Mechanisms of soil carbon accrual and storage in bioenergy cropping systems

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Abstract

Annual row cropping systems converted to perennial bioenergy crops tend to accrue soil C, likely a function of increased root production and decreased frequency of tillage; however, very little is known about the mechanisms governing the accrual and stability of this additional soil C. To address this uncertainty, we assessed the formation and stability of aggregates and soil organic C (SOC) pools under switchgrass, giant miscanthus, a native perennial grass mix and continuous corn treatments in Michigan and Wisconsin soils differing in both texture and mineralogy. We isolated different aggregate size fractions, >2 mm, 0.5–2 mm, and <0.5 mm, using a procedure intended to minimize alterations to aggregate biological and chemical properties. We determined SOC, permanganate oxidizable C (POXC), and microbial activities (i.e. enzyme activities and soil respiration rates) associated with these aggregates. Soil type strongly influenced the trajectory of aggregate formation and stabilization with differences between sites in mean aggregate size, stability, SOC and microbial activity under perennial vs. corn cropping systems. At the Michigan site, soil microbial activities were highest in the >2 mm aggregates, and higher under the perennial grasses compared to corn. Contrastingly, in Wisconsin soils, microbial activities were highest in the <0.5 mm aggregates and evidence for soil C accrual under perennial grasses was observed only in a fast turnover pool in the <0.5 mm aggregate class. Our results help explain cross-site variability in soil C accrual under perennial bioenergy crops by demonstrating how interactions between below-ground productivity, soil type, aggregation processes and microbial communities influence the rates and extent of SOC stabilization. Bioenergy cropping systems have the potential to be low-C energy sources but first we must understand the complex interactions controlling the formation and stabilization of SOC if we are to maximize soil C accrual.

Keywords: aggregate stability, microbial activity, miscanthus, perennial grass, soil aggregation, switchgrass

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Introduction

Biofuels are an important component of renewable energy strategies in the United States and around the world (McLaughlin & Kszos, 2005; Ragauskas et al., 2006), but the sustainability of biofuel cropping systems remains uncertain (Fargione et al., 2008; Gelfand et al., 2013). The use of perennial grasses as biofuel feedstocks is attractive due to their positive environmental impacts, their ability to thrive in low nutrient or droughty environments, and their soil carbon (C) sequestration potential (Lemus & Lal, 2005; McLaughlin & Kszos, 2005; Anderson-Teixeira et al., 2013; Gelfand et al., 2013). When land is converted from annual grain cropping systems such as corn to perennial bioenergy crops, systems tend to accrue soil organic C (SOC) (Hansen et al., 2004; Lemus & Lal, 2005; Liebig et al., 2005; McLaughlin

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Aggregate size distributions control soil pore space size and connectivity, which in turn is critical for determining microbial activity and SOC mineralization (Tisdall & Oades, 1982; Voroney, 2007; Ananyeva et al., 2013). For example, aggregation can provide larger pore spaces and greater pore connectivity in fine textured soils, which ultimately facilitates the movement of water and gases, provides habitat for soil biota and promotes microbial activity (Yoo et al., 2006; Ruamps et al., 2011; Ananyeva et al., 2013). Contrarily, SOC that is adsorbed to soil mineral surfaces, occluded within aggregates or accessible only though micropores is protected from decomposition.

Aggregation is important in the context of perennial vs. row crop bioenergy systems because increased root production, biomass and exudates, in perennial systems can promote aggregate formation and soil C stabilization. The formation of aggregates and subsequent stabilization of soil organic matter (SOM) proceeds as soil particles are re-oriented through both physical (i.e., advection) and biological (i.e., plant root growth, fungal hyphae, and invertebrate movement) perturbations (Six et al., 2002, 2004; Jastrow et al., 2007). Through these perturbations, soil organic C is thought to be incorporated into and protected within aggregates in an orderly and predictable trajectory, moving from unstable macro-aggregates to stable micro-aggregates contained within stable macro-aggregates (Tisdall & Oades, 1982). Perennial cropping systems likely contribute to aggregation trajectories as their extensive root systems enmesh particles into aggregates and stimulate the production of microbial extracellular polysaccharides that ‘glue’ aggregates together (Traore et al., 2000; Bossuyt et al., 2001; Six et al., 2006). In addition, root exudates are composed primarily of low molecular weight compounds that directly form organo-mineral complexes (Qualls & Haines, 1992; Bradford et al., 2008; Bolan et al., 2011; Fahey et al., 2011) while also promoting the growth and turnover of microbial biomass, both processes that positively contributing to SOC accrual (Grandy & Neff, 2008; Jung et al., 2011; Millner et al., 2011). While the trajectory of aggregate formation and its contributing factors are similar across systems, the timing and speed of these processes is likely to vary greatly in soils with contrasting textures and under different cropping systems because of differences in belowground productivity.

In addition to the positive impacts of belowground productivity on SOC accrual, there are also concomitant counteracting mechanisms that are responsible for SOC losses through destabilization and mineralization. For example, increases in root growth could negatively impact SOC stability by increasing aggregate turnover through physical perturbation, and altering aggregate structure and soil pore sizes such that older, previously protected SOC could be newly exposed to decomposition (Reid & Gross, 1982; Helal & Sauerbeck, 1984; Kuzyakov, 2002). In addition, increased root exudate production and the stimulation of microbial activity may also induce a priming effect that would increase decomposition rates of older SOC (Kuzyakov, 2002; Dijkstra et al., 2006; Bird et al., 2011). Thus, it is important that we better understand how the mechanisms controlling both SOC accrual and losses, such as aggregate formation and stabilization, and microbial activity, interact in bioenergy cropping systems.

