Microbial physiology and necromass regulate agricultural soil carbon accumulation

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Abstract

Strategies for mitigating soil carbon (SOC) losses in intensively managed agricultural systems typically draw from traditional concepts of soil organic matter formation, and thus emphasize increasing C inputs, especially from slowly decomposing crop residues, and reducing soil disturbance. However these approaches are often ineffective and do not adequately reflect current views of SOC cycling, which stress the important contributions of microbial biomass (MB) inputs to SOC. We examined microbial physiology as an alternate mechanism of SOC accumulation under organic (ORG) compared to conventional (CT) agricultural management practices, where ORG is accumulating C despite fewer total C inputs and greater soil tillage. We hypothesized that microbial communities in ORG have higher growth rates (MGR) and C use efficiencies (CUE) and that this relates to greater MB production and ultimately higher retention of new C inputs. We show that ORG had 50% higher CUE (±8 se) and 56% higher MGR (± 22 se) relative to CT ($p < 0.05$). From *in situ* ^{13}C substrate additions, we show that higher CUE and MGR are associated with greater rates and amounts of ^{13}C glucose and phenol assimilation into MBC and mineral-associated SOC pools in ORG up to 6 mo after field substrate additions ($p < 0.05$). ORG soils were also enriched in proteins and lipids and had lower abundances of aromatic compounds and plant lipids ($p < 0.05$). These results illustrate a new mechanism for SOC accumulation under reduced C inputs and intensive soil disturbance and demonstrate that agricultural systems that facilitate the transformation of plant C into MB may be an effective, often overlooked strategy for building SOC in agricultural soils.

1. Introduction

Intensively managed agricultural systems deplete soil organic carbon (SOC), leading to potentially severe land degradation (Rajan et al., 2010), declining soil fertility (Johnston et al., 2009), and increased CO_2 emissions (Paustian et al., 1998). Traditional soil C concepts and models suggest that these trends can be reversed by increasing C inputs through additions of slowly decomposing crop residues, or by reducing C outputs by minimizing soil disturbance to slow decomposition losses (Janzen, 2006; Robertson and Grandy, 2006). Yet, in several long-term agricultural experiments, SOC accumulation rates are paradoxically higher under relatively lower C inputs and more intensive tillage (Drinkwater et al., 1998; Gregorich et al., 2001; Marriott and Wander, 2006). Indeed, enhancing C inputs, increasing the proportion of chemically recalcitrant residue inputs, and reducing soil disturbance have all had inconsistent effects on SOC (Leifeld and Fuhrer, 2010; Pittelkow et al., 2014). These inconsistencies in SOC response to traditional soil C management practices might reflect an inadequate representation of our contemporary understanding of soil microbial controls over SOC dynamics. In contrast to traditional SOC concepts and models, emerging experimental and theoretical evidence show that dead microbial biomass (MB) (i.e. necromass) is a significant fraction of soil organic matter (SOM) (Frey et al., 1999; Grandy and Neff, 2008; Schmidt et al., 2011; Cotrufo et al., 2013; Wieder et al., 2014). If true, microbial physiological processes that regulate MB production and turnover should be strongly related to SOC accumulation (Bradford et al., 2013), and potentially under the control of management practices not exclusive to input rates and chemical recalcitrance.

The continuous and rapid turnover of living MB can produce, over time, a considerable amount of necromass (Liang et al., 2011), now considered a key constituent of stable SOM

(Simpson et al., 2007; Kindler et al., 2009; Miltner et al., 2011; Schmidt et al., 2011; Cotrufo et al., 2013; Gleixner 2013). Although MB can be rapidly mineralized by soil organisms due to its favorable energy yield and low C:Nitrogen (C:N) ratio (Blagodatsky et al., 2000), microbial necromass and other microbial byproducts can also be selectively preserved via interactions with soil minerals and incorporation into soil aggregates (von Lutzow et al., 2006; Heckman et al., 2013; Throckmorton et al., 2014). As a result, stable SOC pools in many soils appear to be enriched in compounds abundant in MB (e.g. lipids and proteins), but not in decomposing plant tissue (Grandy and Neff, 2008; Bol et al., 2009; Heckman et al., 2013; c.f. Kramer et al., 2012). Given microbial necromass contributions to SOC, changes to microbial growth characteristics that affect the size, turnover rate, or biomass yield of the community may influence stable SOC accumulation via changes in the amount of MB available for stabilization (Cotrufo et al., 2013).

