Diversified cropping systems support greater microbial cycling and retention of carbon and nitrogen

Alison E. King¹ and Kirsten S. Hofmockel^{1,2*}

¹ Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, IA 50011, United States

² Pacific Northwest National Laboratory, Richland, WA, 99354 United States

*Corresponding author email: khof@iastate.edu

Keywords: Crop rotation; alternative agriculture; organic nitrogen; extracellular enzyme activity; microbial biomass; soil carbon

Abstract

Diversifying biologically simple cropping systems often entails altering other management practices, such as tillage regime or nitrogen (N) source. We hypothesized that the interaction of crop rotation, N source, and tillage in diversified cropping systems would promote microbially-mediated soil C and N cycling while attenuating inorganic N pools. We studied a cropping systems trial in its 10th year in Iowa, USA, which tested a 2-yr cropping system of corn (*Zea mays* L.)/soybean [*Glycine max* (L.) Merr.] managed with conventional fertilizer N inputs and conservation tillage, a 3-yr cropping system of corn/soybean/small grain + red clover (*Trifolium pratense* L.), and a 4-yr cropping system of corn/soybean/small grain + alfalfa (*Medicago sativa* L.)/alfalfa. Three year and 4-yr cropping systems were managed with composted manure, reduced N fertilizer inputs, and periodic moldboard ploughing. We assayed soil microbial biomass carbon (MBC) and N (MBN), soil extractable NH₄ and NO₃, gross proteolytic activity of native soil, and potential activity of six hydrolytic enzymes eight times during the growing season. At the 0-20cm depth, native protease activity in the 4-yr cropping system was greater

than in the 2-yr cropping system by a factor of 7.9, whereas dissolved inorganic N pools did not differ between cropping systems ($P = 0.292$). At the 0-20cm depth, MBC and MBN the 4-yr cropping system exceeded those in the 2-yr cropping system by factors of 1.51 and 1.57. Our findings suggest that diversified crop cropping systems, even when periodically moldboard ploughed, support higher levels of microbial biomass, greater production of bioavailable N from SOM, and a deeper microbially active layer than less diverse cropping systems.

1. Introduction

The Midwestern United States ranks among the nation's most biologically altered landscapes (Klopatek et al., 1979). It is dominated by annual grain crops fertilized with synthetic N and is linked to multiple environmental costs, including soil erosion (Heathcote et al., 2013; Zaimes et al., 2008), nutrient loss and attendant hypoxia (David et al., 2010; McIsaac et al., 2001), and pesticide runoff (Davis et al., 2012; Gilliom et al., 2006). Incorporating hay, forage, or green manures into cropping systems offers multiple benefits to the producer and to the environment (Boody et al., 2005), including reduced reliance on synthetic fertilizers (Carlsson and Huss-Danell, 2003; Davis et al., 2012), reduced nutrient losses (Randall et al., 1997; Vache et al., 2002) and reduced soil erosion (Burkart et al., 2005; Vache et al., 2002). These legume or manure-based systems also promote greater total and biologically active soil organic matter (SOM) (Drinkwater et al., 1998; Kallenbach and Grandy, 2011; Maillard and Angers, 2014; Poeplau and Don, 2015; Wander et al., 1994). SOM is important for crop N supply because soil

organic N can provide 50% or more of crop N even when fertilizer N applications exceed crop demand (Cassman et al., 2002).

Soil organic N is made up of about 40% proteinaceous material (Schulten and Schnitzer, 1998; Sowden et al., 1977), so the supply of plant-available N from SOM depends in large part on protease enzymes that liberate amino acids (Jan et al., 2009; Schimel and Bennett, 2004) which can be taken up directly by plants (Franklin et al., 2016) or readily mineralized to ammonium (Jan et al., 2009). The extent to which amino acids contribute to plant available N depends on both the microbial production of protease enzymes and soil protein concentrations (Hofmockel et al., 2010). Under native soil substrate conditions, amino acid production can increase linearly with soil protein concentrations (Raab et al., 1999), suggesting that rates of amino acid production are in some cases controlled by soil protein concentration. Therefore, the liberation of bioavailable N is expected to be greater in soils with higher proteinaceous substrate availability, such as legume- and manure-based cropping systems.

Understanding the microbial activities underlying coupled C-N cycling within integrated management systems is necessary to project how interactions between crop rotation, N source, and tillage regime affect soil C and N cycling at different soil depths. To test the effects of management practices on soil C and N cycling and retention, researchers often isolate management practices; for instance, the effects of inorganic compared to organic fertilizers (Jenkinson and Rayner, 1977; Vitosh et al., 1996), non-use compared to use of green manure (Baggs et al., 2000; Tonitto et al., 2006), or conservation tillage compared to conventional tillage consisting of moldboard ploughing (Madejon et al., 2007; Melero et al., 2009). When implementing cropping systems, however, producers often need to vary multiple factors at once (Drinkwater, 2002). For instance, green manures and perennial forages frequently improve soil

organic matter (Amado et al., 2006; McDaniel et al., 2014; Sanford et al., 2012), and often require tillage (SARE, 2012), a management practice generally reported to reduce soil organic matter (Alvarez et al., 1995; West and Post, 2002, although this effect depends on sampling depth; see Luo et al., 2010).