The balance between SOC accrual and mineralization, and thus the potential for soil C sequestration under perennial grasses appears to vary widely across systems and soil types (Anderson-Teixeira et al., 2009). For example, switchgrass systems in the mid-Atlantic region have increased SOM stocks from 20 to 125% after 10 years (McLaughlin & Kszos, 2005), while soils under miscanthus in Europe had increases in SOM of only 0.2–0.5% after 6–8 years (Beuch et al., 2000). Because very little is known about the complex interactions between soil texture, soil aggregation and the soil microbes that control SOC accrual under bioenergy crops, we address the following fundamental questions:

1. How do perennial biofuel cropping systems influence aggregate formation and stability compared to more traditional annual biofuel cropping systems such as continuous corn?
2. Do the activities of soil microbes related to C and nutrient acquisition differ under annual vs. perennial biofuel cropping systems?
3. How does soil aggregation and stability interact with microbial activity under perennial vs. annual cropping systems to influence soil C storage?

We address these questions and the mechanisms of SOC accrual in two locations with contrasting soil texture and mineralogy, but with similar climates and identical bioenergy cropping systems, by assessing the progress of aggregate formation, the stability of soil aggregates and the associated microbial activities that ultimately control SOC formation and accumulation.

**Materials and methods**

**Study sites**

Our study included two Biofuel Cropping System Experiments located at the W. K. Kellogg Biological Station, Michigan (42°24′N, 85°24′W) and the Arlington Agricultural Research Station, Wisconsin (43°18′N, 89°21′W). Both sites are part of the Great Lakes Bioenergy Research Center. Annually, the Michigan site averages 810 mm yr⁻¹ of precipitation and
9.7 °C with soils predominantly of the Kalamazoo series, which are sandy loam mesic Typic Hapludalfs (Munoz & Kravchenko, 2011). The Wisconsin site averages 833 mm yr⁻¹ precipitation and 7.4 °C (Midwest Regional Climate Center [MRCC], 2012) with soils primarily of the Plano series, which are silt loam mesic Typic Argiudolls (National Resource Conservation Service Web Soil Survey [NRSC WSS], 2012). In 2008, at each site, five replicate plots of continuous corn (Zea mays L.), switchgrass (Panica virgatum), miscanthus (Miscanthus x giganteus), and a mix of five North American native grass species (Andropogon geradi, Elymus canadensis, Schizachyrium scoparium, Sorghastrum nutans, Panica virgatum) was established. Prior to 2008, these sites were annual row cropping systems. The plots for each of these bioenergy cropping systems are 40 x 28 m, are separated by a mowed field of 15 m width, and are randomly distributed among five blocks. We collected soils at the conclusion of the fourth growing season and after biomass had been harvested (November 15–16, 2011) at each site by removing three soil cores, 15 cm deep by 7.6 cm diameter, from each of the five replicate plots of the above treatments. Soil cores were sealed in airtight bags, leaving bags and soil were submersed in water for 5 min before the soils were continually monitored over several days until they reached a gravimetric water content of ca. 100 g kg⁻¹. Stability was calculated as the proportion of water stable macro-aggregates (aggregates >0.25 mm) remaining on the sieve after correcting for sand content. Sand content (all particles >0.053 mm) was determined on all aggregate size fractions >0.125 mm by dispersing aggregates in a 5% sodium metaphosphate solution before sieving through a 53 μm mesh.

**Soil aggregates**

Upon arrival in New Hampshire, each soil core was weighed for determination of soil bulk density before being gently broken along natural fracture planes and gently passed through an 8 mm mesh sieve. Subsamples of the sieved soil were weighed and dried at 60 °C to determine gravimetric water content. The three replicate soil cores from each plot were then combined into one sample per plot for further analyses. For separation of soil aggregates by size, we first weighed 100 g soil from each plot, spread each of the soils out in large weigh boats, then placed them in a walk-in cooler set at 4 °C. Soils were continually monitored over several days until they reached a gravimetric water content of ca. 100 g kg⁻¹ for the Michigan soils and ca. 80 g kg⁻¹ for the Wisconsin soils, which were determined by trial to be the optimal soil moistures (i.e., maximum soil friability) for dry sieving (Kristiansen et al., 2006). A dry-sieving approach was used to minimize impacts on microbial communities (Schutter & Dick, 2002). In addition, this aggregate separation method is optimal for producing aggregates that have fractured along natural planes while simultaneously minimizing changes in aggregate SOM composition (Kristiansen et al., 2006). Wet sieving methods, for example, release water soluble SOM and colloids thus changing the chemical composition of aggregates (Elliott, 1986; Six et al., 2000). Dry sieving was accomplished on a rotary sieve shaker (Retsch AS 200) using seven sieves to obtain seven aggregate size fractions; >4 mm, 2–4 mm, 1–2 mm, 0.5–1 mm, 0.25–0.5 mm 0.125–0.25 mm, and <0.125 mm. We sieved three replicate aggregate replicates, 100 g soil samples from each experimental plot for 2 min, and then averaged these replicate values for statistical analyses. Aggregates and bulk soil samples were air dried after sieving was completed. Subsamples of these air dried soils were ground to a fine powder then used for determination of total soil C and N on an elemental analyzer (Costech ECS 4010; Costech Analytical Technologies Inc, Valencia, CA, USA). We also used these air dried aggregates to measure permanganate oxidizable C (POXC) per Weil et al. (2003). POXC represents a dynamic and highly active SOC pool, similar to particulate organic C and microbial biomass C due to its relatively high accessibility to microbes and their extracellular enzymes (Weill et al., 2003; Culman et al., 2010).

