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

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1   **Abstract**

2   Declines in plant diversity will likely reduce soil microbial biomass, alter microbial

3   functions, and threaten the provisioning of soil ecosystem services. We examined whether

4   increasing temporal plant biodiversity in agroecosystems (by rotating crops) can partially reverse

5   these trends and enhance soil microbial biomass and function. We quantified seasonal patterns

6   in soil microbial biomass, respiration rates, extracellular enzyme activity, and catabolic potential

7   three times over one growing season in a 12-year crop rotation study at the W.K. Kellogg

8   Biological Station LTER. Rotation treatments varied from one to five crops in a three-year

9   rotation cycle, but all soils were sampled under a corn year. We hypothesized that crop diversity

10   would increase microbial biomass, activity, and catabolic evenness (a measure of functional

11   diversity). Inorganic N, the stoichiometry of microbial biomass and dissolved organic C and N

12   varied seasonally, likely reflecting fluctuations in soil resources during the growing season.

13   Soils from biodiverse cropping systems increased microbial biomass C by 28-112 % and N by

14   18-58 % compared to low diversity systems. Rotations increased potential C mineralization by

15   as much as 53 %, and potential N mineralization by 72 %, and both were related to substantially

16   higher hydrolase and lower oxidase enzyme activities. The catabolic potential of the soil

17   microbial community showed no, or slightly lower, catabolic evenness in more diverse rotations.

18   However, the catabolic potential indicated that soil microbial communities were functionally

19   distinct, and microbes from monoculture corn preferentially used simple substrates like

20   carboxylic acids, relative to more diverse cropping systems. By isolating plant biodiversity from

21   differences in fertilization and tillage, our study illustrates that crop biodiversity has overarching

22   effects on soil microbial biomass and function that last throughout the growing season. In

23   simplified agricultural systems, relatively small increases in crop diversity can have large

24   impacts on microbial community size and function, with cover crops appearing to facilitate the

25   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

49   for ecosystem services such as sequestering C and soil fertility (Carpenter-Boggs et al. 2000,

50   Kallenbach et al. 2015), but also for ecosystem "dis-services" such as emission of soil-to-

51   atmosphere greenhouse gases (McDaniel et al. 2014*b*). Furthermore, the partitioning of

52   resources used in catabolism of residue and formation of SOM will affect long-term soil fertility

53   (Lange et al. 2015; Kallenbach et al. 2015).

54   Soil microbial catabolism can be assessed using many different methods. The two most

55   common measures are soil extracellular enzyme activities, microbe-produced catalysts for

56   catabolism of soil substrates, and respiration response when supplying microbes with a source of

57   C. The latter method, when multiple C compounds are added to the same soil, is commonly

58   referred to as a community-level physiological profiles (CLPP), or as catabolic response profiles.

59   The basic method for measuring soil CLPP involves adding a suite of C substrates to soils and

60   measuring the catabolic response as CO_2 production or O_2 consumption with redox indicators

61   (e.g. Biolog; Guckert et al. 1996). These C substrates are typically ecologically-relevant

62   compounds found in soils, and are intended to represent root exudates, microbial or plant cell

63   structures, or other more-processed soil organic molecules. Other studies have used CLPPs to

64   establish a catabolic "fingerprint" to distinguish soil microbial communities from one another by

65   how they utilize different C substrates (Lupwayi et al. 1998; McDaniel et al. 2014*b*). The CLPP

66   data can also be used to derive measures of metabolic diversity including substrate-use richness

67   or catabolic evenness.

68   What can catabolic potential, and even catabolic evenness, tell us about soil microbial

69   functioning in agroecosystems? Previous studies have shown that these metabolic diversity

70   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.

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, 2015). 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

took place on April 27th, 2012; July 19th, 2012; and November 1st, 2012 – hereafter referred to as spring, summer, and autumn. Corn was planted in all plots on June 11th, 2012. Three 5 cm diameter soil cores (0-10 cm deep) were collected between rows from each plot, homogenized in the field, and then put on ice and shipped to the University of New Hampshire. In the lab, field-moist soils were immediately sieved using a 2 mm sieve. A sub-sample was taken from sieved soil and dried at 105 °C to determine gravimetric water content. Water-holding capacity was determined as the water content after soils were saturated and drained for 6 h.

Soil carbon and nitrogen parameters

Five g of field-moist soil were extracted for inorganic N with 40 ml of 0.5 M K_2SO_4. The soil slurries were shaken for 1 h before the extracts were filtered on Whatman GF/C (5) filters and filtrate frozen and stored until analysis. Soil nitrate (NO_3^-) and ammonium (NH_4^+) were measured using the methods detailed in McDaniel et al. (2014c). We also used the same extracts to measure dissolved organic C and N (DOC and DON). The extracts were run on a TOC-TN analyzer (TOC-V-CPN; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). Total C and N were analyzed by sieving soils through 2 mm sieve, grinding and analyzing on an ECS 4010 CHNSO Elemental Analyzer (Costech Analytical Technologies, Inc., Valencia, CA).

