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LETTER

Crop rotational diversity enhances belowground communities and functions in an agroecosystem

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Abstract

Biodiversity loss, an important consequence of agricultural intensification, can lead to reductions in agroecosystem functions and services. Increasing crop diversity through rotation may alleviate these negative consequences by restoring positive aboveground–belowground interactions. Positive impacts of aboveground biodiversity on belowground communities and processes have primarily been observed in natural systems. Here, we test for the effects of increased diversity in an agroecosystem, where plant diversity is increased over time through crop rotation. As crop diversity increased from one to five species, distinct soil microbial communities were related to increases in soil aggregation, organic carbon, total nitrogen, microbial activity and decreases in the carbon-to-nitrogen acquiring enzyme activity ratio. This study indicates positive biodiversity–function relationships in agroecosystems, driven by interactions between rotational and microbial diversity. By increasing the quantity, quality and chemical diversity of residues, high diversity rotations can sustain soil biological communities, with positive effects on soil organic matter and soil fertility.

Keywords

Crop rotation, microbial community, soil carbon, soil nitrogen, soil organic matter.

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INTRODUCTION

Row crop agriculture's disruption of soil ecosystems threatens long-term food security, enhances atmospheric trace gas emissions, accelerates soil erosion and reduces water quality. Efforts to mitigate these negative environmental effects have targeted two important properties of cultivated agroecosystems, frequent disturbance to the soil structure and excess nutrient inputs, with limited success. Managing another common characteristic of agroecosystems, low plant diversity, may offer greater promise, based on ecological theory linking biodiversity and ecosystem function. Greater biodiversity has been shown to increase productivity, resource use efficiency and nutrient availability, and to lead to greater ecosystem stability (Hooper *et al*. 2005; Tilman *et al*. 2006). These relationships have been tested primarily in prairie grasslands and whether cultivated agroecosystem soils show similar responses to aboveground biodiversity is unclear, given fundamental differences in the structure of natural and agricultural systems (Zak *et al*. 2003; Hooper *et al*. 2005; Tilman *et al*. 2006).

In row crop agricultural systems, there are no inter-species interactions between plants as most fields are planted with one species. When crop rotations (i.e. sequential planting of individual crops through time) are used to increase crop diversity, nutrient competition and the chemistry and quality of plant inputs to soils change more across time than space. Still, belowground benefits of rotational diversity, such as increases in soil organic matter (SOM) stocks, have been observed across a multitude of studies (West & Post 2002; McDaniel *et al*. 2014b). In natural systems, the belowground benefits of plant diversity have been linked to changes in microbial communities (Zak *et al*. 2003; Hooper *et al*. 2005; Tilman *et al*. 2006) and this is also true in agroecosystems, potentially operating at different spatial and temporal scales (Van der Putten *et al*. 2009). In a recent meta-analysis of 122 studies, McDaniel *et al*. (2014b) showed that crop rotations increase microbial biomass by an average 21%. Changes in microbial community structure with differences in rotational diversity have also been reported (Alvey *et al*. 2003; Johnson *et al*. 2003; Yin *et al*. 2010); in particular, rotational diversity increases microbial community diversity and the relative abundance of fungi vs. bacteria (Bunemann *et al*. 2004; González-Chávez *et al*. 2010; Suzuki *et al*. 2012). These changes are important because microbial community diversity is linked to functional resilience or resistance to disturbance (Griffiths *et al*. 2000). Fungal-dominated communities have been associated with both qualitative and quantitative enhancement of SOM in agroecosystems (Six *et al*. 2006). Thus, changes in microbial communities, including the enhancement of soil microbial biomass and function, are likely related to changes in SOM accumulation with crop

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rotation (Govaerts *et al*. 2007; Hungria *et al*. 2009; McDaniel *et al*. 2014b). Despite these findings, uncertainty remains about how changes in microbial community structure influence SOM responses to crop diversification.

The process of soil aggregation, which is an important regulator of SOM dynamics and soil fertility, is expected to be closely related to changes in microbial communities. Fungal hyphae and microbial production of extracellular polymeric substances affect aggregate formation and stabilisation processes, which are directly linked to SOM persistence (Six *et al*. 2004; Abiven *et al*. 2007; Jastrow *et al*. 2007). Furthermore, microbial community functions, such as extracellular enzyme production, decomposition and production of aggregate binding agents, have previously been linked to aggregate formation and SOM accrual (Six *et al*. 2004; Tiemann & Grandy 2015). These microbial functions are governed by the structure of the community (Johnson *et al*. 2003; Six *et al*. 2006); therefore, we must also assess if and how community structure is changing if we are to determine whether or not shifts in function are likely to be long term.

The goal of this study was to determine whether increasing agricultural plant diversity through time via crop rotation, independent of other changes in management, enhances soil ecosystem structure and functions. In agricultural systems, management practices are typically bundled so that changes in plant communities are accompanied by changes in fertiliser or other inputs to maximise yields. In fact, we are unaware of any previous studies in which tillage and other management practices (e.g. fertiliser and pesticide use) have not co-varied with plant diversity, preventing the isolation of rotational diversity effects on microbial communities and SOM dynamics. We used a unique experimental crop diversity gradient in which increases in crop diversity were accomplished by adding crops sequentially in rotation. We sampled soils from plots ranging in diversity from monoculture to a five crop rotation that included two cover crops, or non-harvested crops grown principally to benefit the soil. We separated the soils into two aggregate size fractions that capture both the earliest stages of SOM formation and stabilisation [macro-aggregates (> 2 mm)] and longer term, more stable SOM that is protected from microbial attack (micro-aggregates 0.053–0.25 mm; Six *et al*. 2004; Jastrow *et al*. 2007). Here, for the first time, we use these aggregate fractions to simultaneously assess linkages between rotational diversity, soil structure, microbial community structure, microbial activity and SOM chemistry. To assess microbial community structure we used phospholipid fatty acid (PLFA) analysis, which is a sensitive measure for detecting broad changes in microbial community structure in response to changes in plant communities (Ramsey *et al*. 2006; Smith *et al*. 2014). We hypothesised that increases in crop diversity through rotation would alter microbial community structure leading to positive effects on microbial activity. Furthermore, these changes in microbial community structure and increases in microbial activity would be linked to enhanced aggregate formation and stabilisation processes, which would in turn lead to SOM accumulation and increases in soil fertility.