Aggregate stability was determined on all dry-sieved aggregate size fractions >1 mm (>4 mm, 2–4 mm, 1–2 mm fractions) following Kemper & Rosenau (1986). Briefly, each size fraction was subjected to a wet sieving procedure that consisted of placing 25 g dry aggregates into a 0.25 mm mesh sieve, the cutoff for macro-aggregates in most aggregate hierarchies. The sieve and soil were submersed in water for 5 min before the sieve was raised and lowered 50 times with strokes of 3 cm length over the course of 2 min. Stability was calculated as the proportion of stable macro-aggregates (aggregates >0.25 mm) remaining on the sieve after correcting for sand content. Sand content (all particles >0.053 mm) was determined on all aggregate size fractions >0.125 mm by dispersing aggregates in a 5% sodium metaphosphate solution before sieving through a 53 μm mesh.

**Microbial activity**

To assess microbial activity in the dry-sieved aggregate size fractions, we first pooled aggregates in order to produce just three size fractions, >2 mm, 0.5–2 mm, and <0.5 mm. We pooled aggregate size classes (e.g. >4 mm and 2–4 mm aggregates were pooled to form a >2 mm fraction) in the same proportions as they were found in the bulk soils. These three aggregate size fractions were then used to determine extracellular enzyme activities (EEA) associated with C, nitrogen (N), and phosphorus (P) acquisition including β-glucosidase (BG); N-acetyl-β-glucosaminidase (NAG); Tyrosine amino peptidase (TAP); Acid phosphatase (PHOS); Phenol oxidase; and Peroxidase. To assess EEA, we first homogenized soils in 50 mM sodium acetate buffer that was adjusted to a between-sites average pH of 6.5. The resulting soil slurries were pipetted into 96-well microplates into which we added fluorescently labeled substrates corresponding to the above enzymes. Enzyme activity was measured via the accumulation of the fluorescent label, either methylumbellif erone (MUB) or methyl coumarin (MC) or the color change associated with the break-down of the substrate 3,4-dihydroxy-L-phenylalanine (L-DOPA) over time (Tiemann & Billings, 2011). Soils were incubated at 25 °C for ca. 18 h and just prior to fluorescence measurement, we added 10 μl 0.5 M NaOH to each well to maximize MUB and MC fluorescence, which was determined on a Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA) with 365 nm excitation and 460 nm emission filters. For the L-DOPA based colorimetric measures of phenol oxidase and peroxidase activities, absorbance was measured on the same instrument at 460 nm.

As additional measures of both microbial activity and SOC stability, we conducted a short-term (50 days) incubation of soil aggregates. Briefly, 2 g dried soil aggregates (the same
three size classes used for EEA) were added to 100 ml serum bottles and brought to 50% water holding capacity. To avoid the initial respiration pulse due to re-wetting the soils we began measuring respiration rates after the first 2 days of the incubation, with subsequent measures every 1–2 days for the first 14 days and every 3–4 days thereafter. To measure soil respiration rates, bottles were capped and a gas sample was removed, then immediately injected into an infra-red gas analyzer (LI-820, Li-Cor Inc., Lincoln, NE, USA). The bottles were then incubated at 25 °C for at least 2 h before a second gas sample was removed and (CO₂) measured again as above. Before and after gas sampling events, bottles were opened and flushed with air. Between gas sampling events, soils were incubated in the dark at 25 °C with the bottles loosely capped. Soil moisture was monitored over the course of the incubation and did not change significantly. Final respiration rates were calculated as the amount of C respired between the first and second gas sampling time points. We use measurements of EEA and C mineralized during the incubation to evaluate treatment effects on microbial activity and SOM stability. These assays were conducted under optimized conditions and therefore represent only potential microbial activity, but because all soils were treated in the exact same manner, the assays provide a robust means to test for treatment and site effects on microbial communities.