Potential mineralization rates of C (PMC) and net N (or PMN) estimate the quantity of potentially-mineralizable SOM at an optimal temperature and soil moisture, and reflect both the activity of the microbial community and availability of SOM (Paul et al. 1999, Robertson et al. 1999). These mineralization assays provide a good indicator of the potential for a soil to provide plants with N (Stanford and Smith 1972, Robertson et al. 1999). Both PMC and PMN were measured on 10 g of air-dried soils in Wheaton serum vials and brought to 50% water-holding capacity, which is near optimal water content for respiration in these soils (Grandy and

139 Robertson 2007), and incubated for 4 months. During this 4-month period CO_2 efflux was

140 measured on a LI-820 infrared gas analyzer (LI-COR, Lincoln, NE). Efflux was measured using

141 the change in headspace CO_2 concentration measured between two time points. Each soil efflux

142 measurement began by aerating jars, capping, and injecting a time-zero sample and then a second

143 sample between 5 h to 2 d later. Efflux was calculated as the difference in CO_2 concentration

144 between the two time points divided by time. Measurements of PMC occurred more frequently

145 at the beginning of the experiment (daily), and became less frequent toward the end (once every

146 other week), for a total of 19 sampling events over 120 d. High frequency measurements are

147 required during the beginning of these incubations, when respiration rates are high, to prevent

148 build-up of CO_2 (and lack of O_2). The PMN was assessed by extracting the inorganic N (NH_4^+ +

149 NO_3^-) produced at the end of the incubation, measuring it with the methods described above,

150 then subtracting this final value from the initial inorganic N extracted before the incubation

151 began.

152 *Soil microbial parameters*

153   Soil microbial biomass C (MBC) and N (MBN) were determined using the modified

154 chloroform fumigation and extraction method (Vance et al. 1987), but modified for extraction in

155 individual test tubes (McDaniel et al. 2014c). Briefly, two sets of fresh, sieved soil (5 g) were

156 placed in 50 ml test tubes, and 1 ml of chloroform was added to one set of tubes and capped.

157 The tubes sat overnight (24 h) and were then uncapped and exposed to open air in a fume hood

158 to allow chloroform to evaporate. Soils were then extracted in the tubes with 25 ml of 0.5 M

159 K_2SO_4. The chloroform fumigated and non-fumigated extracts were run on a TOC-TN analyzer

160 (TOC-V-CPN; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). We used 0.45

161 (Joergensen 1996) and 0.54 (Brookes et al. 1985) for the C and N extraction efficiencies.

162   Soils were analyzed for 8 extracellular enzyme activities (EEAs): β-1,4-glucosidase

163   (BG), β-D-1,4-cellobiohydrolase (CBH), β-1,4-N-acetyl glucosaminidase (NAG), acid

164   phosphatase (PHOS), Tyrosine aminopeptidase (TAP), Leucine aminopeptidase (LAP),

165   polyphenol oxidase (PO), and peroxidase (PER). Given the large number of samples (60) and

166   variety of measurements made at each of 3 sampling dates, soil EEAs were conducted on frozen

167   samples within 4 weeks of sampling. While some studies show freezing has minor effects on

168   EEAs (Peoples & Koide 2012), others show no effects (Lee et al. 2007, Deforest 2009), and we

169   assume that any effects of freezing will be consistent among treatments. Extracellular enzyme

170   activity assays were carried out following previously published protocols (Saiya-Cork et al.

171   2002, German et al. 2011), but with some modifications. Briefly, 1 g of soil was homogenized

172   with a blender in 80 ml of sodium acetate buffer at pH 5.6 (the average pH at the site). Soil

173   slurries were pipetted into 96-well plates and then analyzed on a Synergy 2 plate reader (BioTek

174   Instruments, Inc., Winooski, VT). For oxidoreductase enzymes, the supernatant from the slurry

175   plates were pipetted into a clean plate to avoid interference with soil particles. Hydrolase assays

176   were read at 360/40 and 460/40 fluorescence and oxidoreductases at 450 nm absorbance. For

177   more details on the extracellular enzyme methods see McDaniel et al. (2014c).

178   Community-level physiological profiles (CLPP) were conducted using the MicroResp^{TM}

179   system (Chapman et al. 2007, Zhou et al. 2012, McDaniel et al. 2014b). The MicroResp^{TM}

180   system allows for high-throughput measurement of soil catabolic responses to multiple C

181   substrates. Each soil was loaded into 96 deep-well plates using the MicroResp^{TM} soil dispenser,

182   and then brought to 50% water-holding capacity. Thirty-one substrates were used at

183   concentrations ranging from 7.5 to 30 mg C per g of soil H_2O, as recommended by the

184   MicroResp^{TM} 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 (qCO_2) was calculated simply as the basal respiration

199   over 6 h (determined in the MicroResp[TM] method) divided by the MBC. Almost all the soil data

200   were non-normal, including: DOC, DON, PMC, PMN, microbial biomass, enzymes, and

201   catabolic evenness. All these data were log-normal transformed, except for catabolic evenness

202   which was 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} $\pm$

218   SE) in each treatment was as follows: mC $= 2846 \pm 152$, CS $= 4208 \pm 575$, CSW $= 4107 \pm 220$,

219   CSW1 $= 4015 \pm 187$, CSW2 $= 5219 \pm 1180$ (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

229   spring and 13 times higher than summer. Soil NO_3^--N was the only variable that showed a

230   significant Season × Rotation interaction ($P < 0.001$). There were significant main effects of

231   crop rotation on DOC and DON (Table 1). During the summer the two cover crop treatments

232   had the highest NO_3^--N concentrations (16.68 ± 0.87 and 12.14 ± 4.03 mg kg^{-1}), which was 67 %

233   greater than CSW and CS treatments, and 158 % greater than mC. The CSW1 treatment had 112

234   % greater DOC concentrations than mC ($P < 0.001$), and two cover crop treatments had 107 %

235   greater DON than non-cover crop treatments and 211 % more than the mC treatment.