Under conservation or zero tillage, crop residues accumulate in surface soils and increase microbial activity and SOM in that layer relative to soils that are mixed with moldboard ploughing (Alvear et al., 2005; Franzluebbers, 2002; Madejon et al., 2007). This increase has been quantified as the stratification ratio, or the proportion of soil organic carbon (SOC) at the soil surface relative to SOC in a deeper soil layer (Franzluebbers, 2002), and has been extended to microbial biomass (Alvear et al., 2005; Madejon et al., 2007). Franzluebbers (2002) proposed higher soil stratification ratios as unit-less, relative indicator of soil quality. Most researchers use stratification ratios to compare cropping systems that are identical except for their tillage regime and find that no-till or conservation tillage increases stratification ratios compared to conventional tillage (Alvear et al., 2005; Madejon et al., 2007; Melero et al., 2009; Moreno et al., 2006; Pu et al., 2015; Zhao et al., 2015). To our knowledge, no studies have reported on stratification ratios under whole-systems comparisons in which both tillage and crop rotation vary. Since cropping systems must often vary by both tillage regime and crop rotation, testing stratification ratio in a whole-system comparison may be useful in determining its generalizability as an indicator.

To test the effects of cropping system diversification - and associated changes in tillage regime and N source - on microbial cycling of C and N, we studied three corn-based cropping systems (Table 1) within a long-term cropping systems experiment. These represent conventional (2-yr) and diversified (3-yr and 4-yr) cropping systems in the Midwestern United States. We

hypothesized that more diverse cropping systems would support 1) more microbial biomass and higher rates of both native protease and potential enzyme activities, but 2) coincide with smaller pools of dissolved inorganic nitrogen (DIN), and 3) display lower stratification ratios. In order to hold the effect of crop on soil C and N cycling constant, we studied soils only under corn. This whole-systems approach is not designed to isolate particular management practices as they influence soil biological processes, but rather to study the impacts of integrated cropping systems as they are likely to appear on farms.

2. Methods

2.1. Field Site

Field work was conducted at the Iowa State University Marsden Farm in Boone County, Iowa (42°01' N; 93°47' W; 333 m above sea level) during the 2013 growing season (May – October). Soils vary across the experimental site and are predominantly Clarion loam (fine-loamy, mixed, superactive, mesic, Typic Hapludolls, 2%–5% slope), Nicollet loam (fine-loamy, mixed, superactive, mesic, Aquic Hapludolls, 1–3% slope) and Webster silty clay loam (fine-loamy, mixed, superactive, mesic, Typic Endoaquolls, 0–2% slope), with smaller areas of Harps loam (fine-loamy, mixed, superactive, mesic Typic Calciaquolls, 0–2% slope), and Canisteo silty clay loam (fine-loamy, mixed, superactive, calcareous, mesic Typic Endoaquolls, 0–2% slope). Soil properties 0–20 cm are as follows: soil organic matter, 51 g kg⁻¹, Bray P, 31 mg kg⁻¹ (Liebman et al., 2008), and soil particle size distribution is 35% sand, 38% silt, and 26% clay. Soil pH was 7.5 in May 2013. Weather conditions were measured about 1 km from the site: fifty

year average annual rainfall from May to October at the site is 592 mm and mean temperature is 19°C for the same period. During 2013, total rainfall from May to October was slightly lower than the long-term average, 436 mm, and average air temperature was 19°C. Prior to establishment of experimental plots, the site had been managed for at least 20 years in corn-soybean rotation receiving conventional fertilizer inputs.

2.2 Cropping systems experiment

Cropping systems are described in Table 1. Plots were established in 2002 in a randomized complete block design, with each crop phase of every crop rotation present every year. Plots were 18 x 85 m. The 2-yr cropping system was representative of conventional cropping systems in the Midwestern US, and the 3-yr and 4-yr cropping systems were representative of diversified farming systems in the Midwest. The small grain in both 3-yr and 4-yr systems was triticale (*Triticosecale* Wittmack) from 2003-2005 and oat (*Avena sativa* L.) after 2006. In the 3-yr cropping system, red clover was used as a green manure (non-harvested), and in the 4-yr cropping system, alfalfa was harvested for hay. All other crops were harvested for grain and residues were left on the field. In all cropping systems, the late spring nitrate test (Blackmer et al., 1997) was used for corn to determine rates for post-emergence side-dress N applications, so N applications varied by year (Table 1). All N was in form of 32% liquid urea ammonium nitrate (UAN), urea, or monoammonium phosphate. Composted manure was applied to 3-yr and 4-yr cropping systems in red clover and established alfalfa, respectively, at a rate of 10.2 Mg ha⁻¹ (dry weight).