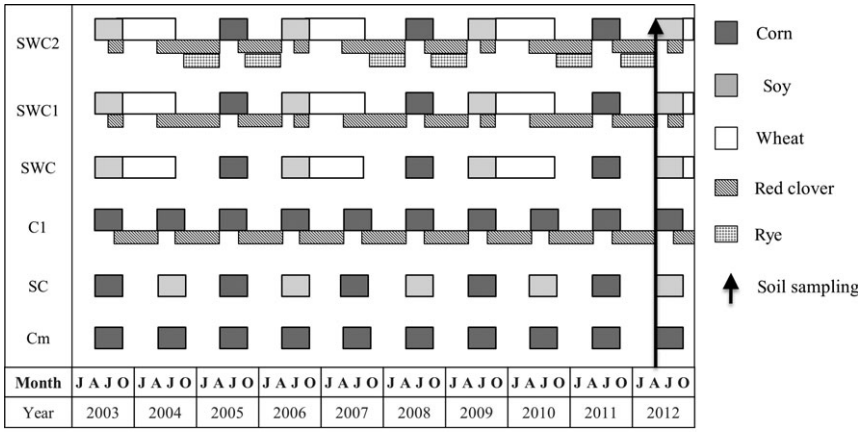
METHODS

Study Sites

The study was conducted at the W.K. Kellogg Biological Station in Michigan, USA (42°24′ N, 85°24′ W, elevation 288 m), which averages 1005 mm $year^{-1}$ precipitation and 10.1 °C with soils developed on glacial till that are sandy loam mesic Typic Hapludalfs. The experiment (http://lter.kbs.msu.edu/research/long-term-experiments/biodiversity-gradient) consists of four replicated randomised treatment blocks, with 9 × 27 m experimental plots. Each plot is chisel ploughed annually to a depth of 15 cm and receives no external inputs (chemical or organic fertilisers; pesticides). Treatments used in this study include corn monoculture (Cm), corn and soy rotation (SC), corn with a late summer/winter leguminous cover crop, red clover (C1) and rotations of corn, soy, wheat with no cover (SWC), with red clover (SWC1) and with both red clover and rye cover crops (SWC2; Fig. 1). The crop species chosen represent common crop combinations across the Midwestern U.S.; therefore, the experimental design does not include all combinations of species for each level of diversity. This design allows us to test the effects of specific sets of species on belowground processes, and these 'sets' are indicative of rotational diversity in agroecosystems. At this site, increased rotational diversity has led to increased corn yields, with highest diversity rotation yields 100% greater than monoculture corn (Smith *et al*. 2008). In the 3 years prior to the current study, biomass inputs in the form of grain crop residues and cover crops was greatest in SWC2, SWC1 and C1 compared to other rotations and lowest in monoculture (Cm; Fig. S1). To reduce the possibility of 'hidden treatment' effects, which could confound rotation and current crop effects, we sampled soils when all treatments were under the same species, corn (Fig. 1). Soil cores, 10 cm deep by 7.6 cm diameter, were collected on April 27, 2012 in triplicate in each of the four replicate plots, packed in insulated boxes and shipped on ice and stored at 4 °C until further processing.

Soil aggregate separation

Each soil sample was weighed for bulk density determination, then passed through an 8-mm mesh sieve by gently breaking soils along natural fracture planes. We dried 100 g of each soil at 4 °C until it reached a gravimetric water content of ~ 100 g kg^{-1}, previously determined to be the optimal soil moisture for dry sieving these soils (Tiemann & Grandy 2015). We employed dry sieving to minimise effects on microbial communities and SOM, while producing aggregates fractured along natural planes (Kristiansen *et al*. 2006). Using a rotary sieve shaker (Retsch 200; Verder Scientific Inc., Newton, PA, USA) we separated soil into three aggregate size classes: > 2 mm mega-aggregates, 0.25–2 mm macro-aggregates and 0.053–0.25 mm micro-aggregates. To determine aggregate stability we used the same sieves and sieve shaker, but applied a lid to the top, fitted with a nozzle and hose attached to a deionised water tap (Tiemann & Grandy 2015). We determined sand content (> 0.053 mm) by dispersing aggregates in a 5% (w/v) sodium hexametaphosphate solution



Treatment	Total Species	Species per year	Abbreviation	Crop diversity index (CDI)
Soy-Wheat-Corn, 2 cover crops	5	3	SWC2	15
Soy-Wheat-Corn, 1 cover crop	4	2-3	SWC1	10
Soy-Wheat-Corn rotation	3	1-2	SWC	4.5
Corn with 1 cover crop	2	2	C1	4
Soy-Corn rotation	2	1	SC	2
Corn monoculture	1	1	Cm	1

Figure 1 Experimental design of the crop diversity gradient experiment at the W.K. Kellogg Biological Station in Michigan, USA, including description of rotations and calculated crop diversity index (CDI).

and sieving through a 0.053-mm mesh sieve. After correcting for sand content, the percentage of field moist mega-aggregates that remain as water stable mega-aggregates is used to represent aggregate stability.

Soil C and N and microbial activity

We measured total soil C and N on finely ground sub-samples on an elemental analyser (Costech ECS 4010; Costech Analytical Technologies Inc., Valencia, CA, USA). We used three methods to assess the stability of soil C (see Supporting information for detailed descriptions). First, we determined permanganate oxidisable C (POXC), a relatively easy to access and highly active pool of soil C (Weil *et al*. 2003). Second, we assessed potentially mineralisable C via a short (56 days) soil incubation (Robertson *et al*. 1999). Finally, we determined the chemical composition of SOM in mega- and micro-aggregates via pyrolysis-gas chromatography and mass spectrometry (py-GC/MS; Wickings *et al*. 2012).