Statistical analyses

Initial measures of soil C and N, POXC, aggregate proportions and stability were made on all seven original size fractions, but visual inspection of the data indicated similar treatment effects when data were pooled into just three aggregates fractions, with the exception of aggregate stability. Therefore, we analytically pooled soil C and N, POXC and aggregate proportion data into three fractions, >2 mm, 0.5–2 mm and <0.5 mm, in the same way we pooled the actual aggregates for measurement of microbial activities, using the relative bulk soil proportions of each original fraction. At each site treatment effects on aggregate proportions, aggregate mean weight diameter (MWD), aggregate stability, total C and N, POXC, EEA, and total C respired were determined using one-way ANOVA (SAS PROC MIXED, SAS Institute, Cary, NC, USA) with cropping system as the fixed effect and block as a random effect (when significant). These data were analyzed separately by aggregate size fraction. Aggregate MWD was calculated as the sum of each aggregate fraction’s average size times its proportion of bulk soil by weight. Aggregate stability data were analyzed by two-way ANOVA with cropping system, site and cropping system*site as the main effects. We analyzed respiration rates over the course of the incubation using two-way repeated measures ANOVA (SAS PROC MIXED) with treatment and day as fixed effects and a spatial model, sp(POW), for the covariance structure. We then integrated respiration rates over the entire incubation time period to determine cumulative CO₂-C respired. For multivariate analyses, we normalized EEA and cumulative CO₂-C data by dividing each measurement by the highest measured activity level for each enzyme or total CO₂-C respired (Sinsabaugh et al., 2002). We performed a factor analysis on these normalized microbial activity data and then analyzed the final factor scores by site and by aggregate size fraction via ANOVA as described above. Correlation analyses (SAS PROC CORR) were used to assess relationships between soil aggregation, aggregate MWD, stability of all aggregates >1 mm, soil C, POXC, EEA, respiration rates, and total C respired.

Results

Soil aggregation and SOC

In both MI and WI soils, the majority of aggregates separated by dry-sieving were >2 mm (Fig. 1). The level of aggregation was significantly influenced by cropping system at the MI site with more >2 mm aggregates found in switchgrass and native grass soils compared to miscanthus and corn soils (Fig. 1a; Table 1). In contrast, we saw no significant effects of cropping system on aggregation in soils from the WI site (Fig. 1b; Table 2). Aggregate MWD was significantly greater in MI switchgrass compared to corn soils (Fig. 2a; P = 0.046) but not different by crop at the WI site (Fig. 2a). Aggregate
higher concentrations of soil C that aggregates was greater in corn and miscanthus all treatments, compared to native grass soils (Table 1; Fig. 4a). Across gates, but the concentration of SOC in the miscanthus grass and native grass soils, and corn reduced aggregate stability compared to switchgrass (Fig. 3b). In the 0.5–2 mm fraction, corn reduced aggregate stability compared to switchgrass (Fig. 3b). These WI SOC increases were significantly correlated with aggregate MWD (r = −0.49; P = 0.030) and aggregate stability (r = 0.51; P = 0.023). Cropping system had no effect on SOC in any aggregate size class at the WI site. The only cropping system effect on SOC we observed at the WI site was in the <0.5 mm aggregates in the relatively dynamic POXC pool where these aggregates under corn had a significantly lower concentration of POXC compared to all other cropping systems (Table 2). Across treatments we saw higher concentration of soil C in <0.5 mm aggregates compared to >2 mm and 0.5–2 mm aggregates (P = 0.002; Fig. 4b).

At the MI site, we saw no change in bulk SOC by cropping system (Fig. 2b) and SOC was not related to aggregate stability or MWD. Cropping system at MI did not influence SOC in the >2 mm and 0.5–2 mm aggregates, but the concentration of SOC in the <0.5 mm aggregates was greater in corn and miscanthus soils compared to native grass soils (Table 1; Fig. 4a). Across all treatments, >2 mm and 0.5–2 mm aggregates had higher concentrations of soil C that <0.5 mm aggregates (P < 0.0001; Fig. 4a). We did find significantly greater bulk SOC in WI under miscanthus and switchgrass compared to corn (Fig. 2b). These WI SOC increases were significantly correlated with aggregate MWD (r = −0.49; P = 0.030) and aggregate stability (r = 0.51; P = 0.023). Cropping system had no effect on SOC in any aggregate size class at the WI site. The only cropping system effect on SOC we observed at the WI site was in the <0.5 mm aggregates in the relatively dynamic POXC pool where these aggregates under corn had a significantly lower concentration of POXC compared to all other cropping systems (Table 2). Across treatments we saw higher concentration of soil C in <0.5 mm aggregates compared to >2 mm and 0.5–2 mm aggregates (P = 0.002; Fig. 4b).

### Microbial activity

Mineralizable C was significantly impacted by cropping system at both sites. Total C respired from MI >2 mm aggregates was marginally higher in miscanthus compared to corn and switchgrass soils (Table 1; Fig. 5a) and there were higher respiration rates with

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Table 1 Results of one-way ANOVA analyses for effects of cropping system on Michigan (MI) soil physical and chemical properties and microbial activities by aggregate size fraction