At the most basic level, the production of MB is governed largely by microbial physiological traits such as microbial C use efficiency (CUE) and microbial growth rate (MGR), both of which affect the rate and efficiency at which plant C is used to build MB. Microbial CUE represents the proportion of new biomass produced from substrate-C consumed, and MGR characterizes the rate of bacterial cell duplication or fungal hyphal extension over time. Both have direct influences on net MB production (Russell and Cook, 1995) and thus potentially on the rates of SOC accumulation (Bradford et al., 2013). In soils, CUE estimates range from 0.10 to 0.80 (Manzoni et al., 2012), while MGR varies from hours to weeks (Rousk and Bååth, 2011). This variability in both CUE and MGR arises from differences in the intrinsic physiology of specific bacterial and fungal species (Dethlefsen and Schmidt, 2007; Molenaar et al., 2009; Beardmore et al., 2011) and also microbial community sensitivity to the soil environment, i.e. soil temperature and resource quality (Thiet et al., 2006; Frey et al., 2013; Lee and Schmidt,

2014). At the individual species-level, variability in CUE and MGR are manifested based on differences in life history traits (Russell and Cook, 1995; Beardmore et al., 2011; Resat et al., 2012). Copiotrophic microbes, selected under resource-rich environments, are typically characterized by rapid growth rates but lower CUE relative to slower-growing oligotrophs (e.g. some fungi, actinomycetes) with a higher CUE (Fierer et al., 2007; Maharjan et al., 2007; Lipson et al., 2008; Beardmore et al., 2011). At the community-level, the manifestation of these traits will then depend on the diversity and relative abundance of active microbial species present in the community.

Even without differences in the active microbial community composition between soils, CUE and MGR can also vary depending on the soil environment. For example, assuming no changes to microbial community composition, both CUE and MGR typically increase within a microbe as resource concentrations (Eichinger et al., 2010; Roller and Schmidt, 2015) and resource quality (e.g. available free energy, or C:N) (Sinsabaugh et al., 2013) increase. An individual microbe that consumes a resource with more available free energy (*e.g.* low oxidation state) and nutrients (*i.e.* lower C:N) has more resource-C remaining for biomass synthesis after meeting their energy requirements (Gommers et al., 1988; Chiellini et al., 2007) and will lose less C to spill-over metabolism and enzyme production when nutrient limitations are minimized (Russell and Cook, 1995; Sinsabaugh et al., 2013). As such, when community-level life history traits are unchanging, labile inputs with more available energy and lower C:N ratios may theoretically encourage both a higher CUE and MGR (Eiler et al., 2003; Manzoni et al., 2012).

Agricultural management strategies that increase CUE and MGR and subsequently facilitate a faster and more efficient conversion of plant inputs to MB, may enhance stable SOC accumulation. Cover crops, which alter the overall quality and quantity of soil resource inputs,

may generate this response. Often possessing low lignin concentrations and C:N ratios, cover crops should promote microbial growth and high CUE, leading to increased soil C retention. This could explain frequently reported increases in SOC with cover crops (Kallenbach et al., 2010; McDaniel et al., 2013; Tieman et al., 2015). Examples include long-term cover-cropped agroecosystems in Pennsylvania (Drinkwater et al., 1998) and Michigan (Grandy and Robertson, 2007; Syswerda et al., 2011), where reported soil C accumulation rates were 46 g C m^{-2} y^{-1} and 50 g C m^{-2} y^{-1} despite intensive tillage and relatively fewer total soil C inputs due to lower annual net primary productivity. Here, we examine if differences in microbial physiology could be a potential mechanism for greater SOC accumulation rates under organic, cover crop management where C inputs are reduced and soil disturbance is more intense. We hypothesize that 1) distinct agricultural practices are associated with different microbial physiological growth traits; 2) cropping systems with greater CUE and/or MGR will convert relatively more new C inputs into stable microbial-derived SOC pools; and 3) management systems associated with higher CUE and MGR, and thus a potentially greater input of MB to stable SOC pools, will have a greater abundance of SOM compounds associated with MB (*i.e.* proteins and short-chain lipids).

2. Materials and Methods

2.1 Overview

We assessed differences in microbial physiology due to agricultural management (H_1) by comparing CUE and MGR across an organic and conventional cropping system over two cropping cycles. We then examined how observed differences in microbial physiology are linked with microbial allocation of new C inputs into various SOC pools using *in situ* field additions of isotopically labeled C substrates (H_2). Finally, biomarker and soil chemistry analyses were used

to relate differences in observed C allocation strategies to the abundances of SOC compounds associated with microbial-derived SOC (H_3).

2.2 Study site

We conducted our experiment at the W.K. Kellogg Biological Station (KBS) Long-Term Ecological Research Site in Hickory Corners, MI, established in 1988 (http://kbs.msu.edu). Following initial declines in SOC immediately after establishment, the organic cropping system at KBS has been accumulating SOC (Syswerda et al., 2011), while conventional management is maintaining or exhibiting slight losses in SOC (SI Figure 1; Table 1). This is despite greater tillage (for weed control) and slightly lower total C inputs under organic management compared to conventional management (Table 1). The site has a mean annual precipitation of 1027 mm y^{-1} and consists of fine–loamy Kalamazoo and coarse–loamy Oshtemo soils, both Typic Hapludalfs formed on glacial till (Robertson and Hamilton, 2015). Our field experiment and soil sampling were carried out in the organic (ORG) and conventional (CT) treatments, each with six 1-ha replicated plots arranged as a complete randomized block design. Both ORG and CT treatments are under a corn-soybean-winter wheat rotation. CT is managed based on typical conventional management practices of the region, including tillage and fertilizer application rates with adjustments made based on soil tests (Robertson and Hamilton, 2015). ORG receives no chemical inputs and has a fall-planted red clover (*Trifolium pretense*) cover crop following wheat and corn. ORG weed management is accomplished via mechanical cultivation. Neither ORG nor CT receives compost or manure.