236   The potentially mineralizable pools of C and N showed significant main effects of both

237   Season and Rotation ($P < 0.03$), but no interactions. The PMC was highest during the autumn

238   (636 ± 105 µg CO_2-C g $soil^{-1}$), while PMN was highest during the summer (89 ± 105 µg

239   NH_4^++NO_3^- g $soil^{-1}$). Generally, both PMC and PMN increased with increasing number of crops

240   in rotation (Fig. 1), and the incorporation of cover crops appeared important in regulating both

241   PMC and PMN. For example, the PMC average of both cover crop treatments (CSW1 and

242   CSW2) were 53 % and 41 % greater than mC and CS treatments ($P < 0.042$), respectively. The

243   PMN average from the cover crop treatments was 36 %, 48 %, and 72% greater than the mC, CS,

244   and CSW treatments, respectively ($P < 0.015$). The potentially mineralizable C-to-N ratio

245   (PMC:PMN), considered an index of the quality of accessible SOM (Schimel et al. 1985; Clein

246   & Schimel 1995), showed a significant Season × Rotation interaction ($P = 0.045$, Fig. S3). The

247   PMC:PMN was markedly higher in the autumn than in summer and spring, indicating a greater

248   demand for N in autumn. For summer and spring more diverse rotations had less CO_2 produced

249   per unit of net inorganic N mineralized. However in the autumn, after harvest, the crop rotation

250   effects on the PMC:PMN were reversed; meaning the more diverse crop rotations had greater

251   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

275   glucosamine from chitin (NAG) and the lignin-reducing enzyme that uses peroxide (PER) were

276   the only enzymes that showed a significant Season × Rotation interaction ($P < 0.001$). Spring

277   had the greatest activities of LAP, 175% greater than the average of the other seasons (Fig. 3,

278   Table 2). In summer, we see a shift to the highest PHOS activity – 25% greater than autumn and

279   99% greater than spring. There were no main effects of Season on BG or CBH, but Rotation

280   main effects were significant, with the CSW1 treatment having an average of 42 and 50 % higher

281   BG and CBH activity than CS and mC soils, respectively. The majority of the hydrolase

282   enzymes were higher in the cover crop treatments compared to that of the non-cover crop

283   treatments, especially mC (Table 2, Fig. 3). The two oxidoreductase enzymes (PO and PER)

284   decreased with crop diversity. There were no significant main effects on the enzyme ratio used

285   to assess C-versus-N demand (BG to NAG+LAP).

286   The community-level physiological profile (CLPP), a catabolic profile of the soil

287   microbial communities, showed both significant Season ($P < 0.001$) and Rotation ($P = 0.003$)

288   main effects (Figs. 4, S4; Table 3). A principal components analysis of the CLPP data showed

289   that the summer soils corresponded with highest carboxylic acid utilization (Fig. 4), as Season

290   was the strongest discriminating factor along principal component 1 (PC1, Table 3). However,

291   when rotating and examining PC2 and PC3, there was a strong treatment gradient from the

292   bottom-right to upper-left quadrants of the graph (Fig. 4, right panel). The lower-diversity

293   treatments corresponded with greater use of carboxylic acid substrates. Across seasons, summer

294   exhibited the lowest catabolic evenness (12.9 ± 1.4), but there was no crop rotation effect on

295   catabolic evenness using all substrates (i.e. Full, Table 4).

296   Due to the overwhelming influence of carboxylic acids in the PCA variation, and their

297   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-

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

322   evenness.

323   We used the soil microbial responses of EEA and the CLPP because we assumed they

324   would be complementary. For example, adding N-acetyl glucosamine in the CLPP should be

325   related to ß-1,4-N-acetyl glucosamindase (NAG) enzyme activity.   Indeed, this was the case.

326   Measuring NAG enzyme and adding the Nag amine to the soils showed a somewhat tight

327   relationship, but this changed during autumn (Fig. S6). Additionally, when the CLPP substrates

328   were grouped by guild they were significantly correlated with EEAs (Fig. S7). For instance,

329   total amino acid catabolic response positively correlated well with LAP+TAP enzymes ($r^2 =$

330   $0.35, P < 0.001$) meaning that high activity of these enzymes in soils corresponded with high

331   relative use of these substrates when added to soils, compared to other substrates added to the

332   soil. This suggests that the LAP and TAP enzymes strongly reflect demand for N-bearing amino

333   acids in soils. However, the catabolic response of the 'Complex' guild was negatively correlated

334   with PO ($r^2 = 0.29, P < 0.001$). Soil PMN was better correlated with crop yields ($r^2 = 0.61, P <$

335   0.001) than NO_3^- in early spring (Fig. S8), highlighting the importance of PMN-like

336   measurements being used as soil fertility tests.

Discussion

338   Increasing biodiversity in this long-term crop rotation experiment has altered the soil

339   microbial dynamics across an entire growing season. This occurred even though the soils in our

340   study were all in the same crop phase (corn) for the season, indicating that observed differences

341   among soils reflect long-term rotation effects. Microbial biomass C, N, potential mineralization,

342   and catabolic potential were all altered by crop rotations, although the rotation effect for some of

343   these indicators of microbial functioning also depends upon the season. Soil microbial biomass

344   and activity are now widely recognized as pillars of soil health (Doran and Zeiss 2000). Our

345   results clearly indicate that diversifying agroecosystems (through crop rotations) enhances this

346   aspect of soil health, and is also likely linked to changes in SOM dynamics (Tiemann et al. 2015)

347   as well as the observed differences in yield among crop rotations (Smith et al. 2008, Fig. S8).

348   *Crop biodiversity and soil microbial functioning*

349   Both soil microbial biomass and functioning were strongly affected by increased crop

350   diversity through rotation. This rotation effect was largely independent of the season, as

351   indicated by the limited number of observed Season × Rotation interactions. The exception to

352   this was microbial biomass C/N ratio (Fig. 2), potentially mineralizable C-to-N (Fig. 1 and S3),

353   and two extracellular enzyme activities (NAG and PER, Table 2), which together are likely

354   indicative of the enhanced ability of soil microbes under diverse rotations to process, provision,

355   and retain soil N. The stoichiometric shifts in microbial biomass and potentially mineralizable

356   SOM suggest seasonal changes in microbial communities and/or how microbes shift between C

357   and N resources among crop rotations. For instance, the MBC:MBN ratio is only significantly

358   wider in the two cover crop treatments than those without during the summer when inorganic N

359   was plentiful, and labile C might have been limiting. On the other hand, during the autumn when

360   the soils were most N-limited, the potentially mineralizable C-to-N ratio widened in all

361   treatments but was widest among diverse crop rotations (Figs. 1 and S3). Together these

362   findings suggest that labile C might be a major regulating factor of soil N cycling, and that crop

363   rotations change these dynamics.