<table>
<thead>
<tr>
<th>MI soil variable</th>
<th>&gt;2 mm</th>
<th>0.5–2 mm</th>
<th>&lt;0.5 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value</td>
<td>Treatment effects</td>
<td>P-value</td>
</tr>
<tr>
<td>Proportion of bulk soil (g aggregates kg⁻¹ soil) SOC</td>
<td>0.005</td>
<td>N = S &gt; C = M*</td>
<td>0.001</td>
</tr>
<tr>
<td>(mg C g⁻¹ soil)</td>
<td>NS</td>
<td>NS</td>
<td>0.049</td>
</tr>
<tr>
<td>POXC (ng C g⁻¹ soil)</td>
<td>NS</td>
<td>NS</td>
<td>0.07</td>
</tr>
<tr>
<td>Total C respired (mg C g⁻¹ soil)</td>
<td>0.058</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BG (nmol activity g⁻¹ soil h⁻¹)</td>
<td>&lt;0.001</td>
<td>N &gt; M = S &gt; C</td>
<td>NS</td>
</tr>
<tr>
<td>NAG (nmol activity g⁻¹ soil h⁻¹)</td>
<td>&lt;0.001</td>
<td>N &gt; M = S &gt; C</td>
<td>NS</td>
</tr>
<tr>
<td>TAP (nmol activity g⁻¹ soil h⁻¹)</td>
<td>0.008</td>
<td>N &gt; M = S = C</td>
<td>0.001</td>
</tr>
<tr>
<td>PHOS (nmol activity g⁻¹ soil h⁻¹)</td>
<td>0.003</td>
<td>M = N &gt; S = C</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phenol oxidase (nmol activity g⁻¹ soil h⁻¹)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Peroxidase (nmol activity g⁻¹ soil h⁻¹)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Factor 1 scores</td>
<td>&lt;0.001</td>
<td>N &gt; M &gt; S &gt; C</td>
<td>0.012</td>
</tr>
<tr>
<td>Factor 2 scores</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Treatment effects of corn (C), miscanthus (M), switchgrass (S), and a five species native grass mix (N) were assessed using post hoc testing of differences between least-squares means.

** P-values in bold represent marginally significant results with no post hoc testing.

*Treatment effects of corn (C), miscanthus (M), switchgrass (S), and a five species native grass mix (N) were assessed using post hoc testing of differences between least-squares means.

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miscanthus compared to all other treatments ($P = 0.018$). Cropping system did not impact respiration rates or total C respired in any MI $< 2$ mm aggregates (Table 1; Fig. 5a). WI $> 2$ mm aggregates had higher total C respired in all soils from perennial grass cropping systems compared to corn (Fig. 5b; Table 2) and the highest respiration rates in native grass compared to all other crops ($P < 0.001$). In WI 0.5–2 mm aggregates from native and miscanthus treatments we observed higher total C respired compared to corn (Fig. 5b; Table 2) and higher respiration rates than both corn and switchgrass ($P = 0.002$). In the WI $< 0.5$ mm fraction

Table 2 Results of one-way ANOVA analyses for effects of cropping system on Wisconsin (WI) soil physical and chemical properties and microbial activities by aggregate size fraction

<table>
<thead>
<tr>
<th>Variable</th>
<th>&gt;2 mm P-value</th>
<th>Treatment effects</th>
<th>0.5–2 mm P-value</th>
<th>Treatment effects</th>
<th>&lt;0.5 mm P-value</th>
<th>Treatment effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of bulk soil (g aggregates kg$^{-1}$ soil)</td>
<td>NS</td>
<td>NS</td>
<td>0.09**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOC (mg C g$^{-1}$ soil)</td>
<td>NS</td>
<td>NS</td>
<td>0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POXC ($\mu$g C g$^{-1}$ soil)</td>
<td>NS</td>
<td>0.088</td>
<td>0.012</td>
<td>S = M = N &gt; C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total C respired (mg C g$^{-1}$ soil)</td>
<td>0.001</td>
<td>N &gt; S = M &gt; C*</td>
<td>0.043</td>
<td>N = M &gt; C</td>
<td>0.034</td>
<td>N = M &gt; C</td>
</tr>
<tr>
<td>BG (nmol activity g$^{-1}$ soil h$^{-1}$)</td>
<td>NS</td>
<td>0.063</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAG (nmol activity g$^{-1}$ soil h$^{-1}$)</td>
<td>0.059</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAP (nmol activity g$^{-1}$ soil h$^{-1}$)</td>
<td>0.083</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHOS (nmol activity g$^{-1}$ soil h$^{-1}$)</td>
<td>0.003</td>
<td>C &gt; M = S &gt; N</td>
<td>0.047</td>
<td>C &gt; M = S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol oxidase (nmol activity g$^{-1}$ soil h$^{-1}$)</td>
<td>0.027</td>
<td>N &gt; S = M &gt; C</td>
<td>0.014</td>
<td>S &gt; C = M = N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxidase (nmol activity g$^{-1}$ soil h$^{-1}$)</td>
<td>0.017</td>
<td>C &gt; M = S = N</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Factor 1 scores</td>
<td>NS</td>
<td>0.024</td>
<td></td>
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<tr>
<td>Factor 2 scores</td>
<td>0.001</td>
<td>C &lt; N, M, S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Treatment effects for corn (C), miscanthus (M), switchgrass (S) and a five species native grass mix (N) were assessed using post hoc testing of differences between least-squares means.

**P-values in bold represent marginally significant results with no post hoc testing.

Fig. 2 Michigan (MI) and Wisconsin (WI) soil (a) aggregate mean weight diameter (MWD) and (b) bulk SOC. Points represent means ± 1 SE ($n = 5$).
under all perennial grass systems compared to corn we found greater total C respired (Fig. 5b; Table 2) and higher respiration rates ($P < 0.001$).