2.3 Plot-level soil sampling

We collected soils from ORG and CT (n =6) four times between 2013 and 2014 to determine microbial carbon use efficiency (CUE), growth rate (MGR) and total SOC, and once

in 2014 for characterizing SOC molecular chemistry and soil lipid profiles. We began soil sampling on July 3rd, 2013 just prior to wheat harvest and when red clover was flowering in ORG (SI Table 1). Subsequent sampling occurred on Oct 2013, June 2014, and Sept 2014 coinciding with changes in above ground plant cover (SI Table 1). At each sampling time, 10 soil cores were collected randomly across each treatment plot using a 2.5-cm dia. soil probe to a 10-cm depth, combined in the field, and stored on ice until same-day transfer to a 4˚C refrigerator. All soils used for biological measurements were homogenized by wet sieving to 2 mm and stored at 4˚C for no longer than 3 days.

2.4 In situ ^{13}C isotope tracer experimental design

To assess how physiological traits correspond to differences in microbial allocation of new C into SOC pools, we established an isotope tracer experiment in both ORG and CT plots using ^{13}C-labeled glucose and phenol. These substrates represent typical plant C inputs from root exudates and crop residues (Jones et al., 2009) and differ in their available free energy. We used two different substrates to better understand the effects of substrate quality on potential interactions between substrate quality and management-induced differences in microbial growth. The ^{13}C-labled cores were then used to determine the amount of ^{13}C substrate recovered in microbial biomass C and different SOC pools over time (see below).

We initiated the isotope tracer experiment Sept 2013, approximately 1 mo after wheat harvest and just prior to mowing red clover in ORG (SI Table 1). In each of the 6 replicated blocks, 7 10-cm dia. PVC collars, open at the top and bottom, were inserted 7 cm into the soil until the top of the PVC was flush with the soil. Within each block, 3 collars received ^{13}C-labeled phenol, 3 received ^{13}C-labeled glucose, and 1 served as a control and only received water. In Sept 2013, we syringe-injected 15 ml of 99-atom% ^{13}C-glucose or ^{13}C-phenol mixed

with deionized water to a 5-cm depth into each core. Substrate solutions contained 3 mg C ml^{-1} and, after injection, amounted to ~3 µg C g^{-1} dry soil (<0.0001% of bulk soil C). One of the three cores for each substrate in each treatment plot were destructively sampled on each of three separate harvest dates, at 1, 6 and 12 mo after substrate additions. In June 2014, soil in all the cores was hand mixed to simulate spring tillage. At this time, 3.7 g of above and below ground clover biomass (~2:1 mass wt) was also mixed into ORG cores to parallel spring incorporation of clover biomass at the plot-level (SI Appendix 1).

2.5 Soil microbial physiology

We characterized CUE on soils collected at the plot-level by measuring the amount of a ^{13}C-labeled substrate incorporated into microbial biomass carbon (MBC) and respired as $^{13}CO_2$-C using previously described protocols (Brant et al., 2006; Frey et al., 2013). This approach estimates the amount of C allocated to biomass per unit of C resource consumed and represents a proxy for microbial CUE. Soils were amended in the lab with 50 µg C g^{-1} dry soil of 25 atom% labeled ^{13}C glutamic acid (<1% total soil C) and incubated for 22 hrs at 25˚C. The 22-hr incubation time was based on preliminary respiration curves to determine the time at which the majority of substrate had been utilized but before substrate recycling began. After incubation, soils were extracted for ^{13}C-glutamic acid incorporation into MBC (see below for MBC extraction) and a 12-ml CO_2 sample was collected for determining $^{13}CO_2$ respiration (SI Appendix 1). CUE was calculated as: [$MB^{13}C$/ ($MB^{13}C$+$^{13}CO_2$-C) *100], where $MB^{13}C$ and $^{13}CO_2$-C is the amount of substrate incorporated into MBC and the substrate C respired as CO_2. The amount of $^{13}CO_2$-C and $MB^{13}C$ from substrate was determined as: [µg C g $soil^{-1}$ *(δ_c-δ_s)/(δ_c-δ_L)], where µg C g^{-1} soil is the total pool of MBC or respired CO_2, δ_C is the $\delta^{13}C$ value of the

207 respired CO_2 or MBC from the control, δ_S is the $\delta^{13}C$ of respired CO_2 or MBC in the soils with
208 added substrate, and δ_L is the $\delta^{13}C$ of the labeled substrate.