Working depth for moldboard plow used to incorporate red clover and alfalfa in the

diversified cropping systems was 23 cm, and working depth for chisel plow used for partial incorporation of corn residue in all cropping systems was 33 cm, however carbon measurements from this site show that most corn residue was incorporated only to the 0-10 cm depth (Lazicki, 2011). More complete site descriptions, crop yields, and economic characteristics of cropping systems are reported elsewhere (Davis et al., 2012; Liebman et al., 2008).

2.2. Soil sampling

Soils were collected from the corn phase of each cropping system eight times throughout the 2013 growing season (SI Table 1), an intensive sampling schedule designed to produce accurate generalizations of system effects. At each sampling date, a set of 10 cores was taken from each plot using a 2.2-cm diameter core, and soil cores were divided into 0-10 and 10-20 cm depths before compositing. Sampling locations were randomized but were taken in a ratio of 3 row:1 inter-row to control for strong variability of root and N locations (e.g., Buczko et al., 2008). Soils were sieved to 4 mm and stored at 4°C until subsampling (within 48 hours) for subsequent analyses. Gravimetric moisture content was measured as water mass loss upon drying at 105 °C to a constant weight and was used to determine wet-weight to dry-weight ratios. Response variables are presented on a dry weight basis.

2.3. Microbial biomass and inorganic N

Microbial biomass was estimated using direct chloroform-fumigation-extraction (modified from Vance et al., 1987 and Witt et al., 2000). Extracts were analyzed for non-

purgeable organic C and total N via combustion catalytic oxidation (Shimadzu TOC-L analyzer, Shimadzu Corporation, Columbia, Maryland, USA). Microbial biomass C and N were calculated as the difference between fumigated and unfumigated extracts, with conversion factors of 0.45 for C (Vance et al., 1987) and 0.54 for N (Brookes et al., 1985). Unfumigated extracts were used to measure NO₃ and NH₄ concentrations via spectrophotometry (BioTek Synergy HT plate reader, BioTek Instruments, Inc., Winooski, VT, USA) (Hood-Nowotny et al., 2010).

2.4. Native protease activity

Proteolytic activity was measured with a method modified from Lipson et al. (1999) on subsamples of soil stored at -80°C (Brzostek et al., 2012). Soils were preincubated at 23°C for 12 hours. All soil samples (~3.0-3.5 g) received 10 mL 0.02 M MOPS buffer at pH 7.5. All samples also received 0.4 mL toluene, which inhibits microbial uptake (Skujins, 1967). Initial samples, used to determine standing pool of free primary amines, received 3 mL trichloroacetic acid (TCA) mix immediately in order to halt proteolytic reactions. Incubated samples were shaken lengthwise at 120 rpm for 4 hours at 23°C before addition of 3 mL TCA mix. All samples were centrifuged at 2300 rpm for 5 min and the supernatant filtered through Whatman #42 papers. Three technical replicates per plot and depth combination were run for both initial and incubated samples, giving a total of 144 samples per sampling date. Extracts were stored at -20°C until analysis.

The concentration of total free primary amine-N (TFPA-N) in soil extracts was evaluated using the o-phthaldialdehyde (OPA) and β-mercaptoethanol method, with an L-leucine standard curve (Darrouzet-Nardi et al., 2013). Three analytical replicates per sample and incubation time

combination were run on each plate, along with buffer blanks and an OPA standard curve. The interference of NH_4 with OPA reagent was subtracted using NH_4^+ concentrations in MOPS extracts determined via colorimetric analysis (Darrouzet-Nardi et al., 2013; Hood-Nowotny et al., 2010). Native proteolytic rate ($\eta\text{mol TPFA-N g soil}^{-1} \text{ hr}^{-1}$) was calculated as the difference between incubated and initial samples.