Microbial community function across rotations and soil aggregates was assessed by measuring extracellular enzyme activities (EEA) including: the labile C acquisition enzymes β-glucosidase and cellobiohydrolase; N acquisition enzymes N-acetyl-β-glucosaminidase and leucine amino peptidase; acid phosphatase; and recalcitrant C acquisition enzymes phenol oxidase and peroxidase, which break down compounds with aromatic rings and amorphous structures (Tiemann & Grandy 2015). Following Tiemann & Grandy (2015), we homogenised soils in buffer adjusted to average soil pH of 6.5 and incubated with labelled substrates at 25 °C for ~ 18 h before fluorescence or absorbance was determined on a Synergy HT plate reader (BioTek, Winooski, VT, USA).

Microbial Community Structure

Lipids were extracted from *c*. 3 g of freeze-dried and ground soil aggregates using a hybrid version of PLFA and fatty acid methyl ester (FAME) analysis (see Supporting information). Extracted lipids were analysed on an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) and peaks identified and quantified by running a mixed FAME standard (EUKARY; 9 : 0 and 19 : 0; Sigma, St. Louis, MO, USA) with Sherlock microbial identification software (MIDI, Inc., Newark, DE, USA). Only fatty acids ≤ 20 C chain length, identifiable and present at > 0.5 mol %, were used (Table S1). We calculated fungi : bacteria ratios and the ratio of total saturated to total monounsaturated fatty acids, which can indicate resource limitation, with greater ratios indicating more resource limited microorganisms (Fierer *et al*. 2003; see Supporting information and Table S1).

Statistical analyses

One-way ANOVA analyses by aggregate size class, with cropping system as a fixed effect and 'block' as a random effect were performed using PROC MIXED (SAS Institute, Cary, NC, USA). We used Waller–Duncan *k*-ratio *post hoc* tests to evaluate differences between cropping systems. We calculated a crop diversity index (CDI) for each rotation by multiplying the average number of crop species per year by the total number of species across a 3-year period to simultaneously capture differences in spatial and temporal diversity (Fig. 1). The CDI ranged from 1 (Cm) to 15 (SWC2; three species per year × five total species). We used SAS PROC CORR for correlation analyses of CDI and soil or microbial variables

(Table S2). The Shannon-Weiner diversity index (H) was calculated using EEA and PLFA data. These diversity metrics are useful for examining how microbial communities changed as rotational diversity increased, but are not 'true' measures of diversity because maximum species richness was dictated by EEA and PLFA experimental protocols *a priori*.

RESULTS

Soil chemistry and aggregate size distribution and stability

Aggregate size distributions varied with rotation, with more water stable mega-aggregates in the high diversity rotations ($P < 0.001$; Fig. 2a). We found no significant differences due to rotation in the proportions of field moist aggregates. We found the greatest mega-aggregate stability in SWC1 compared to C1, SC and Cm ($P = 0.032$; Fig. 2a). Mega-aggregate stability was significantly correlated with the CDI ($r = 0.63$; $P = 0.001$), SOC ($r = 0.62$; $P < 0.001$), TN ($r = 0.68$; $P < 0.001$) and fungal abundance ($r = 0.59$; $P = 0.002$). Soil bulk density did not vary across rotations (Fig. S3).

SOC in bulk soils ($P = 0.01$) and sand free bulk soils ($P = 0.07$) was greater in the high diversity rotations (Fig. 2b). Concentrations of SOC and TN, after correcting for sand content, did not differ across rotations in mega-aggre-