209 For brevity, we refer to glutamic acid-CUE as simply CUE throughout the text but it
210 should be noted that this method does not consider the efficiency of other C sources in the soil.
211 Glutamic acid was selected for our CUE measurements since it is a common root exudate and
212 also requires little, if any, enzymatic breakdown for uptake (Moe, 2013). Thus, glutamic acid
213 CUE should better approximate C utilized for biomass growth relative to more complex
214 substrates (*i.e.* cellulose or organic acids). Substrate C not accounted for in the CO_2 or MBC
215 measurements was assumed to be remaining in the soil and likely included unutilized substrate C
216 and microbial excretions.

217 We measured MGR on the same soils as CUE within four days of field collection at the
218 plot-level, using a recently developed method (Aanderud and Lennon, 2011; Blazewicz and
219 Schwartz, 2011). This method is a proxy for MGR and follows microbial uptake of heavy water
220 (labeled $H_2{}^{18}O$) into microbial DNA. Cell doubling is linearly related to DNA production and
221 since microbes universally use water in DNA synthesis, all newly formed cells exposed to $H_2{}^{18}O$
222 during a given incubation period should contain ^{18}O labeled DNA (Aanderud and Lennon, 2011).

223 Twenty µl of 99-atom% universally-labeled $H_2{}^{18}O$ combined with 100 µl of unlabeled
224 deionized water was injected and mixed into 3 g dry wt field moist soil. This small volume of
225 water relative to native water content is not expected to induce microbial growth responses to
226 water additions. Soils were then incubated at 25˚C for 0, 24 and 48 hrs to determine the rate of
227 $H_2{}^{18}O$ incorporation into DNA. At 0, 24, and 48 hrs, soils were moved to a -80˚C freezer to halt
228 growth. DNA was extracted from frozen samples using the MoBio PowerSoil kit (MoBio Labs,
229 Inc., Carlsbad, CA) within a week of incubation and quantified fluorometrically (Qubit Systems,

Ontario, Canada). Extracted DNA (200 µl; 4-12 µg DNA μl^{-1}) was then mixed in silver capsules with 10 µg of salmon sperm DNA to meet molecular oxygen requirements for analysis. The ^{18}O of sample DNA ($\delta^{18}O_{sample}$) was quantified on a TC/EA (Thermo Scientific, Austin, TX) coupled with Thermo Finnigan Delta Plus XL IRMS. The ^{18}O in microbial DNA ($\delta^{18}O_{mDNA}$) was calculated as: $[(\delta^{18}O_{sample} - F_{SS} \times \delta^{18}O_{SS})/F_{MDNA}]$, where F_{ss} and F_{MDNA} are the mass fractions of salmon sperm and microbial DNA, respectively, and $\delta^{18}O_{ss}$ is the $\delta^{18}O$ of salmon sperm relative to an internal ^{18}O standard. MGR is expressed as the slope of $\delta^{18}O$ of microbial DNA at time 0, 24, and 48 hrs, where the greater the slope, the higher the estimated MGR.

2.6 Microbial community structure and metabolic activity

Phospholipid fatty acids (PLFAs) were extracted (Bligh, E.G. and Dyer, 1959; SI Appendix 1) from the ^{13}C-labeled soil PVC cores sampled in Oct 2013, 1 mo after ^{13}C substrate additions in the field. Methylated PLFAs were analyzed on a GC-FID (Agilent Technologies model 6890) and IRMS (PDZ Europa 20-20) and identified with the MIDI® Sherlock system (MIDI; Newark, DE, USA) (SI Appendix 1). PLFA biomarkers were assigned to six microbial groups: universal saturated PLFAs, gram positive bacteria (G+), gram negative bacteria (G-), actinomycetes, total fungi, and arbuscular mycorrhizal fungi (AMF) (SI Appendix 1). Bacterial biomass was estimated as the sum of G- and G+ and did not include universal saturated PLFAs. Only one fungal biomarker (18:1ω9c) was identified.

PLFA enrichment levels from ^{13}C-labeled substrates were used to indicate metabolically active microbial groups. The proportion of PLFA-C derived from either ^{13}C glucose or phenol (ng PLFA-^{13}C g $soil^{-1}$) was corrected for C introduced during transesterfication (SI Appendix 1) and calculated as: ng PLFA-C g $soil^{-1}$ $*(A\%_C - A\%_S)/(A\%_C - A\%_L)$, where $A\%_C$ is the atom