2.5. Potential enzyme activities

Potential enzyme activities were measured on subsamples of soil stored at -20°C . A suite of six enzymes was measured, three involved in organic matter decomposition (BG, BX, and CB) as well as three aminopeptidases (LAP, Ala, AAP), which liberate amino acids or tripeptides from polypeptides. BG, BX and CB were tested with MUB (Methylumbelliferone) - linked substrates: CB, 4-MUB- β -D-cellobioside; BG, 4-MUB β -D-glucopyranoside; BX, 4-MUB β -D-xylosidase. Aminopeptidases were tested with MUC (7-amino-4-methylcoumarin) - linked substrates: LAP, L-leucine-7-amido-4-MUC; Ala, L-alanine-7-amido-4-MUC; AAP, L-alanine-alanine-phenylalanine-7-amido-4-MUC. Briefly, 1 g of soil was homogenized with 125 mL of 100 mM tris maleate buffer, pH 7.5. MUB- or MUC- linked substrates were added at saturating concentrations of $400\mu\text{M}$ for all substrates. Plates were incubated at 23°C for 3 hours and read using a fluorometer (360 nm excitation and 460 nm emission; BioTek Instruments, Inc., Winooski, VT, USA) without the addition of NaOH. Eight analytical replicates per sample and substrate combination were run and each plate included a MUB or MUC standard curve, substrate controls, and homogenate controls. Enzyme activity was calculated as $\eta\text{mol enzyme g}^{-1} \text{ soil hr}^{-1}$ based on MUB or MUC standard curves and accounting for the quench of each sample

(German et al., 2011). The linearity reaction of the 3-hour incubation was confirmed in preliminary work.

2.6. Statistical analyses

We performed all analyses using repeated measures mixed effects ANOVA in JMP Pro 11. We averaged technical replicates from the same plot and depth before analysis, and we calculated values for the whole soil profile (0-20 cm) by averaging values for samples of both depths (0-10 and 10-20 cm). We transformed data where necessary to meet assumptions of normality. In the ANOVA mixed model, we used a full factorial of fixed effects for all response variables except stratification ratios, with sampling date, depth, and cropping system as main effects, as well as each of their interactions. Random terms included an 'ID' term, coded by plot and depth (12 plots x 2 depths; total 24 'ID'), a plot term, and a block term. For stratification ratios, we used an ANOVA mixed model with sampling date and cropping system as main effects, their interaction, and block as a random effect. We used the residual covariance structure. Subsequent pair-wise comparisons were made using Tukey's HSD. Significance was determined at $\alpha = 0.05$.

3. Results

3.1. Microbial biomass

At the 0-20 cm depth, MBC in the 4-yr cropping system exceeded that in the 2-yr cropping system by a factor of 1.51 and ($P = 0.023$, Fig. 1), and MBN in the 4-yr cropping system exceed that in the 2-yr cropping system by a factor of 1.57 ($P = 0.025$). MBC and MBN concentrations in the 0-20 cm depth of the 3-yr year cropping system did not differ from either 2-yr or 4-yr cropping systems. MBC concentrations in the 0-10 cm depth did not differ between any cropping systems, however the 10-20 cm depth in the 2-yr cropping system was lower than the same depth of 3-yr or 4-yr cropping systems. MBN concentrations responded similarly to depth and cropping system as did MBC concentrations (Fig. 2). The effect of cropping system on MBC and MBN did not differ across sampling times ($P = 0.959$).

3.2. Native protease and potential enzyme activities

Proteolysis of native soil in the 0-20 cm depth of the 4-yr cropping system exceeded proteolysis under the 2-yr cropping system by a factor of 8 ($P = 0.019$, Fig. 1), with the 3-yr cropping system intermediate. Within cropping systems, proteolytic rates were similar between depths (Fig. 2). In the 0-20 cm depth, activities of BG and CB in the 3-yr cropping system exceeded those in the 4-yr cropping system ($P = 0.045$ and $P = 0.047$, respectively, Fig. 1), with the 2-yr cropping system intermediate; activities of LAP and AAP exhibited a similar trend ($P = 0.102$ and 0.076 , respectively, Fig. 1). Activities of BX and Ala did not differ significantly between cropping systems when averaged over the 0-20 cm depth.

Most enzyme activities responded similarly to cropping system and depth, with some variation in statistically significant differences (Fig. 2). In the 0-10 cm depth, all enzyme activities were greatest in the 2-yr cropping system and least in the 4-yr rotation by factors

ranging from 1.83 (BG) to 4.26 (AAP), with the 3-yr cropping system intermediate. In the 10-20 cm depth, enzyme activities in the 3-yr and 4-yr cropping systems were similar to or greater than those in the 2-yr cropping system. The effect of cropping system on native proteolytic and potential enzyme activities did not differ over the growing season.

3.4. Specific enzyme activity

Potential enzyme activity was normalized by MBC to determine biomass-specific activity. For all enzymes in the 0-20 cm depth, the 3-yr cropping system trended toward higher specific activity than the 4-yr cropping system, although this relationship was significant only for CB ($P = 0.029$, Fig. 3). Similarly, for all enzymes the 2-yr rotation trended toward higher specific activity than the 4-yr cropping system, although this relationship was significant only for CB ($P = 0.046$). Specific activities of the 2-yr and 3-yr cropping systems were not statistically different for any enzyme.

For all enzymes, potential activities in the 0-10 cm depth were highest in the 2-yr cropping system and lowest in the 4-yr cropping system by factors ranging from 2.20 (BG) to 5.50 (CB), with the 3-yr cropping system intermediate (Fig. 4). In the 10-20 cm depth, specific enzyme activities in the 3-yr and 4-yr cropping systems were similar to or greater than those in the 2-yr rotation.