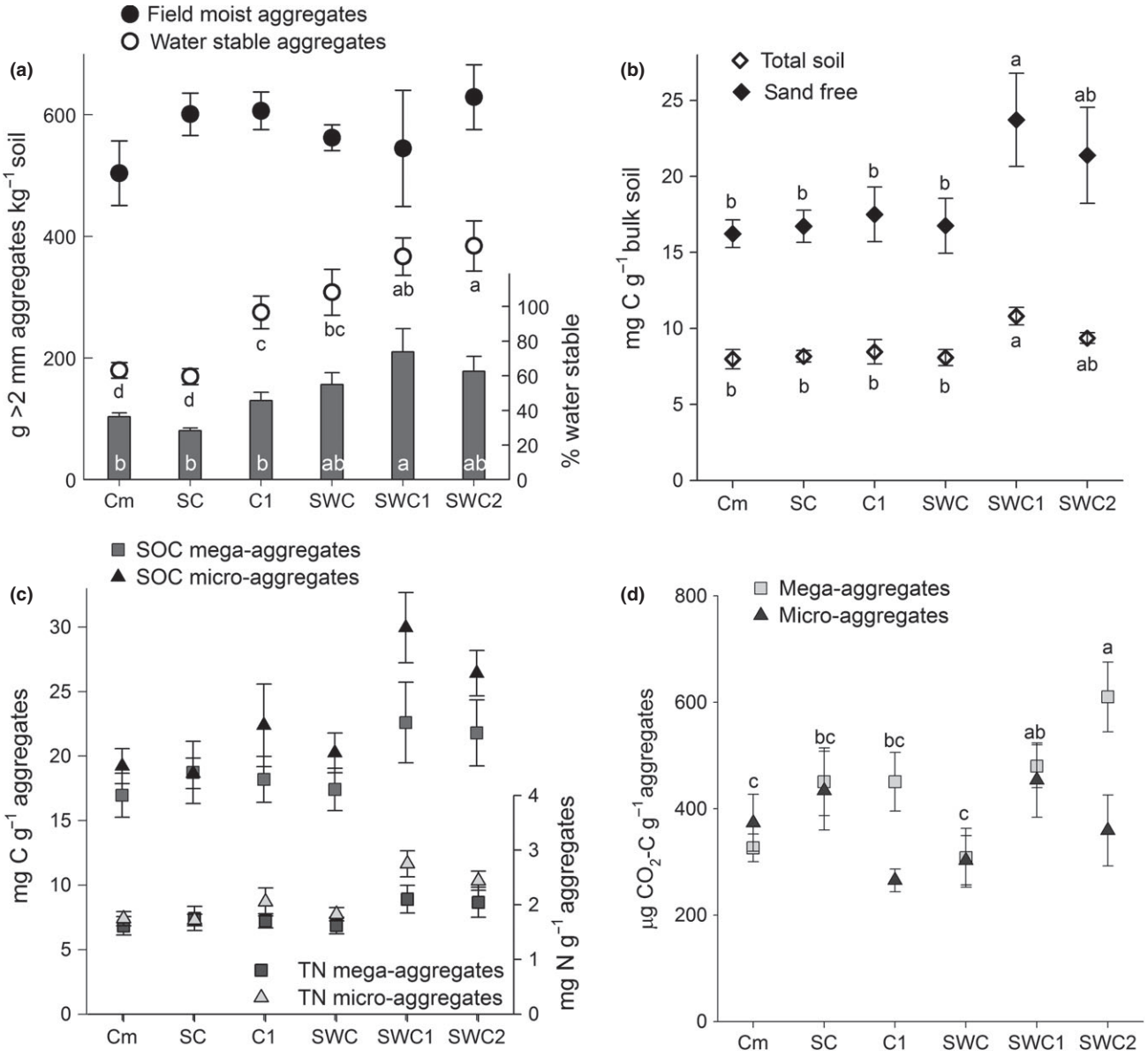


Figure 2 Impacts of crop rotational diversity on (a) aggregation (circles) and aggregate stability (bars, which represent percentage of > 2 mm field moist aggregates that were water stable); (b) bulk soil C (total soil and sand-corrected soil); (c) concentrations of soil organic carbon (SOC) and total N (TN) in mega- and micro-aggregates; and (d) potentially mineralisable carbon in mega- and micro-aggregates. Points and bars represent means ± SE ($n = 4$) and letters indicate significant differences ($P < 0.05$) between rotations. Treatment effects on SOC and TN are presented in Table 1; otherwise an absence of letters indicates no significant treatment effect.

Table 1 One-way ANOVA analyses for the effects of crop diversity on soil C and N pools and microbial activities by aggregate size fraction

	Mega-aggregates (> 2 mm)		Macro-aggregates (0.25–2 mm)		Micro-aggregates (0.053–0.25 mm)	
	Mean ± SD	Treatment effects	Mean ± SD	Treatment effects	Mean ± SD	Treatment effects
SOC (mg C g^{-1} sand free soil)	19.3 ± 4.6		19.6 ± 4.5	C, C1, SC < SWC1, SWC2*	22.5 ± 5.4	C, SC, SWC < SWC1, SWC2; C1 < SWC1*
Soil N (mg N g^{-1} sand free soil)	1.8 ± 0.4		1.8 ± 0.4	C, SC < SWC1, SWC2*; SWC<SWC1**	2.1 ± 0.5	C, SC<SWC1, SWC2; SWC < SWC1*
POXC (μg C g^{-1} soil)	0.35 ± 0.1		0.36 ± 0.1	C, SC, C1, SWC < SWC1, SWC2**	0.64 ± 0.2	C, C1, SC, SWC SWC1 < SWC2*
Total C respired (mg C g^{-1} soil)	440.6 ± 140.2	C, C1, SC SWC < SWC2; C, SWC < SWC1**	407.6 ± 106.3		362.6 ± 107.2	
Labile C acquisition EEA (nmol activity g^{-1} soil h^{-1})	101.5 ± 32.4		79.3 ± 16.9		134.2 ± 20.8	
N acquisition EEA (nmol activity g^{-1} soil h^{-1})	44.5 ± 23.9	C< C1, SWC2 SC < SWC2*	29.4 ± 8.9		47.4 ± 9.5	
P acquisition EEA (nmol activity g^{-1} soil h^{-1})	116.8 ± 22.1	C < SWC2*	112.5 ± 15.6	SC, SWC2 < C1*	146.7 ± 13.9	
Recalcitrant C acquisition (nmol activity g^{-1} soil h^{-1})	1087.5 ± 416.3		753.8 ± 257.6	SWC2, SWC1, SWC < C1, SC, C*	709.8 ± 249.7	

Presented are means ± SDs across treatments for each aggregate size class and results of *post hoc* analyses of crop rotation treatment effects. See text and Fig. 1 for definition of crop rotation abbreviations.
Significance of treatment effect: *$P \leq 0.05$; **$P = 0.001$ to 0.01.

gates, but increased in macro- and micro-aggregates with increasing rotational diversity (Table 1; Fig. 2c). Increases in both SOC and TN were correlated with increasing crop diversity in all aggregate size classes (Table S2). In the macro- and micro-aggregates, POXC was greatest in the most diverse rotations (Table 1). Rotation effects on potentially mineralisable C were dependent on respiration rates early in the incubation; we found significant rotation effects in all aggregate sizes only days 3–6 of the incubation. Specifically, the mega-aggregates on days 3–6 (rotation × day $P < 0.0001$) and micro-aggregates day 6 (rotation × day $P < 0.003$) had greater respiration rates in high diversity rotations. Respiration rates integrated over the 56 days indicated more C respired from higher diversity rotations from mega-aggregates and marginally more from macro-aggregates (Fig. 2d; Table 1).

SOM chemistry differed by rotation and among the aggregate size classes (Table S3). In particular, the concentration of lignin relative to other compound classes was greatest in micro- compared to mega-aggregates ($P = 0.034$) and in the most diverse rotation, SWC2, compared to Cm ($P = 0.054$). In addition, we observed greater abundance of proteins ($P = 0.036$) and N-bearing ($P = 0.080$) compounds in the smallest sized aggregates compared to mega-aggregates.

Microbial community function and structure

Statistically significant differences in EEA were difficult to detect due to high variability, however, we did find generally greater N-acquisition and lower recalcitrant C acquisition EEA in the higher diversity rotations in the mega- and macro-aggregates (Table 1; Fig. 3a). In addition, we found significant and positive correlations between the CDI and labile C, N and P EEA ($r = 0.54$, 0.54 and 0.57, respectively; $P < 0.004$) and a negative correlation between the CDI and recalcitrant C EEA ($r = -0.57$, $P = 0.004$) in the mega-aggregates. Furthermore, we found a decrease in the ratio of C : N acquisition EEA as crop rotation diversity increased in the mega- and micro-aggregates (Fig. 3b; Table 2) with a strong negative correlation between C : N EEA and the CDI ($r = -0.75$; $P < 0.001$). The ratio of labile to recalcitrant C acquisition EEA was marginally greater in mega-aggregates and macro-aggregates from SWC2 compared to SC and Cm (Fig. 3c; Table 2). Finally, across all levels of crop diversity, we found labile C, N and P EEA to be greatest in the micro-aggregates (all $P < 0.001$) and recalcitrant C EEA to be greatest in the mega-aggregates ($P < 0.001$).