364   With regards to provisioning of N, the PMN, MBN, and NAG enzyme activity were

365   greater in soils under more diverse crop rotations during the spring (Fig. 1, 2 and Table 2). NAG

366   has been shown to be strongly related to net N mineralization (Ekenler & Tabatabai 2002),

367   therefore the alignment between these two measures of microbial function were not surprising.

368   Taken together, though, these data indicate that soil microbes from diverse rotations might be

369   able to better supply crops with N via mineralization, at this critical stage when corn crop N

370   demand is high (Blackmer et al. 1989). Thus, in this severely N-limited cropping system, it

371   makes sense that spring PMN was better related to yield than soil inorganic N concentrations

372   because these crops are relying almost exclusively on SOM-derived N. Most importantly, it also

373   suggests that the greater provisioning of N from SOM to plants in more diverse cropping systems

374   is a likely factor for the higher yields in our study (Fig. S8).   These findings are consistent with

375   plant biodiversity studies that find increased aboveground diversity enhances soil microbial

376   biomass and functioning in natural (Stephan et al. 2000, Zak et al. 2003, Lange 2015) and

377   agricultural ecosystems (Lupwayi et al. 1998, Xuan et al. 2012, McDaniel et al. 2014c).

378   While there were some significant differences in soil microbial dynamics between the

379   non-cover-crop rotations (CS and CSW) and monoculture corn (Table 1, Fig. 1 and 2), the

380   largest differences were between the two cover crop treatments and monoculture. This was

381   particularly the case for the red-clover-only cover crop treatment (CSW1). A growing number of

382   other studies show the large positive impact cover crops have on soil microbes and their activity

383   (Mendes et al. 1999; Kabir & Koide 2000; McDaniel et al. 2014c; Mbuthia et al. 2015). The

384   reason cover crops consistently increase soil microbial biomass and activity is likely due to the

385   increased quantity and quality of crop residue inputs, but cover crops also have been shown to

386   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). Both Jiang et al. (2012) and McIntosh et al. (2013) used the same

410 MicroResp™ method used in this paper, while Bartelt-Ryser et al. (2005) used Biolog.

411   In our study, when a subset of the C substrates were analyzed (all non-carboxylic acids,

412 or carboxylic acids only), we found that increased crop diversity decreased catabolic evenness

413 (Table 4). This is unusual considering soils from this same study, but collected a year prior,

414 showed increases of soil biodiversity (Shannon-Weiner index or H') with increased crop

415 diversity when measuring phospholipid fatty acids (Tiemann et al. 2015); and diversity has been

416 found to be strongly, positively related to species evenness in plants and animals (Stirling &

417 Wilsey 2001). In this study, our findings of a lack of an effect (or even a negative effect) of crop

418 biodiversity on catabolic evenness is also contradictory to the findings of Degens et al. (2000),

419 who showed that management practices that decreased soil C are associated with low catabolic

420 evenness. Yet, evidence from these same soil samples showed that crop diversity significantly

421 decreased H' for bacterial 16S rRNA by as much as 5 % compared to monoculture corn (Peralta

422 et al. *in review*). Taken together, the decrease of functional and structural diversity of soil

423 bacteria with crop diversity indicates that crop diversity might decrease bacterial diversity in this

424 crop rotation experiment.   Nevertheless, a recent meta-analysis showed that crop rotations tend

425 to increase soil biodiversity by 3 % and richness by 15 % (Venter et al. 2016), but there was

426 large variability around these estimates. Regardless of aboveground-belowground diversity

427 trends, crop rotations did create functionally distinct microbial communities in our study (Fig. 4).

428 We still do not have a good understanding of how crop rotations alter soil microbial dynamics,

429 nor (arguably more importantly) how these changes in belowground communities might provide

430 beneficial soil ecosystem services like increasing soil C or mineralizing more N to increase crop

431 yields.

432   One trend that emerges across the suite of 31 C substrates is that crop rotations altered

433   soil microbial preference for C substrates (i.e. complex versus simple C substrates). The soils

434   from monoculture corn corresponded to greater use of simple C substrates (especially carboxylic

435   acids), and showed less response to the suite of N-containing and complex substrates (Fig. 4).

436   This finding corroborates a previous study we conducted using whole-plant residues, in which

437   we showed diverse crop rotations resulted in greater decomposition of low quality crop residues

438   (e.g. corn and wheat, McDaniel et al. 2014*c*). Further, when looking only within the relatively

439   labile carboxylic acid substrates, microbial communities in the less diverse crop rotations (mC,

440   and to a lesser extent CS) responded to more labile, low-molecular weight carboxylic acids (e.g.

441   citric, malonic, and malic acid), while soil microbes from more diverse crop rotations responded

442   more to complex, higher-molecular weight carboxylic acids (e.g. caffeic, tartaric, and vanillic

443   acids - Fig. S5d). The strong effects of crop diversity on catabolism of carboxylic acids is not

444   surprising due to the small, yet dynamic, pool of these compounds in soil (Strobel 2001). Since

445   soil microbial function (as measured by CLPP) is an aggregate measure of both the community

446   composition and available resources, it is impossible to tease out which (or both) have changed

447   due to increased crop biodiversity. However, our overall findings indicate that increased

448   aboveground biodiversity through crop rotations and cover crops appears to facilitate soil

449   microbial communities' use of complex C substrates relative to simple ones.