3.5. Stratification ratios of MBC and MBN

Stratification ratios of MBC and MBN were greater in the 2-yr cropping system than either the 3-yr or 4-yr cropping system ($P < 0.0001$ for all comparisons, Fig. 5); stratification ratios in the 2-yr cropping system exceeded one whereas stratification ratios in the 3-yr and 4-yr cropping systems were close to but less than one. Stratification ratios did not differ between 3-yr and 4-yr cropping systems for either MBC or MBN ($P > 0.17$).

3.5. Inorganic nitrogen

Temporal dynamics of DIN were similar across depths and cropping systems (Fig 6). Nitrogen concentrations were low at both depths early in the season, in May and June. After side dress N, on 23 July, the 0-10 cm depth showed a more marked increase in DIN concentrations than did the 10-20 cm depth. Across all cropping systems, DIN concentrations in the 0-10 cm depth were greater than those in the 10-20 cm depth, and ammonium showed a similar trend ($P = 0.062$, Table 3). Ammonium was the only N form to differ significantly by cropping system, irrespective of depth (Table 3). Ammonium concentrations in the 4-yr cropping system were 38% lower than in the 2-yr cropping system ($P = 0.042$), with the 3-yr cropping system intermediate. There was no significant time by cropping system interaction for ammonium, nitrate or DIN.

4. Discussion

Our analysis of microbially mediated C and N cycling at a 10-year cropping systems experiment showed diversified cropping systems support higher rates of soil organic matter

depolymerization and greater retention of C and N in microbial biomass. Our results support the notion that through their metabolic activity and biomass dynamics, it is soil microbes' response to cropping system diversification that plays a key role in improving soil quality (Bach and Hofmockel, 2015; Kallenbach et al., 2015). The 3-yr and 4-yr cropping systems compared to the 2-yr cropping system supported higher levels of MBC and MBN. Although increases in soil C pools are often attributed to increases in C inputs (Kong et al., 2005; Rasmussen et al., 1980), at Marsden Farm levels of C inputs did not predict those of microbial biomass C. The 4-yr cropping system provided the least C input annualized over the full rotation period (Table 2, Lazicki et al., 2016). Despite lower C inputs, the 4-yr cropping system supports enhanced microbial biomass compared to other cropping systems, as discussed below.

Cropping system management can alter microbial physiology to increase microbial carbon use efficiency (CUE), that is, the efficacy with which microorganisms convert available organic substrates into microbial biomass (Kallenbach et al., 2015). The microbial biomass C and N pools will increase in one cropping system relative to another only if microbes have access to sufficient substrates and non-growth requirements have been satisfied. Therefore, greater microbial biomass in diversified cropping systems may be a consequence of increased substrate availability, where greater retention and recycling of C and N enhance available substrate to support microbial growth and biosynthesis (Geyer et al., 2016). This is supported by greater pools of decomposable substrate in diversified systems at Marsden Farm. Despite lower C inputs, the diversified systems at Marsden Farm accumulate particulate organic C (Lazicki, 2011), which correlates positively with both microbial biomass and extracellular enzyme activity (Fernandes et al., 2011). Greater substrate availability in the diversified cropping systems is evidenced by higher rates of native proteolytic enzyme activity, where the 4-yr cropping system

increased native protease activity by 8 fold compared to the 2-yr cropping system. Diversified cropping systems support this increase in proteolytic rates despite receiving similar levels of total N input (including biological nitrogen fixation, Lazicki et al., 2016). Because these are native rates (no substrate additions), rates of amino acid liberation most closely reflect differences in bioavailable N and C between cropping systems. Together, greater proteolytic activity coupled with greater microbial biomass C and N suggest enhanced cycling and retention of C and N in the diversified systems.

In response to cropping systems intensified with inorganic N fertilizer, such as the 2-yr cropping system, microbial communities can also increase biomass-specific enzyme activity, producing more enzymes per unit of microbial biomass (Lagomarsino et al., 2009). In the 2-year cropping system, biomass-specific enzyme activity was greatest in the 0-10cm increment, which may reflect N fertilizer applications. In the 10-20cm soil increment of the 2-yr cropping system, very low specific rates likely reflect low overall investment in enzymes; here resources were primarily allocated to microbial maintenance and survival, as evidenced by significantly lower microbial biomass. These dynamics contrast the 3-yr and 4-yr cropping systems, where specific rates of enzyme activity do not differ with depth and tend to be lowest in the 4-year cropping system where particulate organic C (Lazicki, 2011) and microbial biomass are greatest.

