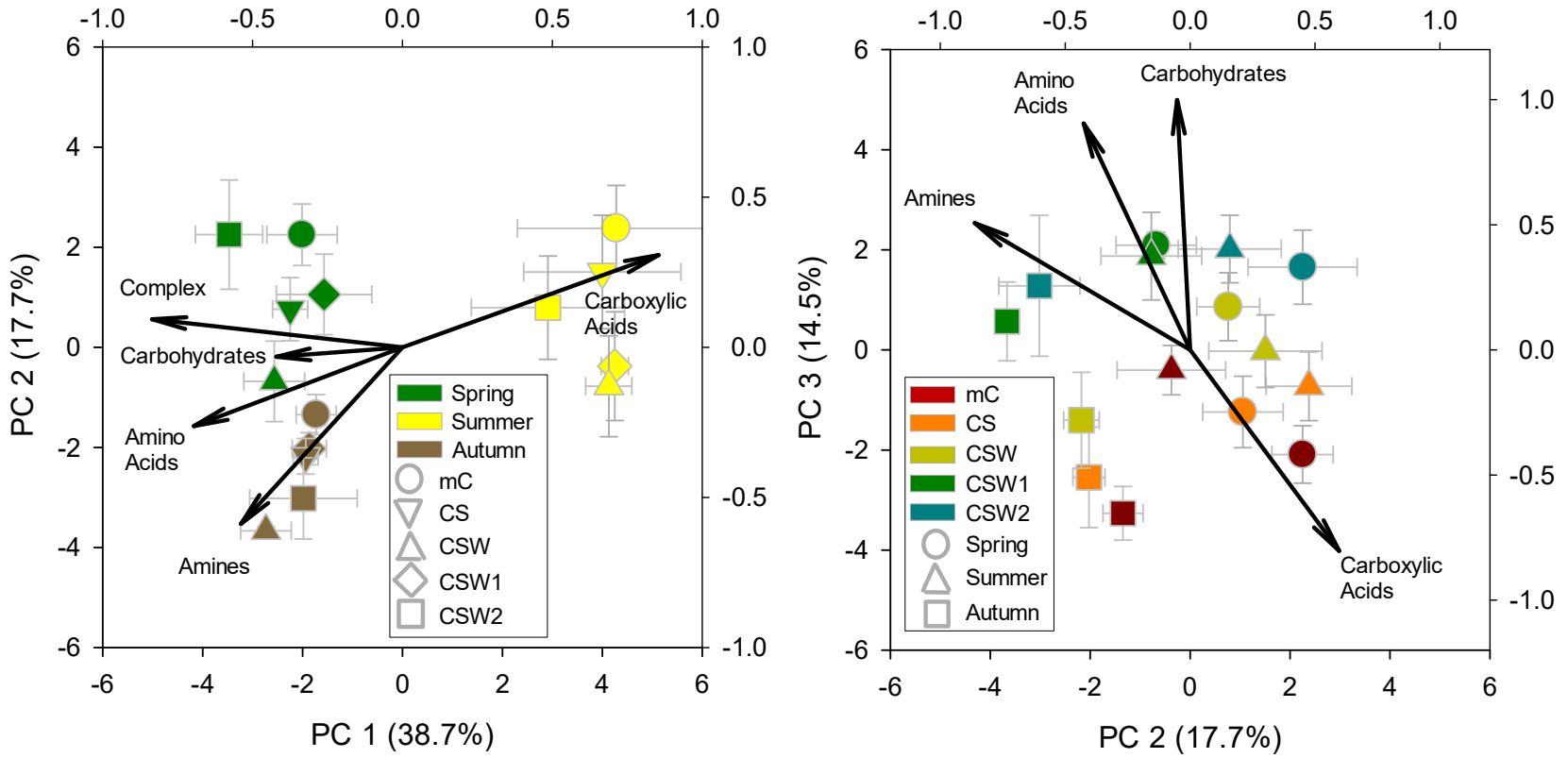

Figure 4. Principal components analysis (PCA) on all 31 substrates. *Left Panel:* Principal components 1 and 2, where Season is dominant discriminating factor ($P < 0.001$) and *Right Panel:* Principal components 2 and 3 where Rotation is highlighted as a dominant discriminating factor. See also Table 5 for PCA and ANOVA results. Means are shown and error bars are standard errors (n = 4). See Fig.1 for crop rotation abbreviations.