450   *Seasonal dynamics and N limitation*

451   Season strongly influenced the measured pools of labile C and N (Table 1), as well as the

452   microbial biomass size and functioning within this agroecosystem (Figs. 1-4).   We hypothesized

453   that soil microbial function would converge over the growing season, as the current crop exerted

454   greater influence over soil microbes. We did find some support for this hypothesis. Both

455 multivariate measures of extracellular enzyme activities and CLPP showed treatments becoming

456 more similar over the growing season (Figs. 3 and 4). This is based on three time points,

457 however, and we do not know for sure if this convergence was due to the influence of the corn

458 crop or other factors (like microclimatic). Some studies have shown that the current plant

459 species identity often trumps biodiversity legacy in controlling belowground microbial structure

460 and functioning (Stephan et al. 2000, Wardle et al. 2003, Bartelt-Ryser et al. 2005). Conversely,

461 several studies have pointed to weak or no influence of current plant species on soil microbial

462 structure and functioning (Costa et al. 2006, Kielak et al. 2008). The question of whether plant

463 species identity versus spatial and temporal diversity has a stronger control on soil biota remains

464 a critical question in terrestrial ecology.

465   The greatest microbial biomass and activity occurred in autumn, but potential N

466 mineralization peaked in summer. In perennial and annual cropping systems in Iowa, potentially

467 mineralizable N declined from spring to late summer; in addition, extracellular enzyme activities

468 peaked in July but there was little effect of the cropping system (Hargreaves and Hofmockel

469 2013). In another study, season was shown to affect microbial biomass and potentially

470 mineralizable C and N pools in a wheat-sorghum-soybean rotation in south-central Texas

471 (Franzluebbers et al. 1994, 1995, Franzluebbers 2002), but timing for peak values differed

472 depending on the study and cropping systems, likely reflecting different climates and soil types.

473 The frequently observed late-summer spike in microbial biomass and activity may be related to

474 higher temperatures during this time period; however, even within agroecosystems, the timing

475 for maximal microbial biomass varies substantially, although few microbial maxima are reported

476 in winter (Wardle, 1992). Our findings highlight the dynamic nature of soil microbial biomass

477 and activity, especially with regards to the supply and demand of N (e.g. microbial C:N,

478   substrate utilization, and extracellular enzyme activities), which is likely a limiting nutrient in

479   these agroecosystems that are receiving no exogenous N inputs.

480   The summer warrants discussion because the sample was collected after a prolonged

481   period of hot and dry days, but right after a large rainfall event. This rainfall event (> 18 mm d^{-1},

482   Fig. S2) increased the volumetric water content in the 0-10 cm of a nearby soil by over 54%

483   from the lowest value of the year (0.1 m m^{-3}, data shared from Hamilton et al. 2015); and we

484   know from previous research that drying-wetting cycles are important soil biogeochemical

485   drivers (Borken and Matzner, 2009) and can alter microbial structure and functioning (Fierer et

486   al. 2003, Schimel et al. 2007, Tiemann and Billings 2011, McDaniel et al. 2014*b*). Indeed, the

487   summer showed several signs of the soil microbial community being impacted by a rapid dry-

488   wet event: lower overall microbial biomass C, high NO_3^--N concentrations (Table 1), high

489   potential N mineralization (Fig. 1), high extracellular enzyme activities per unit of microbial

490   biomass (Fig. S9, presumably a result of lysed intracellular enzymes, Burns et al. 2013), and the

491   particularly strong response of the summer soils to carboxylic acids (a highly-labile class of

492   compounds used by fast-growing, opportunistic microbes, that would be found after a

493   disturbance such as a dry-wet event, Figs. 4 and S3). Dry-wet cycles may drive microbial C and

494   N to be reallocated to stress-response compounds instead of growth or reproduction, making C

495   and N more vulnerable to loss from soils (Schimel et al. 2007). We captured one of these dry-

496   wet events during one of the driest summers in the Kellogg Biological Station LTER's history

497   and we show high soil inorganic N concentrations and altered microbial dynamics relative to the

498   other dates. Climate change may increase the frequency and magnitude of these rapid dry-wet

499   cycles (Groffman et al. 2001, McDaniel et al. 2014*d*), and thus may have long-term impacts on

500   soil microbial functioning and biogeochemistry.

501   In the autumn we found several lines of evidence that indicate soil microbes are N, rather

502   than C, limited. These lines of evidence include: lowest soil inorganic N concentrations, low

503   potentially mineralizable N, high microbial biomass C:N and DOC:DON ratios, and high TAP

504   and NAG enzymes relative to other enzymes (although interestingly not LAP), and finally strong

505   respiration response to the addition of amines and amino acids (Fig. 4). The unusually wide

506   microbial biomass C:N in autumn was very surprising (mean of 24 versus 10 and 8 in spring and

507   summer, respectively), but microbial biomass C:N has been known to be as high as 30 in

508   laboratory conditions (Schimel et al. 1989). Additionally, the few days before and after the

509   collection of the autumn sample were unusually cold (Fig. S2), and cold temperatures and

510   freezing can cause accumulation of carbohydrates in fungi (Tibbett et al. 2002), which could also

511   widen microbial C:N ratio. While environmental conditions may be a factor in the microbial

512   biomass C:N, it is likely that N limitation is a major factor in these long-term, unfertilized,

513   agroecosystems .