252 percent (AP) of the unlabeled control field cores, $A\%_S$ is the AP of the ^{13}C labeled cores and
253 $A\%_L$ is the AP of the substrate solution (97 A%).
254 *2.7 Soil chemistry and lipid profiling*
255 We determined soil organic C chemical structure using pyrolysis-gas
256 chromatography/mass spectrometry (py-GC/MS) following previously described protocols
257 (Grandy et al., 2009; Wickings et al., 2012) (SI Appendix) on dry soil samples collected at the
258 plot-level in June 2014. Organic matter constituents, expressed as the relative abundance of total
259 sample peak area were grouped as lipids, lignin derivatives, polysaccharides, proteins, non-
260 protein N-bearing, aromatics and phenolics.
261 We also quantitatively analyzed soils and plant tissue for solvent-extractable lipids (SI
262 Appendix 1) for a more comprehensive quantitative assessment of multiple lipid groups than is
263 otherwise provided by the PLFA or py-GC/MS analyses. Soil lipids were extracted from 15 g of
264 lyophilized soil, collected at the plot-level June 2014, with 2:1 dichloromethane/methanol using
265 an accelerated solvent extractor (Dionex ASE 350) (Giri et al., 2015). Extracts were then base
266 saponified and converted to trimethylsilyl (TMS) esters and ethers. Lipids were identified and
267 quantified (GC-MS/FID) (Giri et al., 2015) using internal standards, a calibration mix of known
268 polar analytes, mass spectra, and retention times and grouped into four classes: alkanoic acids
269 (FA), alkanes, alkanols, or sterols (SI Appendix 1). In addition to soil lipids, corn and red clover
270 above and below ground tissue (n =1) lipids were also extracted, characterized, and quantified in
271 order to assist in the interpretation of potential soil lipids originating from plant tissue.
272 *2.8 Soil C pool concentrations and ^{13}C retention*
273 The PVC cores with the ^{13}C label were removed from the field at 1, 6, and 12 mo after
274 ^{13}C glucose and phenol additions and brought back to the lab for analysis of ^{13}C-MBC and ^{13}C-

SOC concentrations in different soil fractions (SI Appendix 1). Soil MBC was measured using the chloroform-fumigation extraction method (Vance et al., 1987) and the amount of recovered ^{13}C glucose or phenol into MBC and SOC fractions was determined using the same equation applied for CUE substrate incorporation.

The substrate ^{13}C in SOC fractions was quantified for bulk soil and the more stable aggregate clay fractions to determine the retention of new C inputs in SOC pools. We determined the amount of ^{13}C retained in bulk soil for all time periods and at 1 and 12 mo for the clay+silt intra-aggregate SOC pools. We used physical fractionation to isolate the soil clay+silt macroaggregate ($M_a$clay) and the clay+silt microaggregate ($M_i$clay) fractions to target substrate retention in the more stable microbial-derived SOC pools (Courtier-Murias et al., 2013; Spielvogel et al., 2008). Approximately 100 g air-dry 8 mm-sieved soil was mechanically wet-sieved through a 250 and then a 53 µm sieve to capture macroaggregates (>250 µm), microaggregates (53-250 µm), and free clay and fine silt not associated with aggregates (<53 µm). Aggregate fractions were then sonicated and passed through a <53 µm sieve to isolate the intra-aggregate clay+silt fractions for determining ^{13}C retained in mineral-associated soil C pools (SI Appendix 1). The amount of ^{13}C glucose and phenol substrate retained in the individual pools is reported relative to the total amount of substrate added to the soil cores (µg of added ^{13}C g-soil in core).

2.9 Data analyses

Data were analyzed as a complete randomized block design with six replicates. A linear mixed-model one-way analysis of variance (ANOVA) was used to determine treatment differences among soils collected at the plot-level (i.e. CUE, MGR, and soil chemistry), where replicate was used as a random effect and agricultural management (ORG and CT) was treated as

298 a fixed effect. Data from the ^{13}C soil core experiment were analyzed as a two-way ANOVA with
299 substrate as an additional fixed effect. The effect of agricultural management on lipid
300 abundances and ^{13}C recovery in various C pools was tested separately for each individual
301 biomarker and C pool. Pair-wise comparisons between treatments sampled the same day were
302 determined by Tukey's HSD ($p < 0.05$). We used Pearson's correlation analysis to examine
303 relationships between SOC pools and hypothesized predictors of SOC accumulation, including
304 microbial physiology. All ANOVA and correlation analyses were performed in SAS v.9.3 (SAS
305 Institute, 1999) using PROC MIXED and PROC CORR. Significance for all analyses is
306 determined at a probability level of $p < 0.05$ unless otherwise stated.
307 We also evaluated PLFA community data using non-metric multidimensional scaling
308 (NMDS) in PC-ORD; version 4.14 (McCune and Mefford, 1999). The Sorensen (Bray-Curtis)
309 index was used as a distance measure among treatment PLFA relative abundances and
310 relativized ^{13}C PLFA. Final NMDS solutions were considered acceptable if Monte Carlo
311 simulations had stress values <20 and a solution stability of <0.005 (McCune and Grace, 2002).
312 Following NMDS, we determined significant PLFA groupings by agricultural management and
313 ^{13}C-substrate with a multi-response permutation procedure (MRPP) calculated using a Sorensen
314 (Bray-Curtis) dissimilarity matrix. PLFA groupings between management and between substrate
315 treatments, using MRPP, were considered significant if $p < 0.05$. The magnitude of difference
316 between groups were evaluated with the MRPP effect size (*A*)- a descriptor of within-group
317 homogeneity compared to the random expectation where observations within a group are
318 identical when A=1, and the *T* test statistic, where a more negative *T* suggests a stronger
319 separation (McCune and Grace, 2002).
320 **3. Results**