Microbial biomass may also be suppressed by higher rates of N fertilizer in the 2-yr cropping system relative to 3-yr and 4-yr cropping systems. N fertilizer can reduce soil pH, which is positively correlated with microbial biomass (Serna-Chavez et al., 2013). Previous studies demonstrate that microbial biomass may be depressed by inorganic N inputs (Liebig et al., 2002; Treseder, 2008), potentially offsetting the effect of greater C inputs and contributing to lower MBC in the 2-yr cropping system.

Metabolic shifts in microbial communities have important implications for SOM accrual, as microbial resource allocation toward enzyme production diverts resources from microbial growth and reproduction (Hargreaves and Hofmockel, 2013; Schimel and Schaeffer, 2012). Microbial growth is positively correlated with SOM accrual (Bradford et al., 2013), and much of SOM is thought to be comprised of microbial residues (Schmidt et al., 2011; Simpson et al., 2007). While SOM in the 0-20 cm does not differ at this site (Lazicki et al., 2016), changes in microbial community physiology can explain why the 4-yr rotation is able to maintain similar total C stocks as the 2-yr cropping system despite receiving on average only three quarters the C input. This is not mutually exclusive with other factors influencing C retention that were not measured in our study, such as soil temperature (Frey et al., 2013), bulk density (Wang et al., 2015), or C input quality (Cotrufo et al., 2013). Specifically, the lower C:N and lignin:N ratios of alfalfa biomass compared to corn and soybean (Johnson et al., 2007) may also be implicated in promoting carbon retention in the 4-yr cropping system. Overall our findings suggest that cropping systems with less frequent tillage, reduced fertilizer N inputs, and/or those diversified with perennial forages can decrease the specific enzyme activity of microbial communities and promote microbial biomass C and N, which in turn enhances the potential for SOM accumulation.

In addition to promoting greater proteolytic activity and microbial biomass, the microbially active layer extended deeper in the soil profile in the diversified cropping systems. In the 10-20cm increment, microbial biomass C and N and native proteolysis were substantially enhanced in the diversified systems. These depth dynamics were similar for potential rates of hydrolytic enzymes, which measure enzyme pools given unlimited substrate. In the 3- and 4-yr cropping systems, enzyme production did not differ with depth, whereas the 2-year cropping system harbored significantly smaller enzyme pools in the 10-20 cm compared to the 0-10 cm

increment, suggesting a reduced zone of microbial activity. This stratification under the 2-yr cropping system, which received conservation tillage, is consistent with enzyme stratification under conservation tillage or zero tillage at other sites (Alvear et al., 2005; Madejon et al., 2007).

Stratification ratios of SOM, and SOM pools themselves, generally respond in the same direction to conservation or no-tillage compared to moldboard ploughing. That is, total SOM in the plough layer of moldboard ploughed systems is usually similar to that under conservation tillage, or somewhat decreased (Alvear et al., 2005; Hermle et al., 2008; Moreno et al., 2006). In most tillage comparisons, however, only annual grain crops are investigated and all aspects of cropping systems are held constant except tillage regimes (Alvear et al., 2005; Moreno et al., 2006; West and Post, 2002; Zhao et al., 2015), eliminating the possibility that crop diversification with small grains, green manures, or perennial forages could interact with tillage to influence both stratification ratios and total SOM pools. We found that the interaction between crop rotation and tillage can enhance soil quality in agroecosystems. At the Marsden Farm, conservation tillage (2-yr rotation) increased stratification ratios of MBC and MBN relative to moldboard ploughing (3-yr and 4-yr rotations), despite supporting significantly lower MBC and MBN throughout the plough layer.

Differences in crop species among cropping systems may have accentuated stratification dynamics promoted by tillage regimes: a crop with high shoot:root ratio like corn (Bolinder et al., 2007) may accentuate depth stratification, whereas lower shoot:root ratios of alfalfa and red clover (Bolinder et al., 2007; Høgh-Jensen and Schoerring, 2001) may support a less stratified layer of microbial abundance irrespective of tillage regime. Our study suggests that stratification ratio is most appropriate as an indicator of soil mixing via tillage, but not necessarily an indicator of overall soil health, as perennial forages and green manures can remediate soil microbial

biomass even under moldboard ploughing. This work highlights the importance of whole systems approaches for accurately assess cropping system impacts on soil quality.

In the season under study, 3-yr and 4-yr cropping systems did not significantly reduce inorganic N pools compared to 2-yr crop rotations (Table 3). These findings contrast those of Tomer and Liebman (2014), who assessed $\text{NO}_3\text{-N}$ concentrations at Marsden Farm at 1.2 m and found that across the 2004-2011 growing seasons, spring $\text{NO}_3\text{-N}$ concentrations in the corn phase of the 4-yr rotation were 37% and 24% of 3-yr and 2-yr rotations, respectively. The lack of cropping system effect on DIN pools in our results may be due to the heterogeneity of DIN pools in the environment (Cambardella et al., 1994), seasonal variations in both DIN production from SOM (Zak et al., 1999) and transport via leaching (David et al., 2010), or the inter-annual variability in fertilizer N application rates at Marsden Farm (Table 1). Historically, corn in the 2-yr cropping system received about four times the fertilizer N of corn in 3-yr and 4-yr cropping systems, but in the season under study here, the 2-yr cropping system received only about three times the fertilizer N of the 3-yr and 4-yr cropping systems.