In MI bulk soils, EEA associated with N or P acquisition (TAP and PHOS) were generally higher under corn than under perennial grasses (Fig. 6a and b), while in WI bulk soils we found generally higher N and P acquisition EEA (NAG and PHOS) under perennial grasses than under corn (Fig. 6c and d). In MI soils, the largest aggregates from perennial grass plots had the greatest EEA, but the smallest aggregates from corn plots had the highest EEA (Fig. S1a–f; Table 1). Specifically,

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>2 mm aggregates from perennial grasses had higher BG, NAG, and PHOS activity than under corn and these same EEAs were highest in the <0.5 mm aggregates under corn (Fig. S1a, b & c; Table 1). In WI aggregates, there were fewer treatment effects on EEA (Fig. S2a–f; Table 2), but WI > 2 mm aggregates had higher PHOS and peroxidase and lower phenol oxidase activities under corn compared to perennial grasses (Fig. S2d, e & f; Table 2).

Factor analysis of normalized microbial activities (EEA and total C respired) revealed two orthogonal factors for MI and WI that explained a total of 91% and 92% of the variation, respectively. Across MI aggregate fractions, Factor 1 was highly correlated with BG, NAG,
TAP, and PHOS activities (all hydrolases), while Factor 2 was highly correlated with decreasing phenol oxidase and increasing peroxidase activities as well as cumulative CO₂ respired (Fig. 7a–c). MI soils Factor 1 scores associated with >2 mm aggregates were significantly influenced by treatment, with the highest scores, and therefore highest hydrolytic EEA, associated with perennial grasses (Fig. 7a; Table 1). In contrast, we

![Graph](image-url)

Fig. 7  Factor analysis results using enzyme activities and total C respired for MI (a) >2 mm; (b) 0.5–2 mm; (c) <0.5 mm and for WI (d) >2 mm; (e) 0.5–2 mm; (f) <0.5 mm soil aggregates. Horizontal arrows refer to correlations of variables with Factor 1 scores and vertical arrows represent correlations of the variables with Factor 2 scores. Arrow lengths are proportional to the standardized scoring coefficient for each variable and indicate the direction of increasing enzyme activity or C respired. Points represent factor score means ± 1 SE (n = 5).
found that Factor 1 scores associated with 0.5–2 mm and <0.5 mm aggregates were higher in corn compared to perennial grasses (Fig. 7b and c; Table 1). The MI Factor 2 scores were not influenced by treatment. In WI aggregates Factor 1 was again dominated by hydrolytic EEA with the addition of cumulative CO₂ respired, while Factor 2 was dominated by contrasting phenol oxidase and peroxidase activities (Fig. 7d–f). WI soils Factor 1 scores for 0.5–2 mm aggregates were higher under the native grass mix compared to all other crops (Fig. 7e; Table 2) and Factor 2 scores associated with the >2 mm aggregates were significantly greater (corresponding to greater phenol oxidase and lower peroxidase activities) in all perennial grass soils compared to corn (Fig. 7d; Table 2). Across all aggregate size classes in both MI and WI soils, we found significant correlations between Factor 1 scores and POXC (MI, r = 0.67; P < 0.001 and WI, r = 0.55; P < 0.001) and total SOC (MI, r = 0.56; P < 0.001 and WI, r = 0.53; P < 0.001).

Discussion

Soil aggregation and stability

We found evidence for changes in soil structure under perennial grasses compared to corn, although the magnitude of these effects differed by cropping system and by site. Perennial cropping systems, specifically switchgrass and the native grass mix, in the MI sandy loam soils increased the proportion of >2 mm aggregates, as well as aggregate MWD and the stability of >4 mm aggregates (Fig. 1a, 2a, 3b). While these increases in aggregation and aggregate stability were not related to SOC accrual in this study, previous work at the MI site has shown a positive relationship between SOC accrual and increases in >2 mm aggregates and aggregate MWD over longer time periods (Grandy & Robertson, 2007). The absence of a relationship between SOC and aggregation at the MI site in this study is likely due to the relatively short time period in which the biofuel cropping systems have been in place (four growing seasons), and the known lag time between aggregate formation and soil C accumulation. In the WI silt loam soils, there was marginal evidence for increasing aggregation, although we observed large increases in macro-aggregate stability under perennial grasses (Fig. 3), and these increases in stability were positively related to SOC. In fact, in WI soils, aggregate MWD was negatively correlated with bulk SOC because C accrual appears to occur primarily in the smallest, but most stable aggregates in these soils. Therefore, aggregate stability may be a more important indicator for SOC accrual than aggregation, particularly in finer textured soils.

When our results are viewed in light of an aggregate formation gradient, such as that proposed by Tisdall & Oades (1982), they are indicative of differences in the speed of aggregate formation/stabilization and SOC accrual in sandy loam vs. silt loam soils following biofuel crop conversion. It appears that increases in SOC accrual under perennial crops are directly linked to the stages of macro-aggregate formation and stabilization, which progress from large, unstable aggregates, to large, moderately stable aggregates containing stable micro-aggregates to large, stable aggregates containing stable micro-aggregates (Fig. 8; Tisdall & Oades, 1982; Elliott, 1986; Six et al., 2000). After four seasons of perennial crop growth, the sandy loam MI soils are not as far along this aggregate formation and SOC accrual trajectory as the silt loam WI soils. It appears that MI soils under perennial grasses are still in the first stages of aggregate formation and stabilization, dominated by large (>2 mm) and relatively unstable aggregates that have not yet experienced increases in C. In contrast, WI soils appear to be in the middle of the aggregate formation trajectory, with greater stability in macro-aggregates, and possibly greater micro-aggregate formation and stability (Six et al., 2000). At the WI site, SOC accrual has begun under miscanthus and switchgrass, in line with the greater aggregate stability, but the additional C appears to still be relatively unstable or easy for microbes to access and mineralize (Fig. 5b and POXC data). The contrast in results between different perennial grass systems and between-sites highlights the importance of both soil texture and cropping system choice in regulating SOC dynamics following bioenergy crop conversion.