3.1 Microbial community growth traits

Organic management was associated with significantly higher community-level CUE as well as MGR relative to conventional management (Fig. 1). CUE was 25%, 35%, and 86% higher in ORG relative to CT during peak clover biomass production (Sept 2013), following clover incorporation (Jun 2014), and at corn maturity (Sept 2014), respectively. CUE was highest in both ORG (55%) and CT (47%) on Sept 2013. MGR was also generally higher in ORG relative to CT, being 45% higher on July 2013 and 67% on June 2013 (Fig. 1). MGR was greatest in both treatments on July 2013 during wheat maturity. On Sept 2014, ORG and CT had a similar MGR when the CUE difference between ORG and CT was greatest.

3.2 Microbial community structure and activity

The ORG treatment had higher PLFA absolute concentrations for all biomarkers relative to CT. However, differences in the relative abundances were only noted for 5 of the 17 group-specific PLFAs (SI Fig. 2) and no differences in the metabolically active community between ORG and CT were detected (Fig. 2, SI Fig. 2). There was also no management effect on the fungal:bacterial ratio. NMDS showed some differences in total community composition between ORG and CT, though the spatial separation by PLFA relative abundances was not robust (Fig. 2) based on MRPP (effect size, *A*, of 0.091; p =0.11). We did not observe any significant groupings in the metabolically active community between ORG and CT based on the NMDS of relative ^{13}C label distribution of either glucose or phenol into the different microbial PLFA groups (Fig. 2) (MRPP p =0.283). There was, however, a strong separation in the active microbial community between substrates, where microbial incorporation of glucose-C was more associated with G+ bacteria and fungi, while phenol uptake was more associated with G- bacteria (Fig. 2).

3.3 Substrate retention in SOC pools

We traced the recovery of ^{13}C-labeled glucose and phenol into MBC and bulk soil at 1, 6 and 12 months following field substrate additions and into the clay+silt fraction within micro ($M_i$clay) and macroaggregates ($M_a$clay) at 1 and 12 mo. Enrichment of ^{13}C was detected for all the SOC pools measured over the 1-yr sampling period for all treatments. ORG had initially greater incorporation of both ^{13}C glucose and ^{13}C phenol into MBC relative to CT (Fig. 3). However, 6 mo following field label additions there was no difference between ORG and CT in ^{13}C glucose recovered in MBC. The ^{13}C phenol incorporations rates were consistently lower relative to glucose, with ORG maintaining higher uptake into MBC compared to CT up to 6 mo after substrate additions. As expected, substrate-C recovery in MBC declined over time. Recovery of ^{13}C glucose in MBC after 1 and 6 mo was 16% and 11%, respectively, but uptake dropped significantly after 12 mo to 2.3%. The ^{13}C phenol recovery in MBC was initially 3%, with significant declines in recovered ^{13}C phenol from 6 (2%) to 12 mo (0.75%).

The amount of glucose-C retained in bulk soil was similar between ORG and CT for all time periods (Fig. 3). Bulk soil glucose-C retention in both management treatments went from an initial 55% to a final 23% retained after 12 mo, corresponding to roughly a 75% loss of the initial glucose-C added. The amount of phenol-C retained in bulk soil was generally greater in ORG, with treatment differences increasing over time. At 6 mo, phenol-C retention in ORG was marginally higher than CT ($p = 0.09$) and at 12 mo was significantly higher in ORG (36%) compared to CT (28%) ($p < 0.05$). When comparing across substrates, the final substrate retention at 12 mo between glucose- and phenol-C in bulk soil were similar in CT but within ORG more phenol-C (36%) was retained than glucose-C.

Within the clay+silt SOC pools, the majority of substrate C was retained in the macroaggregate fraction ($M_a$clay) after 1 and 12 mo (SI Fig. 3). The $M_a$clay fraction was the

largest fraction by mass weight and was also where the greatest proportional difference in SOC occurred between ORG and CT (SI Fig. 3). The retention of glucose-C in $M_a$clay parallels the MBC recovery (Fig. 3). After 1 mo, more glucose-C was retained in the ORG $M_a$clay pool (27%) compared to CT (19%), though no management effect was detected after 12 mo. For phenol, the trend for $M_a$clay substrate retention was reversed. Phenol-C retained in $M_a$clay was similar between ORG and CT at 1 mo but diverged by 12 mo, with greater retention in ORG (25%) compared to CT (18%).

3.4 Soil microbial biomass and chemistry

Soil MBC and PLFAs, generally thought to represent the living MB pool, were both greater in ORG relative to CT by 69% and 95%, respectively (Table 2) and total solvent extractable (TSE) lipids were greater by 52%. There was a trend ($p < 0.1$) towards treatment differences in total lipid relative abundance determined by py-GC/MS. ORG soils also had a greater relative abundance of nitrogen compounds, especially protein-derived compounds, and a lower abundance of aromatics. No treatment effects were detected for lignin-derived compounds, phenols, or polysaccharide relative abundances (SI Table 2).