514   *Conclusions*

515   As the growing population is increasingly reliant on soils for food, fiber, and fuel we will

516   either need to consume less, put more land into production, or better use the land we already

517   have in production.   Putting more land in production will likely result in declines in local and

518   global biodiversity. Thus, it is critical to incorporate biodiversity through any means possible

519   into the existing managed ecosystems – even including biodiversity through time as with crop

520   rotations. Here we show that both microbial biomass and function are strongly influenced by

521   cropping diversity. In fact, the influence of crop rotations on soil microbes and functioning lasts

522   over an entire growing season and even when all soils are under the same crop. Crop rotations

523   clearly enhance soil microbial biomass and activity, which are now considered a pillar of soil

524 health, and it appears from our study that rotations also facilitate microbes in supplying more soil

525 N to crops (Fig. S8). Overall, our study highlights the importance of incorporating biodiversity

526 into agroecosystems by including more crops in rotation, especially cover crops, to enhance

527 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
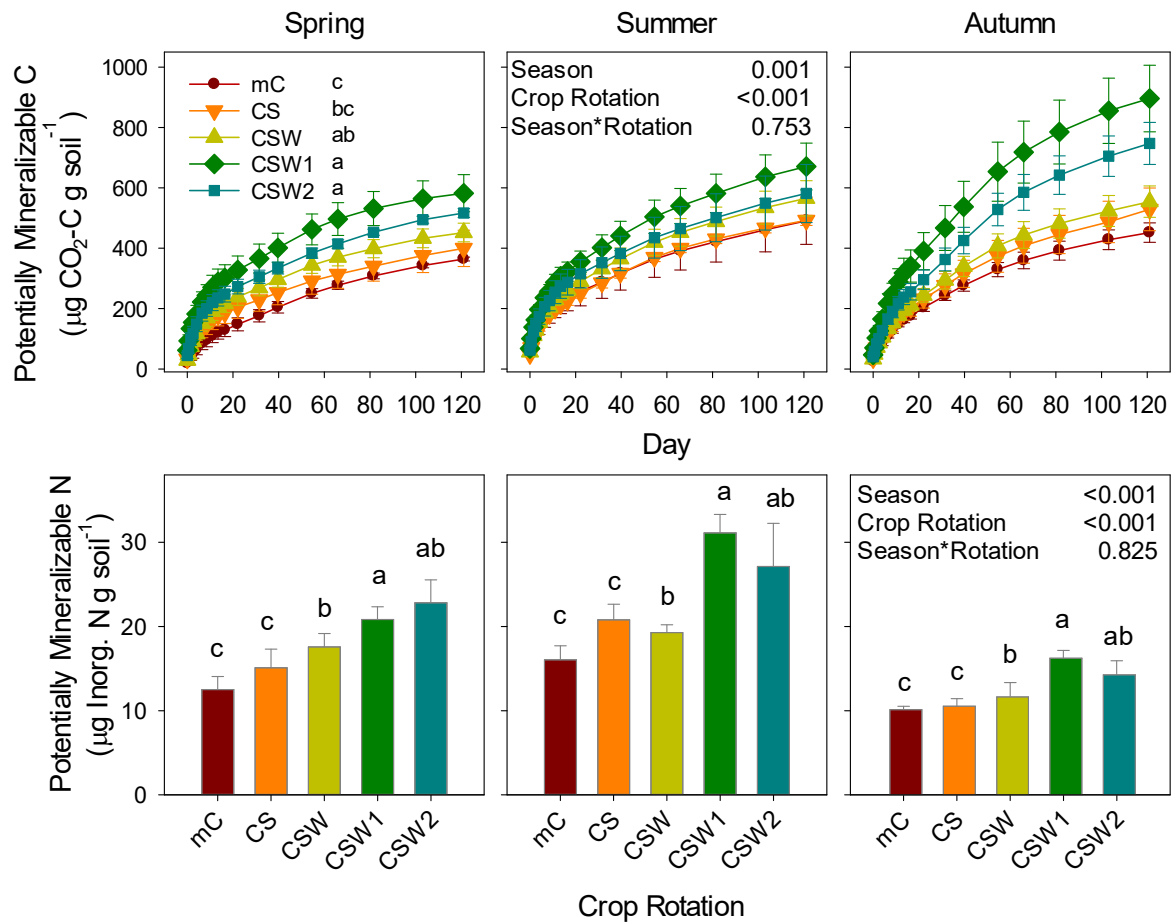
Figure 1. Potentially mineralizable carbon (top row) and potentially mineralizable nitrogen (bottom row). Crop rotation abbreviations are: monoculture corn (mC), corn-soy (CS), corn-soy-wheat (CSW), corn-soy-wheat with red clover cover crop (CSW1), and corn-soy-wheat with red clover + rye cover crops (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.
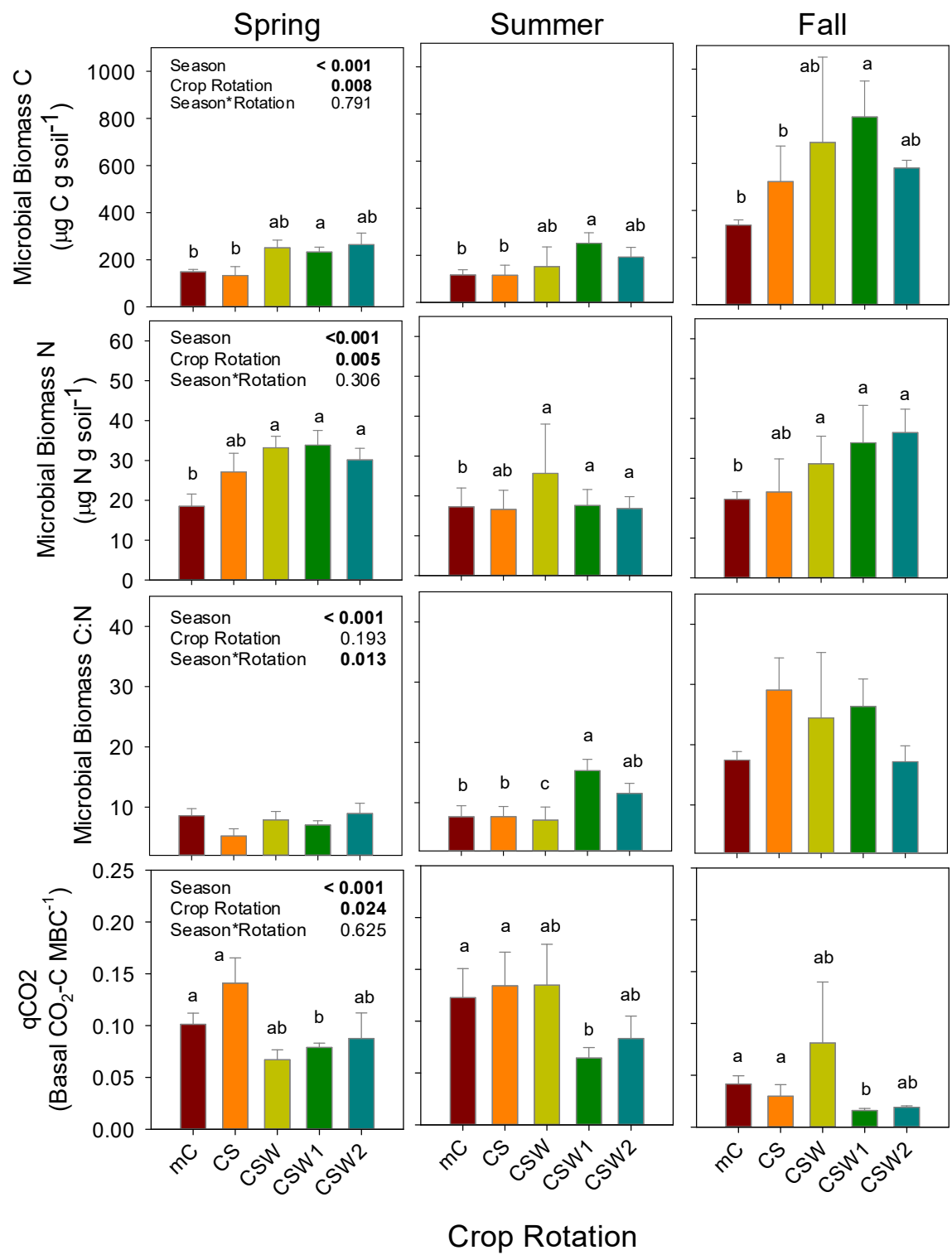
Figure 2. Soil microbial biomass parameters by season and crop rotation. See Fig.1 for crop rotation abbreviations. Means are shown and error bars are standard errors (n = 4). *P* values from ANOVA results are shown for each variable with the main effects (Season and Crop Rotation) and the interaction, as well as significant differences from post-hoc results shown as lowercase letters.