Microbial PLFA biomass varied by rotation in mega- and macro-aggregates, but these differences were not related to increasing crop diversity (Table 2). Across rotations, total microbial PLFA biomass was greatest in the micro-aggregates ($P = 0.001$). In micro-aggregates, bacterial biomass was greater in high diversity (SWC2, SWC1, SWC) compared to low diversity (SC, C1, Cm) rotations and was positively correlated with our CDI (Table S2; $r = 0.71$, $P < 0.001$). The fungi : bacteria biomass ratio varied by rotation in the micro-aggregates (Table 2), with lower ratios found in higher diversity rotations, and a significant negative correlation with the diversity index ($r = -0.64$, $P = 0.001$). When we separated the

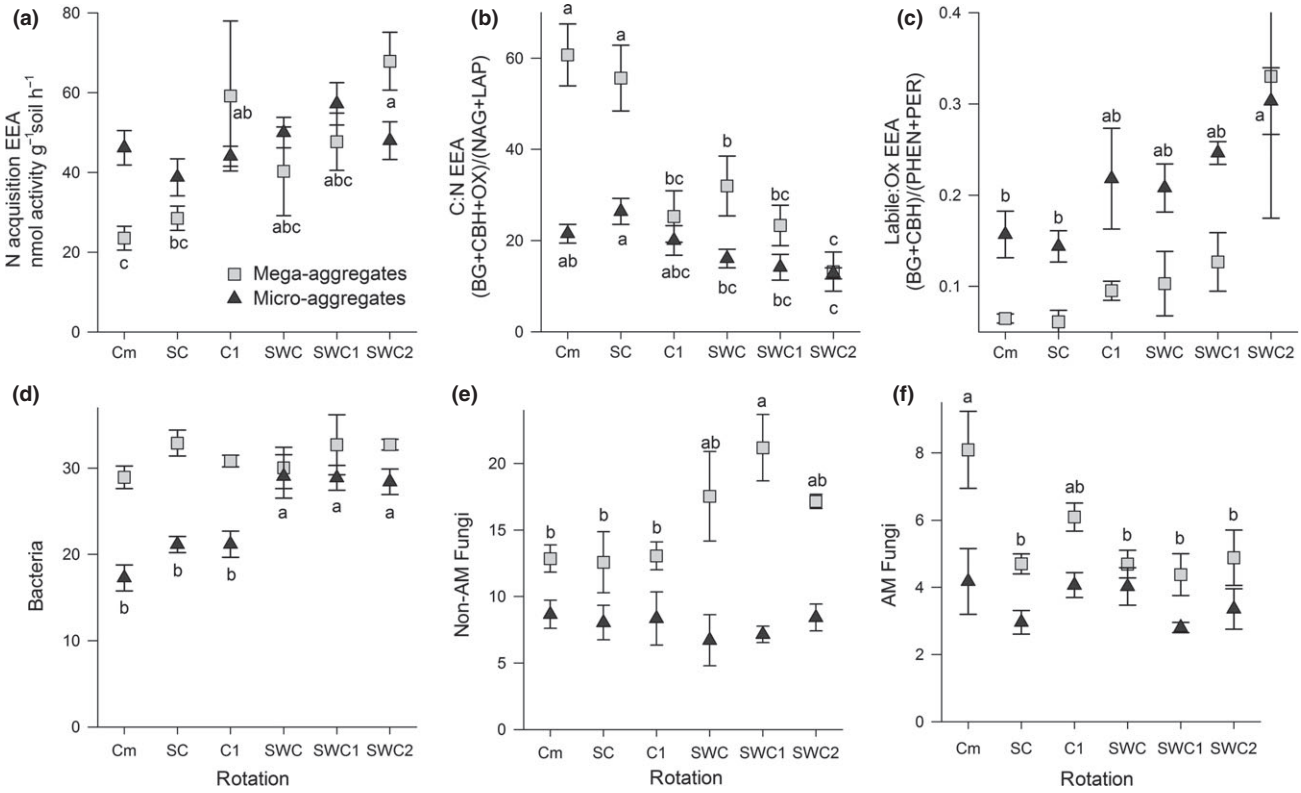


Figure 3 Impacts of crop rotational diversity on (a) activities of nitrogen acquiring enzymes; (b) ratio of carbon/nitrogen acquiring enzymes; (c) ratio of enzymes responsible for labile/recalcitrant SOC breakdown; relative abundance of phospholipid fatty acids (PLFA) associated with (d) bacteria; (e) non-AMF fungi and; (f) arbuscular mycorrhizal fungi (AMF) in mega- or micro-aggregates (μmols PLFA g^{-1} aggregates). Points and bars represent means ± SE ($n = 4$) and letters indicate significant differences ($P < 0.05$) between rotations. An absence of letters indicates no significant treatment effects.

relative abundance of PLFA markers into microbial groups, we found several significant crop rotation effects depending on the aggregate size class (Table 2; Fig. 3 & Fig. S2). In mega-aggregates we saw almost double arbuscular mycorrhizal fungi (AMF) abundance in the Cm compared to all other rotations (except C1). Excluding AMF markers, we found higher fungal abundance in SWC1 compared to SC, C1 and Cm (Table 2; Fig 3e, f). In micro-aggregates we saw higher relative abundance of Gram-positive bacteria and actinomycetes in higher diversity rotations (Table 2; Fig. S3a, c). These results were supported by significant positive correlations between the CDI and micro-aggregate actinomycetes ($r = 0.74$, $P < 0.001$) and Gram-positive bacteria ($r = 0.59$, $P = 0.003$; Table S2). In the mega-aggregates, microbial resource limitation (indicated by the ratio of total saturated to total monounsaturated fatty acids) was higher in SC compared to all other rotations (Table S3), and decreased significantly as the CDI increased ($r = 0.44$; $P = 0.032$).