ORG bulk soils had higher TSE lipid concentrations for 24 of the 46 soil lipids identified compared to CT (SI Table 3). Overall microbial-specific lipid recovery was low, though α-linolenic acid (α-C18:3), a notable fungal lipid, and squalene, a precursor to ergosterol (Toh et al., 2001; Amir et al., 2008), were greater in ORG (SI Table 3) and neither were recovered in plant samples. Although α-C18:2, greater in CT, is found in fungi, we also found that it was the most abundant lipid in both corn and clover tissue (>50%) and thus is not a microbial-specific biomarker (Fig. 4). We used the lipids that had >1% relative abundances in corn tissue (both above and belowground plant parts) and compared them to their relative abundances in ORG and

CT soil (Fig. 4). Generally, the abundant corn lipids were either similar between ORG and CT or greater in CT. Of the 18 abundant plant lipids, 2 (phytol and β-sitosterol) were greater in ORG compared to CT, both of which were also dominant in clover tissue.

3.5 Relationships between soil chemistry, physiology and SOC

In order to explore which variables might best predict SOC, we regressed the SOC fractions showing significant treatment differences (bulk SOC, ^{13}C phenol in bulk soil, and ^{13}C phenol in the more stable $M_a$clay fraction) with microbial physiology, activity, and soil chemistry (Fig. 5; SI Table 4). We found that many of the significant variables that correlated with bulk SOC were similarly strong predictors for ^{13}C phenol retention in $M_a$clay and bulk soil ^{13}C phenol. These three SOC pools also exhibited strong correlations with each other (Fig. 5). The variable exhibiting the strongest relationship with all three SOC pools was the relative abundance of dominant plant extractable lipids, negatively associated with SOC (r = -0.94 to -0.75). CUE, total PLFA concentration, relative abundances of N compounds and microbial lipids were positively correlated with SOC, while aromatic compounds were negatively associated with SOC. There was also a significant positive relationship between microbial activity (MBC^{13}C phenol) one month after substrate additions and ^{13}C phenol retained in bulk SOC 12 mo after substrate additions.

4. Discussion

Based on evidence that microbial necromass is an important constituent of stable SOM, physiological processes that regulate the formation of necromass should influence SOC dynamics (Cotrufo et al., 2013; Wieder et al., 2014; Xu et al., 2014). Our experimental results demonstrate strong relationships between agricultural management, microbial physiology, and the conversion of new C inputs into SOC. More specifically, we show that organic management

supports microbial communities with distinct physiologies, which are associated with more rapid incorporation of new C inputs into MB and greater retention of new C inputs in the clay fraction of soil macroaggregates. Our results provide a potential mechanism to explain the unexpected relationships between plant C inputs and soil C accumulation observed at KBS and other sites (e.g. Drinkwater et al., 1998; Gregorich et al., 2001; Marriott and Wander, 2006).

4.1 Hypothesis 1: Management affects microbial physiology

The organic cropping system supported higher concentrations of MBC and PLFAs and a microbial community that exhibited higher CUE and MGR compared to conventional management. Organic, legume-based cropping systems typically have higher MB, often attributed to higher C inputs via compost and manure additions (Kallenbach and Grandy, 2011) or additional plant inputs provided by a cover crop. Yet, at KBS the cover cropped-ORG treatment does not have additional C amendments and annual above and below ground inputs from cover crops are not enough to exceed the higher biomass productivity in CT (Table 1). Thus, the higher MB concentrations may instead be a result of changes in the quality, timing and diversity of inputs from the addition of a legume cover crop rather than from an increase in C inputs (McDaniel et al., 2013).

The higher CUE and MGR we observed under organic management might arise from a shift in microbial community structure or from shifts in the intrinsic physiology of the existing community. While our results suggest strong effects of management on microbial physiology, differences in community composition at coarse taxonomic resolution were minor (Fig. 2). It is possible that our broad PLFA biomarkers did not capture more nuanced differences in microbial relative abundances and we did detect some differences in the relative abundances of G+ and G-