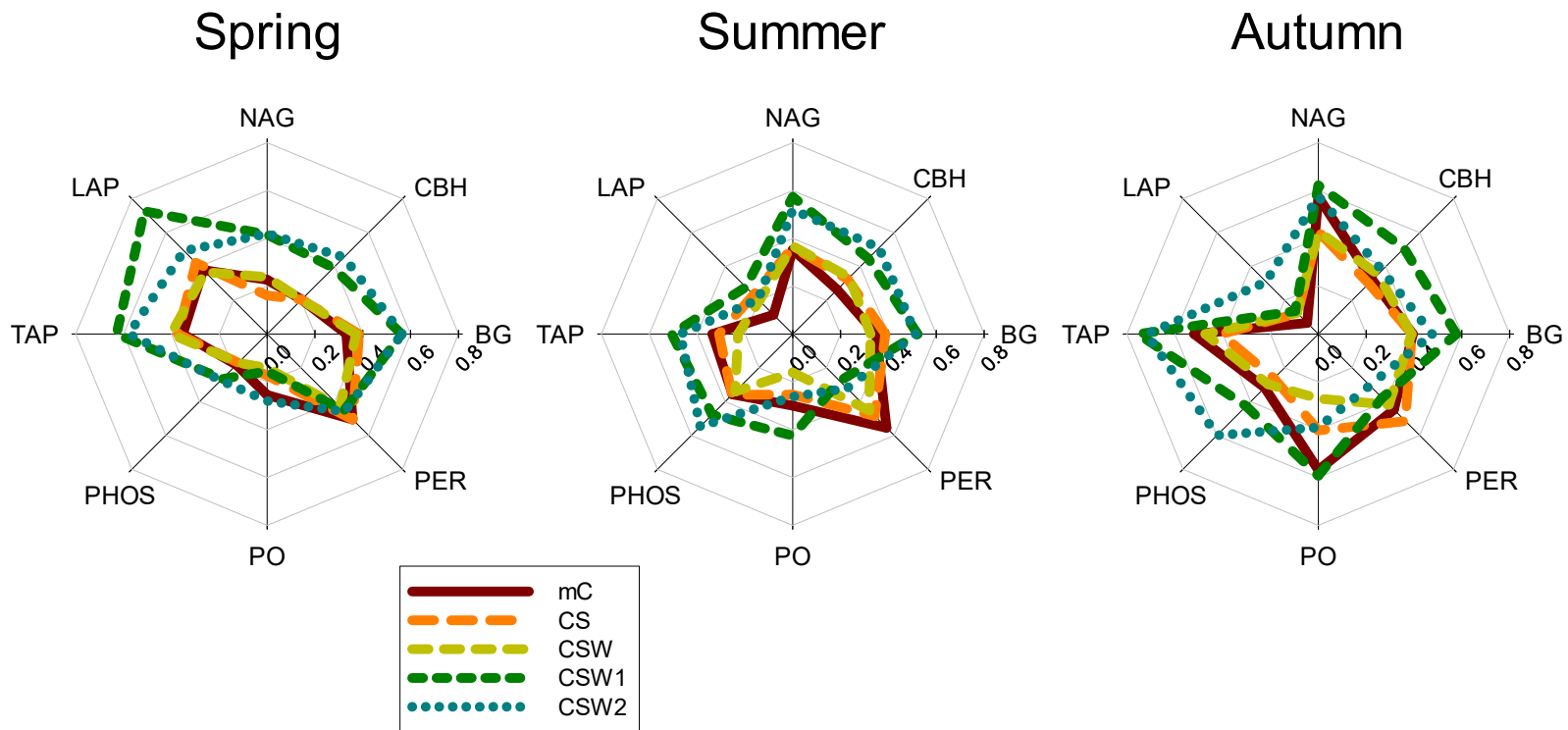
Figure 3. Mean extracellular enzyme activities (EEA) normalized for the maximum value across all seasons. EEA abbreviations are: β-1,4,-glucosidase (BG), β-D-1,4-cellobiohydrolase (CBH), β-1,4,-N-acetyl glucosaminidase (NAG), acid phosphatase (PHOS), Tyrosine aminopeptidase (TAP), Leucine aminopeptidase (LAP), phenol oxidase (PO), and peroxidase (PER). See Fig.1 for crop rotation abbreviations.
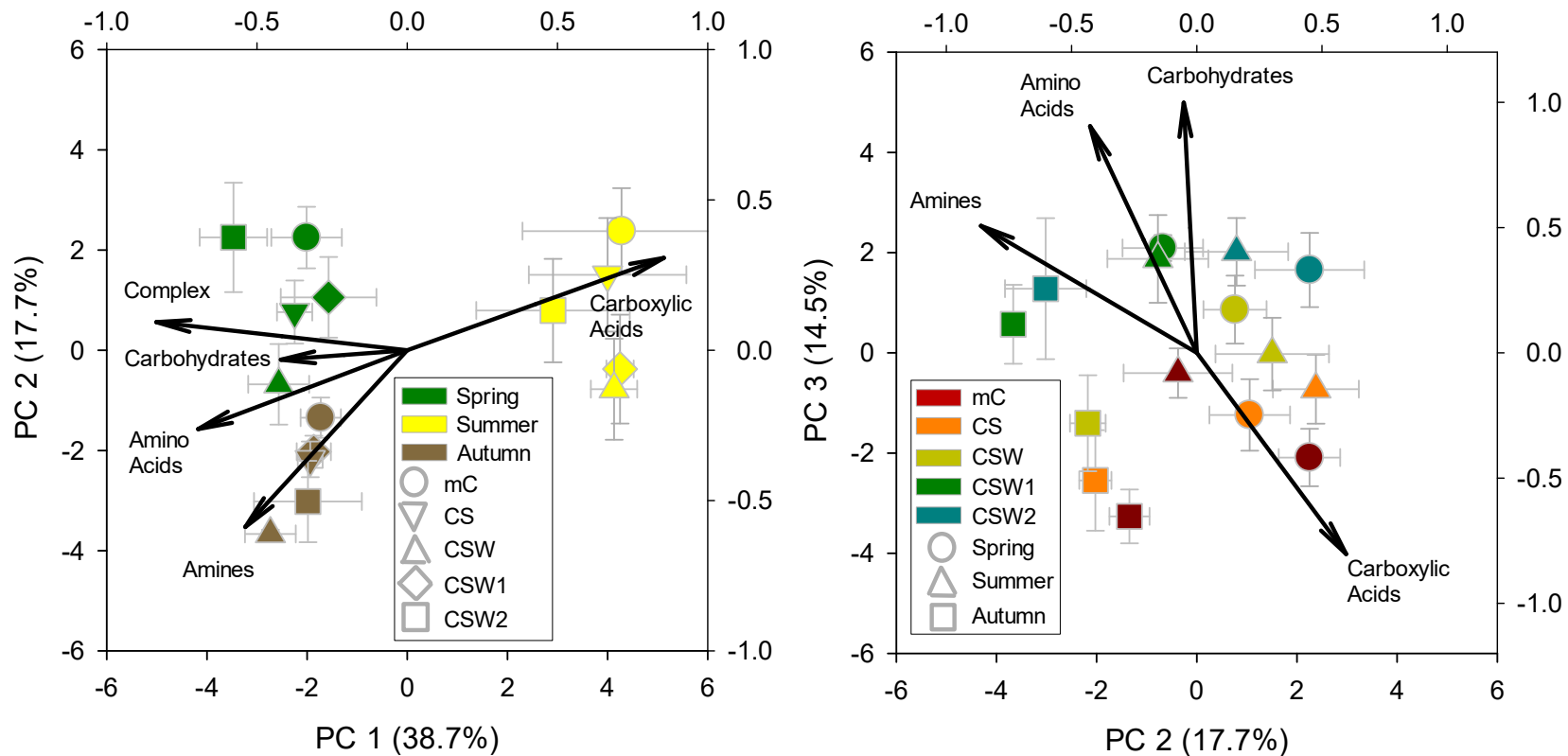
Figure 4. Principal components analysis (PCA) on all 31 substrates. *Left Panel:* Principal components 1 and 2, where Season is dominant discriminating factor ($P < 0.001$) and *Right Panel:* Principal components 2 and 3 where Rotation is highlighted as a dominant discriminating factor. See also Table 5 for PCA and ANOVA results. Means are shown and error bars are standard errors ($n = 4$). See Fig.1 for crop rotation abbreviations.