Microbial functional diversity (H' EEA) was greatest in SWC2 compared to all other rotations in mega-aggregates, while in micro-aggregates, H' EEA was greater in SWC2 and SWC1 compared to Cm and SC (Table 2). PLFA diversity (H' PLFA) in micro-aggregates was greater in SWC2 and SWC1 compared to C1, SC and Cm (Table 2). Both H' EEA and H' PLFA were positively correlated with the CDI in all aggregate size classes (Fig. S4). We also found significant correlations between EEA diversity and soil C and N in the micro-aggregates ($r = 0.43$, $P = 0.043$ and $r = 0.44$, $P = 0.037$, respectively). The PLFA diversity index was positively correlated with soil C and N in the mega- ($r = 0.42$, $P = 0.041$; $r = 0.45$, $P = 0.027$) and micro-aggregates ($r = 0.52$, $P = 0.010$; $r = 0.57$, $P = 0.005$).

DISCUSSION

Modern temperate agricultural systems are characterised by their simplistic plant communities: two-crop rotations are typical and monocultures remain common. While it is broadly accepted that biodiversity influences ecosystem function, relationships between plant diversity and soil ecosystems remain contentious (Kowalchuk *et al.* 2002; Zak *et al.* 2003; Milcu *et al.* 2013), and in agroecosystems, research on plant diversity effects have focused primarily on aboveground insect and disease dynamics (Altieri 1999). Meta-analyses of belowground crop rotation effects show increases in soil fertility factors, such as soil C and N and microbial biomass (West & Post 2002; McDaniel *et al.* 2014b), but these effects cannot be separated from the influence of other land management factors, such as the application of external fertilisers and pesticides. For the first time, we present a comprehensive look at the belowground effects of rotational diversity, in isolation. With increased rotational diversity, we observed a 33% increase in sand-corrected soil C compared to monocultures at this site, which is considerably higher than the average

Table 2 One-way ANOVA analyses for the effects of crop diversity on ratios of enzyme activities, the relative abundance of microbial groups determined via PLFA, and microbial diversity measures by aggregate size fraction

	Mega-aggregates (> 2 mm)		Macro-aggregates (0.25–2 mm)		Micro-aggregates (0.053–0.25 mm)	
	Mean ± SD	Treatment effects	Mean ± SD	Treatment effects	Mean ± SD	Treatment effects
Labile : Oxidative C EEA ratio	0.13 ± 0.15	C, SC < SWC2†	0.12 ± 0.05	SC < SWC2†	0.21 ± 0.08	C, SC < SWC2*
C : N EEA ratio	35.0 ± 20.6	SWC2 < SWC, SC, C SWC1, C1, SWC < SC, C***	29.7 ± 12.7		18.5 ± 6.5	SWC2 < C, SC, SWC1, SWC < SC**
All fungi PLFA	21.2 ± 4.7		25.1 ± 4.8		11.5 ± 2.8	
All bacteria PLFA	31.4 ± 3.8		29.2 ± 4.3		24.3 ± 5.6	C, C1, SC < SWC, SWC1, SWC2***
Fungi : Bacteria PLFA ratio	0.69 ± 0.21		0.88 ± 0.24		0.50 ± 0.17	All < C SWC, SWC1, SWC2 < C1 SWC, SWC1 < SC***
Actinomycetes	3.4 ± 1.1		3.1 ± 0.7		2.3 ± 1.3	C, C1, SC, SWC < SWC2, SWC1**
Gram-positive bacteria	5.2 ± 1.2		5.2 ± 1.0	All < SWC1**	4.1 ± 1.0	C, C1 < SWC, SWC1, SWC2 SC < SWC1, SWC2**
Gram-negative bacteria	14.6 ± 2.1		13.4 ± 2.1		11.0 ± 2.2	
AMF	5.5 ± 1.8	SC, SWC, SWC1, SWC2 < C**	4.9 ± 2.1		3.6 ± 1.2	
Non-AMF	15.7 ± 4.9	C, C1, SC < SWC1*	20.2 ± 4.9		7.9 ± 2.6	
Microbial PLFA Biomass (μmol g^{-1} soil)	0.12 ± 0.04	SC, SWC1 < SWC2, SWC, C1 SC < C**	0.11 ± 0.03	SWC1 < All SC < C1**	0.16 ± 0.07	
H' EEA	0.84 ± 0.25	All < SWC2**	0.87 ± 0.10		1.12 ± 0.10	SC, C < SWC1, SWC2**
H' PLFA	1.60 ± 0.11		1.59 ± 0.11		1.19 ± 0.18	C < SWC, SWC1, SWC2 SC, C1 < SWC1, SWC2**

Presented are means ± SDs across treatments for each aggregate size class and results of *post hoc* analyses of crop rotation treatment effects. See text and Fig. 1 for definition of crop rotation abbreviations.
Significance of treatment effect: *$P \leq 0.05$; **$P = 0.001$ to 0.01; ***$P < 0.001$; marginal $^{\dagger}P < 0.10$.

3.6% gains reported by McDaniel *et al*. (2014b). The use of this novel experimental system may have exaggerated the positive effects of rotational diversity because only rotations with legumes received N-inputs. However, this experimental design has provided insight into the more subtle effects of rotational diversity than have been previously observed in real-world cropping systems (West & Post 2002; McDaniel *et al*. 2014b) even though we cannot directly assess diversity vs. compositional effects *per se*. Furthermore, these results are directly applicable to systems where rotations are particularly important, such as low input and other soil management systems, including organic agriculture, that are striving to optimise internal nutrient provisioning. The data we present support our hypotheses that increasing rotational diversity fundamentally changes microbial community structure and activity, with positive effects on aggregate formation and SOM accrual (Fig. 4), and supports the use of rotational diversity as a viable management practice for promoting soil sustainability.

Irrespective of crop rotation effects, by separating the soils into different aggregate size fractions, we isolated SOM pools with different degrees of physical and chemical accessibility. Relationships between microbial communities and SOM pools suggest that the chemical composition of SOM in aggregates is related to microbial composition and activity (Smith *et al*. 2014). SOM associated with mega-aggregates is generally easier to access by decomposers and relatively unprocessed because it is composed of more recent inputs (Lützow *et al*. 2006; Jastrow *et al*. 2007). In contrast, SOM associated with micro-aggregates is generally older, more microbially processed and primarily mineral bound, and therefore relatively inaccessible. SOM chemistry data obtained via pyrolysis-GC/MS are consistent with these expectations: we observed the greatest abundance of lignin derivatives and N-bearing compounds in the micro-aggregates. The protein and N-bearing compounds found associated with micro-aggregates are likely derived from microbial decomposition and bound to mineral surfaces (Grandy & Neff 2008). With these general differences in SOM accessibility in mind, we examine how crop biodiversity affected SOM through changes in soil aggregation and microbial community structure.