The similarities in corn season DIN concentrations between cropping systems, and thus comparable vulnerability of N to loss via leaching, suggest that long-term N retention demonstrated elsewhere in cropping systems diversified with green manures or perennials is the result of greater time of living soil cover than under cropping systems of only annual grains (Drinkwater et al., 1998; Randall et al., 1997; Syswerda et al., 2012). Nevertheless, that 3-yr and 4-yr cropping systems maintained DIN pools of comparable size to the 2-yr cropping system despite receiving less N fertilizer during the study season suggests that production of N from SOM (e.g., via proteolysis and then mineralization), rather than N fertilizer, maintained a greater proportion of the DIN pools in these cropping systems compared to the 2-yr cropping system.

Indeed, at Marsden Farm, the diversified cropping systems on average support 27% greater gross N mineralization than the 2-yr cropping system, and all cropping systems support similar levels of corn N uptake (Osterholz et al., 2016). These results suggest diversified cropping systems have the potential for reduced reliance on fertilizer N inputs and the potential for tighter coupling of DIN supply and plant demand.

5. Conclusions

We found that cropping systems diversification increases MBC, MBN, and native protease activity throughout the plough layer relative to less diverse systems. The increased retention of organic inputs in microbial biomass in diverse rotations coincided with greater return on enzymatic investment, suggesting microbial efficiency may be promoted by moldboard plough incorporation of a green manure or perennial legume. Increases in SOM depolymerization with diversification are consistent with retention of residue inputs in microbial biomass N (Geisseler and Horwath, 2008) and suggest that depolymerization was primarily limited by soil protein concentration (Raab et al., 1999). Our findings highlight that in contrast to systems relying on inorganic N inputs, diversified cropping systems receiving organic amendments can promote C and N retention. Understanding the microbial ecology of cropping systems is important for predicting their effects on C cycling; substituting small grains or alfalfa hay for cereal grains often decreases C inputs (Bell et al., 2012; Benjamin et al., 2010; Gregorich et al., 2001), but may nonetheless enhance soil quality. The whole-systems approach to site design studied here provides an important comparison of tillage regimes contextualized within cropping systems. When crops are held constant, tillage is generally reported to reduce microbial

biomass (Pandey et al., 2014), and deep tillage like moldboard plowing is discouraged for the sake of conserving soil organic matter stocks (Karlen et al., 2013). Our results suggest that cover crops and perennial forages may remediate soil microbial biomass beyond the detrimental effects of the tillage they often require.

Acknowledgements

This work was supported by USDA National Institute of Food & Agriculture grant number 2014-67019-21628. We thank Don Zak and Matt Liebman for helpful feedback on the manuscript. We thank Queenster Nartey, Sandra Greenwood, Jessica Maciel-Hernandez, and Montana Smith for their assistance in the lab. We are grateful to the late Dave Sundberg for his maintenance of the Marsden farm field plots.

Table 1. Cropping system management at Marsden Farm: cropping system, tillage regime, and nitrogen inputs (excluding biological nitrogen fixation). Nitrogen inputs for year of study (2013) listed separately. Numbers in parentheses represent standard deviation of fertilizer inputs.

Cropping System				Nitrogen inputs 2003-2013		Nitrogen inputs 2013	
				Fertilizer	Manure [‡]	Fertilizer	Manure
				<i>preplant + sidedress</i>		<i>preplant^a sidedress^b</i>	
Tillage regime				kg N ha ⁻¹			
		Spring (prior to planting)	Fall (after harvest)				
2-yr							
corn	-		chisel plow	154 (40)	-	112	-
soybean	field cultivation		-	5 (8)	-	-	-
3-yr							
corn	disking & field cultivation		chisel plow	45 (50)	116 (38)	-	84
soybean	field cultivation		-	4 (7)	-	-	95
small grain + red clover	zero tillage or disking		moldboard plow	26 (5)	-	-	-
4-yr							
corn	disking & field cultivation		chisel plow	34 (38)	116 (38)	-	56
soybean	field cultivation		-	-	-	-	95
small grain + alfalfa	zero tillage or disking		-	26 (5)	-	-	-
alfalfa	-		moldboard plow	18 (*)	-	-	-

*only one fertilizer N application, 2006

[‡]Total N, not available N; manure is applied in the fall preceding corn

a - preplant N fertilizer applied on 15 May 2015

b - sidedress N fertilizer applied on 28 June 2013

Table 2. Carbon inputs to the corn phase and annualized over all crop phases, and SOC stocks by cropping system. Carbon inputs represent the sum of aboveground crop residue, standing summer root biomass, root exudates (estimated as 65% of root biomass, (Bolinder et al., 2007), and manure C inputs in 2008-2009). Standard deviations in parentheses. All values from Lazicki et al., (2016).