Cropping system effects on microbial activities

The observed changes in aggregate stability may be directly related to shifts in microbial community activity and structure in perennial compared to continuous corn cropping systems. Increases in microbial activity that we observed in perennial systems may indicate increased microbial growth and turnover, as well as enhanced production of polysaccharides that serve to bind and stabilize aggregates (Haynes & Beare, 1997; Bailey et al., 2002; Six et al., 2006; Abiven et al., 2007; Le Guillou et al., 2012). Observed shifts in microbial activity may also be indicative of changes in microbial community structure, given the range of relationships between different microbial taxa and aggregation processes (Bailey et al., 2002; Six et al., 2006; Abiven et al., 2007; Le Guillou et al., 2012). For example, fungal hyphae stabilize aggregates through both physical processes, such as enmeshing particles into larger peds, and the production of chemical binding agents such as
glomalin, while bacteria are important contributors of polysaccharides and other more transitory chemical binding agents. Previous studies of the WI soils under corn, switchgrass, and native grass cropping systems have revealed divergent microbial community structures, with the most marked differences being increasing Gram+ bacteria and arbuscular mycorrhizal fungi (AMF) with decreasing saprotrophic fungi abundance moving from corn to native grasses (Liang et al., 2012). Higher levels of aggregate stability observed in WI soils under perennial grasses are likely related to the previously observed increases in AMF abundance in these same perennial cropping systems. Increases in AM fungi would have a large, positive influence on both aggregate formation and stabilization because AMF hyphae act as physical binding agents and produce a number of chemical binding agents such as glomalin (Degens et al., 1996; Haynes & Boare, 1997; Wright & Upadhyaya, 1998). We have no direct evidence that perennial grasses have altered microbial community structure at the MI site, but it is interesting to note that perennial grasses affected microbial activities in MI soils differently than in WI soils with impacts in different aggregate size classes and on different enzymes (Tables 1 and 2; Fig. 7). These differences in microbial community activity between the two sites, in conjunction with differences in soil texture, are likely driving site specific trajectories of aggregate formation and stabilization under perennial crops.

Previous studies have shown equivocal effects of plant communities on soil microbial communities (Zak et al., 2003; Wardle et al., 2004; Berg & Smalla, 2009). Furthermore, the few studies that have investigated microbial communities in relation to aggregate size classes have also observed unclear or no patterns (Schutter & Dick, 2002; Blackwood & Paul, 2003; Allison & Jastrow, 2006; Blackwood et al., 2006). In this study, we similarly found that plant species or aggregate size classes did not have consistent impacts on microbial community activities. Instead, the response of microbial communities to cropping system appeared to be governed by site-specific interactions between plant species, soil type, and aggregation processes (Berg & Smalla, 2009). For example, in MI bulk soils, nutrient (N and P) acquisition enzyme activities were elevated with corn compared to perennial grasses (Fig. 6a and b), and were highest in the largest aggregates (>2 mm) and lowest in the smallest aggregates (<0.5 mm). In contrast, perennial grasses stimulated microbial nutrient acquisition in WI bulk soils and these activities were highest in

![Fig. 8 Trajectory of aggregate formation and stabilization and concurrent SOC accrual under perennial grasses in Michigan (MI) sandy loam vs. Wisconsin (WI) silt loam soils, where time zero represents status of land under continuous corn, prior to conversion to a perennial cropping system. Time zero and the first 5 years of the trajectory are based on data from this study, the trajectory >5 years is based on the model proposed by Tisdall & Oades (1982).](image)
the smallest aggregates (<0.5 mm). Thus, it appears that soil type strongly modifies the spatial patterns of microbial investment in nutrient acquisition in perennial grass vs. corn systems.

While examining enzyme activities can provide us with insight into nutrient acquisition pathways, enzyme activities also strongly regulate the stability of SOM. Microbes in the MI soils appear to be more nutrient limited under corn, investing more in the production of N and P acquisition enzymes. In comparison, microbes in the WI soils appear to be more nutrient limited under perennial grass systems, based upon their greater investment in nutrient acquisition enzymes. These differences in cropping system effects on microbial investment in nutrient acquisition may be the result of trade-offs between root associated increases in soil C availability (Lemus & Lal, 2005; McLaughlin & Kszos, 2005; Anderson-Teixeira et al., 2013; Gelfand et al., 2013), aggregate stability and microbial growth. In WI perennial soils, greater aggregate stability coupled with higher belowground C inputs and microbial activity lead to both increased nutrient demand and decreased nutrient accessibility. Therefore, these microbes must increase their nutrient acquisition efforts, and thus SOM break-down, through enzyme production. In contrast, in MI perennial soils, microbes can invest less in enzyme production for nutrient acquisition because with significantly more large aggregates and lower aggregate stability, SOM is more accessible (Yoo et al., 2006; Ruamps et al., 2011; Ananyeva et al., 2013). Greater SOM accessibility likely enhances nutrient cycling rates but this microbial mining of relatively unstable SOM is not conducive to long-term SOC sequestration (Grandy & Robertson, 2007; Ananyeva et al., 2013). Thus, soil texture is playing a large role in determining soil structure, as well as the location of SOC within large or small aggregates and ultimately its accessibility to microbes.