Soil management practices designed to increase soil biological activity and C concentrations increase the stability of mega-aggregates, which at this study site have the fastest turnover rates and are the most susceptible to changes in management (Grandy & Robertson 2007; Tiemann & Grandy 2015). As crop diversity increased, we found increases in the stability of mega-aggregates, with indications of increasing SOC and TN concentrations. Based on enzyme activity levels and SOC mineralisation rates, the increases in crop diversity appear to have stimulated microbial activity in the mega-aggregates. This increase in microbial activity is likely responsible for the increase in aggregate stability through the production of soil binding agents, such as fungal hyphae, glomalin and polysaccharides (Bossuyt *et al*. 2001). Coupled with the observation that fungi (excluding AMF, which were on average 26% of total fungal biomass) were more abundant in the most diverse

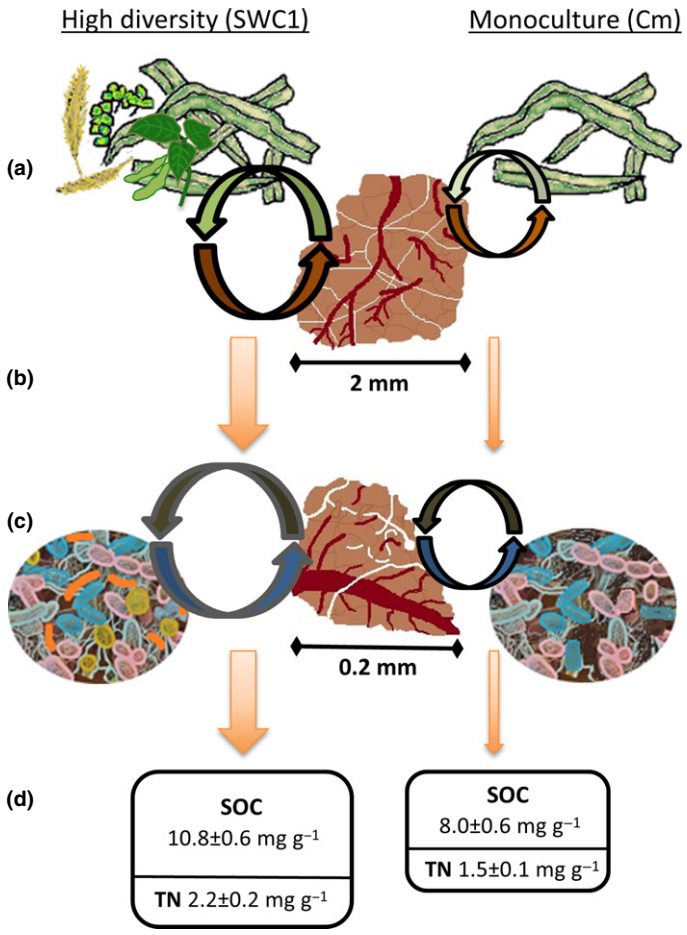


Figure 4 Trajectory of aggregate and SOM formation and stabilization under a high diversity rotation (e.g. SWC2) versus monoculture crop (e.g. Cm). (a) Greater quantity and quality of residues entering soils in high diversity rotations enhances microbial activity with positive impacts on rates and extent of (b) mega-aggregate formation and stabilization. This also leads to (c) enhanced microbial activity in high diversity micro-aggregates and concomitant increases in microbial by-products that accelerates micro-aggregate formation, resulting in (d) increasing stocks of stable SOC and TN.

rotations, a positive correlation between aggregate stability and fungal abundance ($r = 0.59$; $P = 0.002$) is further evidence for a strong link between crop rotational diversity, microbial processes and soil aggregation. The positive correlation between fungal abundance and SOC or TN in the mega-aggregates further supports fungal contributions to soil structural stability and resulting increases in soil C and N stocks (Fig. 4).

While differences in mega-aggregate stability are linked to changes in the microbial community, changes in microbial community structure are likely the result of differences in the quality and quantity of available resources (Fig. 4a). The most recent and higher quality resources reside primarily in the mega-aggregates, which is the first level of protection for newly forming SOM (Jastrow *et al.* 2007). Thus, microbial resource demands in the largest aggregate size classes can forecast how changes in management alter C and N availability and the potential for SOC and TN accumulation. Differences in the ratio of total saturated/total monounsaturated fatty acids in the mega-aggregates suggest increased resource limitation with lower crop diversity. We also found lower relative abundance of lignin and higher lignin-degrading enzyme activities in the monoculture mega-aggregates (Fig. 2c). This finding also suggests microbial C resources were lower in the monoculture. When resource availability is high, plant compounds like lignin are selectively preserved during early stages of decomposition due to high energy costs associated with the production of lignin-degrading enzymes (Ekschmitt *et al.* 2005; Lützow *et al.* 2006; Grandy & Neff 2008).

Microbial enzyme activities are also good indicators of resource demands because their production increases in response to resource limitation (Allison & Vitousek 2005; Sinsabaugh *et al.* 2014). For example, as N demand increases we would expect to see an increase in the production of N-acquiring enzymes. In the current study, we observed increases in both labile C and N-acquiring extracellular enzymes within all aggregate classes; therefore we used enzyme ratios to provide a measure of relative C vs. N limitation among soil microbes (Sinsabaugh *et al.* 2009). Here, the ratio of C : N EEA narrowed as crop diversity increased (Fig. 2b), indicating that either N limitation increased or, more likely, that C limitation decreased with crop diversity. The latter explanation is more likely because we also observed greater POXC and C mineralisation rates early in the soil incubation in high diversity rotations, indicating there is more labile C in those soils. Additionally, the labile to recalcitrant C acquisition ratio (Fig. 2c) increased with crop diversity, suggesting microbes in more diverse rotations are less C limited. A previous study with soils from this site (McDaniel *et al.* 2014a), showed greater decomposition of poor quality (C : N > 38) crop residues and greater N mineralisation in the more diverse crop rotations. This implies that the larger labile SOM pool in more diverse crop rotations is also capable of supplying more N in times of high N demand. The larger labile SOM pool is likely due to increased quantity, quality and diversity of inputs from crops in the high diversity rotations over the 12 years of this experiment. Furthermore, these apparent differences in resource limitations among low and high diversity rotations could provide a mechanism for the observed changes in microbial community dynamics.