Phase	Cropping system	Aboveground biomass			Root biomass			Est. root exudates		Manure		Total C inputs			SOC	
								$g\ C\ m^{-2}\ yr^{-1}$							$Mg\ ha^{-1}$	
Corn	2-yr	374.6	(38.8)	a ^a	14.7	(3.5)	a	14.72	(3.7)			404.5	(40.3)	b		
	3-yr	428	(50.2)	a	12.43	(3.0)	a	13.24	(3.1)	138.2	(0.0)	592.8	(48.9)	a		
	4-yr	413.5	(51.8)	a	12.78	(4.7)	a	12.00	(6.7)	138.2	(0.0)	577.6	(57.6)	a		
Average of all crop years																
	2-yr	293.1	(93.1)	a	13.15	(3.4)	a	12.56	(4.1)			319.2	(97.2)	a	58.98	(6.64) a
	3-yr	304.9	(97.9)	a	11.18	(2.6)	a	9.96	(3.3)	46.1	(68.0)	a	372.6	(166.6)	a	56.05 (7.72) a
	4-yr	175.8	(178.6)	a	12.56	(6.8)	a	11.89	(4.5)	34.6	(61.8)	b	235.6	(231.2)	b	58.4 (7.69) a

a - Different lowercase letters represent differences between systems in a specified crop that were significant at P = 0.05 (Tukey's HSD)

Table 3. Mean inorganic N concentration by crop system and depth, averaged across sampling dates.

Crop System	Depth	NO ₃ ⁻ -N		NH ₄ ⁺ -N		Total DIN	
		<i>ug g⁻¹ soil</i>					
2-yr	0-10 cm	27.89	a ^a	9.93	a	42.53	a
	10-20 cm	11.32	b	4.48	ab	18.74	b
3-yr	0-10 cm	16.17	ab	6	ab	24.23	ab
	10-20 cm	14.45	b	5.23	ab	22.58	b
4-yr	0-10 cm	19.85	ab	5.46	ab	28.74	ab
	10-20 cm	14.55	b	3.25	b	20.85	b
2-yr	0-20cm	18.23	A ^b	6.79	A	28.23	A
3-yr		15.3	A	5.6	AB	23.39	A
4-yr		17.05	A	4.24	B	24.48	A
SE		1.13		1.71		2.59	

a - Entries in a column that share a lowercase letter are not significantly different at P = 0.05 (Tukey's HSD)

b - Entries in a column that share an uppercase letter are not significantly different at P = 0.05 (Tukey's HSD)

Figure legends

Fig 1. Mean soil indicators cropping systems at 0-20 cm depth averaged across eight sampling dates in 2013. Error bars represent standard errors (n = 32: 4 plots x 8 sampling dates). Bars within a frame that share a letter are not significantly different at P = 0.05 (Tukey's HSD). **A)** Native protease activity, or production of total free primary amine-N (TFPA-N). **B)** Soil microbial biomass carbon and nitrogen. **C)** Potential enzyme activities.

Fig 2. Mean soil indicators for cropping systems at 0-10 and 10-20 cm depths, averaged across eight sampling dates in 2013. Error bars represent standard errors (n = 32: 4 plots x 8 sampling dates). Bars within a frame that share a letter are not significantly different at P = 0.05 (Tukey's HSD). **A)** Native protease activity, or production of total free primary amine-N (TFPA-N). **B)** Soil microbial biomass carbon and nitrogen. **C)** Potential enzyme activities.

Fig 3. Mean specific enzyme activity for cropping systems at 0-20 cm depth, averaged across eight sampling dates in 2013. Error bars represent standard errors (n = 32: 4 plots

x 8 sampling dates). Bars within a frame that share a letter are not significantly different at $P = 0.05$ (Tukey's HSD).

Fig 4. Mean specific enzyme activity for cropping systems at 0-10 and 10-20 cm depths, averaged across eight sampling dates in 2013. Error bars represent standard errors ($n = 32$: 4 plots x 8 sampling dates). Bars within a frame that share a letter are not significantly different at $P = 0.05$ (Tukey's HSD).

Fig 5. Stratification ratios (0-10 cm / 10-20 cm) of MBC and MBN.

Fig 6. Concentrations of dissolved inorganic nitrogen (DIN) between cropping systems and depths across the 2013 corn growing season.

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Specific LAP Activity

Specific BG Activity

Specific Ala Activity

Specific BX Activity

Specific AAP Activity

Specific CB Activity