Bioenergy cropping system and soil texture interactions with implications for SOC

After four growing seasons, evidence presented here suggests that any SOC that has accrued under perennial biofuel cropping systems is relatively unstable. The MI soils, in the first stages of aggregate development (Fig. 8), are dominated by large (>2 mm) relatively unstable aggregates in which microbial activities and SOC concentrations are highest. Increases in microbial activity with perennial grasses in the >2 mm aggregates, and more specifically the positive impacts of perennial grasses on hydrolytic enzymes but not oxidative enzymes, suggests there has been some increase in SOC even though it is not yet statistically detectable. Hydrolytic enzymes are generally associated with the breakdown of relatively labile or fresh SOC inputs while oxidative enzymes are typically associated with the breakdown of more recalcitrant SOC, which may include older SOC in the form of aromatic ring structures (Sinsabaugh et al., 2008). These lines of evidence and previous studies of the MI soils (Grandy & Robertson, 2007; Ananyeva et al., 2013) suggest a relatively slow build-up of stable macro-aggregates and therefore long-term or stable SOC. Grandy & Robertson (2007) demonstrated that SOC accrual does occur in MI macro-aggregates, but this SOC is particularly susceptible to microbial attack and rapid turnover when these aggregates are disturbed. Further, Grandy and Robertson (2007) showed that one-time tillage of long-term, grassland soils at the MI site reduced aggregation overall by >30%, while increasing soil CO2 fluxes by >100%. In light of these previous studies, our current research confirms that any SOC accrual in MI perennial bioenergy cropping systems is likely to occur in easily mineralized pools, emphasizing the importance of soil management decisions that minimize disturbance and aggregate destruction.

In the finer textured WI silt loams, we saw a much different response to perennial cropping systems because these soils seem to be further along the aggregate formation and stabilization trajectory (Fig. 8). Taken in conjunction with the higher silt and clay content in WI soils, which makes them better suited to SOC accrual (McConkey et al., 2003; Grandy & Neff, 2008), we expected to find evidence for both SOC accrual and stabilization with perennial grasses at this site. In the <0.5 mm aggregates, we did in fact find evidence of C accrual, with the highest levels of microbial activity (EEA and C mineralization) and in a dynamic SOC pool, represented by POXC. We expected SOC stabilization and accrual to be associated with the smallest, most stable aggregates, where SOC is mineral associated or occluded and therefore inaccessible to microbial attack (Kanazawa & Filip, 1986; Kandel et al., 1999; Stemmer et al., 1999; Grandy et al., 2008). While we do see evidence for this kind of SOC accrual, we did not find evidence for SOC stabilization, 4 years after conversion. In the small WI aggregates, where microbial activity is high, it appears that not enough time has passed for both the build-up and stabilization of microbial by-products and necromass to occur or for the small aggregates to become stabilized and occluded within large aggregates (Fig. 8). In fact, the additional SOC associated with the <0.5 mm fraction under perennial grasses may actually be particulate organic matter that has not yet undergone significant microbial decomposition or modification into recalcitrant forms of SOM. However, as mentioned above, increasing aggregate formation may not be as crucial for SOC accrual in a finer
textured soil, because of the importance of the increased mineral surface area that is associated with more silt and clay sized particles. In the WI soils where microbes are more likely to be in close association with mineral surfaces, because of high concentrations of silt and clay particles, it is likely that under perennial grasses where microbial activity is high and more microbial biomass turns over, stable SOC will accrue as microbial by-products and necromass form stable and long-lived organo-mineral complexes (Simpson et al., 2007; Grandy & Neff, 2008; Liang et al., 2010; Miltner et al., 2011).

The results presented here demonstrate that interactions between soil physical properties and microorganisms determine how different biofuel cropping systems are likely to impact SOC stocks. Spatial patterns of microbial activity and rates of SOC accrual under perennial vs. annual cropping systems likely follow aggregate formation processes along a trajectory of hierarchical development. After four seasons of perennial crop growth, sandy loam MI soils are not as far along this aggregate formation/stabilization trajectory as the silt-loam WI soils (Fig. 8). This study highlights the importance of a complete understanding of soil physical and biological properties when projecting SOC accrual rates under perennial bioenergy crops. While some systems seem to show dramatic increases in SOC under perennial cropping systems, others have more moderate increases that cannot be explained by simple relationships with climate or soil texture alone (Lemus & Lal, 2005). The high variability in SOC accrual under perennial cropping systems is due to the complex interactions between climate, soil texture and the soil biota, and it is these complex interactions that must be further elucidated if we are to accurately predict and maximize the SOC sequestration potential of bioenergy cropping systems.

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