Effects of crop diversity on microbial activities in micro-aggregates were similar to those observed in mega-aggregates. This is surprising as both SOM composition and observed trends in microbial community structure with increasing crop diversity differed among aggregate size classes. For example, in micro-aggregates there was an increase in bacterial, rather than fungal, abundance with increasing crop diversity. Additionally, micro-aggregate SOC appeared to be relatively more inaccessible as measured by less potentially mineralisable C. Inconsistent with this, we observed greater microbial biomass, concentrations of POXC and enzyme activities in the micro-aggregates compared to the mega-aggregates. The enzyme assay is known to activate residual mineral-bound enzymes (Wallenstein & Weintraub 2008) although observed results may also represent increased microbial investment in enzymes in response to more severe resource limitations. In fact, there was a higher ratio of total saturated/total monounsaturated fatty acids in micro- compared to mega-aggregates to support

the idea of greater resource limitation in the micro-aggregates. Even so, EEA indicated relatively high rates of microbial activity in micro-aggregates, particularly with high crop diversity.

While we observed no differences in microbial PLFA biomass among treatments, higher enzyme activities and faster decomposition of labile residues suggest that crop diversification accelerated microbial metabolism in micro- and macro-aggregates (Schimel & Weintraub 2003). Given that microbial by-products are thought to be the primary components of stable SOM, increasing microbial crop residue processing, growth rates and biomass turnover may be driving SOM increases (Fig. 4; Grandy & Neff 2008; Miltner *et al*. 2012). This is particularly true in the micro-aggregates where microbial by-products and necromass from dead cells are more likely to be in close association with mineral surfaces. This mechanism of SOM accrual could account for the link we observed between high microbial activity and SOM accrual in the micro-aggregates in this study. Results from a meta-analysis of crop rotation effects on belowground processes show that increasing crop diversity can have a large and rapid, positive effect on soil microbial biomass (McDaniel *et al*. 2014b). Management strategies that increase microbial biomass and/or activity seem to be the most promising for increasing agricultural SOM stocks and soil fertility (Kallenbach & Grandy 2011; McDaniel *et al*. 2014b).

Cover crops are a common form of rotation diversification, but their effects on soils may be due to both an increase in diversity *per se*, and to their unique management (e.g. grown during a time of year when soils are otherwise bare for the explicit purpose of nourishing the soil). Additionally, legumes, which are common cover crops, have been identified as key species driving overall diversity effects (Fornara & Tilman 2008) and may have a disproportionate effect in the current study, making it difficult to separate crop diversity vs. composition effects. Thus, legume effects may be underlying some of the enhanced microbial activity and SOM accrual with enhanced rotational diversity we observed in this study. Leguminous cover crops are consistently linked to increases in N availability, aggregate formation and aggregate stability (Sainju *et al*. 2003; Liu *et al*. 2005; McDaniel *et al*. 2014b), leaving little doubt that leguminous covers have positive belowground effects. Nonetheless, the effectiveness of a legume, with regards to building SOM, may not be due solely to its functional capacity as a legume (i.e. a compositional effect). There is further evidence for rotational diversity effects above and beyond the compositional effect in the current study and in a meta-analysis, where legume impacts were greater with higher rotational diversity. Across 80 fields, rotations of two crops (one of which was a legume cover crop) had lower SOC gains (8.4%) vs. rotations with more than two crops (with at least one a legume cover crop; 13.9%) compared to monocultures (McDaniel *et al*. 2014b; and unpublished data). In the current study, rotations with a leguminous cover had mixed effects on fungal abundance, aggregate stability and SOC. For example, the soy-wheat-corn rotation that included a leguminous cover (SWC1) had greater aggregate stability and greater SOC than corn with a leguminous cover (C1), even though there appeared to be greater legume biomass in C1 plots (unpublished data). Also, we observed equal amounts of plant biomass inputs into the SWC2, SWC1 and C1 plots (Fig. S1), but the SOC gains were greater in SWC2 and SWC1 than in C1 plots. Furthermore, there was no difference in aggregate stability or SOC between the corn with (C1) or without (Cm) the leguminous cover, suggesting that the diversity of the rotation sequence, or the specific combination of crops in rotation, can be as important as the inclusion of leguminous cover crops. In other words, there are diversity effects beyond just the compositional effects of adding a legume or cover crop to the system. The observed positive and *linear* relationships between CDI and measures of microbial community dynamics (Fig. S4) are additional evidence supporting the importance of plant diversity beyond that of one functional group, such as legumes, and provide evidence for a strong link between plant diversity and soil functioning in agricultural systems where, unlike unmanaged systems, rotational diversity is primarily temporal rather than spatial.

For centuries humanity has observed the aboveground benefits of crop diversity via increased yields, yet we are only beginning to understand the extent of the belowground benefits (Van der Putten *et al*. 2009). In this experimental study, we provide insight into the mechanisms controlling soil system responses to crop diversity. As crop diversity increased through multiple species rotations, we observed significant gains in SOC and TN that were driven by changes in soil and microbial community structure (Fig. 4). The greatest concentrations and most significant gains in SOC and TN occurred in the micro-aggregates, suggesting that these gains are long-lived, because micro-aggregate associated organic matter tends to be relatively more stable (Six *et al*. 2004; Jastrow *et al*. 2007). The positive effects on TN accrual and storage are particularly important in the context of low-input agricultural systems where N amendments are especially limited. With these insights, increasing crop rotational diversity should be considered an important management strategy in the context of soil sustainability and food security.

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AUTHORSHIP

L.K.T., A.S.G. and E.M-S designed the study, M.D.M. and E.E.A. helped with field and laboratory work during implementation of the study. L.K.T. wrote the manuscript and all other authors contributed to revisions.

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