435 biomarkers. However, a recent study at these sites found similar functional gene diversity
436 between ORG and CT (Xue et al., 2013).
437 Moreover, microbes possess trade-offs in life history traits of efficient and rapid growth
438 (Maharjan et al., 2007; Beardmore et al., 2011). As such, we would expect to see trade-offs
439 between CUE and MGR if ORG or CT selected for microbial community structures with distinct
440 life-history traits, constraining the degree to which microbial communities in either ORG or CT
441 can maximize biomass production. Yet we did not observe lower MGR in tandem with higher
442 CUE or vice versa in ORG relative to CT for the time periods we sampled. This, in combination
443 with only slight differences in community PLFA composition, suggests that the observed
444 differences in physiology may be occurring from changes in community metabolism, per se, and
445 less so due to microbial communities with distinct life-history traits. Thus, while we cannot rule
446 out the possibility that variability in microbial community structure underlies the observed
447 differences in microbial growth, it is also possible that these differences reflect physiological
448 shifts within the existing communities (Evans and Wallenstein, 2014; Hargreaves and
449 Hofmockel, 2014), making it possible to optimize both CUE and MGR in ORG.
450 *4.2 Hypothesis 2: Differences in microbial physiology influence the fate of C inputs*
451 Consistent with previous arguments (Allison et al., 2010), we found that higher MBC,
452 CUE and MGR in ORG corresponded with higher extracellular enzyme activities (SI Fig. 4).
453 Given the central role of enzymes in decomposition, and close relationships between enzyme
454 activities and decomposition rates (Sinsabaugh et al., 1991; Rinkes et al., 2013), how can these
455 differences in microbial physiology and activity enhance the persistence of new C inputs in soil?
456 One answer rests with our knowledge that under most temperate and tropical systems plant litter will
457 eventually decompose, and even lignin-rich materials can decompose within a year (Kleber et al., 2011).
458 In contrast, microbial necromass seems to persist due to strong physiochemical interactions within the soil

459 matrix (Dungait et al., 2012). Thus despite increases in extracellular enzyme activity, diverting more plant
460 C into MB might ultimately – over time - result in greater stable soil C accumulation (Cotrufo et al.
461 2013).

462 The higher CUE and MGR we observed in ORG relative to CT (H_1) should minimize
463 respiratory plant-C losses and maximize the rate at which new C inputs are converted into MB,
464 increasing both the amount and rate at which microbial-derived inputs accumulate in the stable
465 SOC pool (H_2). Using *in situ* ^{13}C substrate additions to test this, we expected that ^{13}C substrate
466 recovery in SOC would be faster and retention in SOC pools would be proportionally greater in
467 ORG. Consistent with this, ORG exhibited greater glucose- and phenol-C incorporation into MB,
468 and faster rates of substrate recovery in SOC pools, but only phenol-derived C persisted longer in
469 ORG compared to CT. While unexpected, these interactive effects of agricultural management
470 and substrate quality on substrate assimilation and retention may reflect broad treatment
471 differences in microbial community physiology, and could lead to variations in the temporal
472 dynamics of substrate cycling among SOC pools.

473 The influence of physiology on the fate of glucose should be greatest during periods of
474 active substrate uptake, expected to occur soon after glucose incorporation. Consistent with this,
475 ^{13}C-glucose recovery in MB and $M_a$clay was higher in ORG compared to CT after one month
476 (Fig. 3). Further, more glucose-C was recovered at one month within the $M_a$clay fraction
477 compared to the MBC pool. While the $M_a$clay fraction includes living MBC, this suggests that
478 much of the labeled MBC had already been incorporated into mineral-associated SOC (Fig. 3).
479 However, these differences in MB and $M_a$clay glucose-C retention between ORG and CT did not
480 persist for the entire 12 mo experiment. This might occur if the initially higher label recovery in
481 MB and $M_a$clay in ORG was driven by MGR and MB abundance and less so by CUE. We
482 estimated differences in CUE in the lab using standard approaches with glutamic acid. However,

with glucose, assimilated rapidly by most microbes, CUE differences among microbial communities are not always apparent (Frey et al., 2013), suggesting that differences in MGE between ORG and CT may not be important for long-term glucose-SOC formation dynamics. A higher MGR or an overall higher MB in ORG could increase the rate of microbial ^{13}C glucose uptake and ^{13}C MBC turnover and stabilization in $M_a$clay, but rate-driven differences in ^{13}C glucose allocation and retention among pools alone would not persist long-term in the absence of glucose-CUE variability. However, in our study we followed the fate of only one glucose addition, limiting our ability to capture the iterative process of MB turnover, while, in reality, soil microbes are exposed to repeated inputs of low molecular weight compounds (van Hees et al., 2005). Under frequent C inputs, the manifestation of a distinct MGR between communities may be maintained, sustaining a divergence in the rate of microbial-derived inputs over a longer time period.

Monomeric phenol is also labile, but compared to glucose, has a lower energy yield, and likely selects for a narrower group of decomposers, which would slow incorporation rates and drive greater variability in CUE (Brant et al., 2006; Frey et al., 2013). Indeed, phenol-C was taken up more slowly than glucose-C and ORG exhibited greater MBC enrichment compared to CT even after 6 mo. After 6 and 12 mo, greater microbial utilization of phenol-C in ORG is associated with a greater proportion of phenol persisting in the $M_a$clay fraction in ORG compared to CT. Thus, the community-level differences in CUE, as well as the slight differences in community structure, are therefore more likely having a stronger effect on incorporation rates in phenol- vs. glucose-treated soils. Furthermore, with overall slower incorporation rates of phenol-C, MGR and the temporal dynamics of MBC turnover become important factors in maintaining notable differences in ^{13}C retention throughout the 12 mo. The temporal aspect of

506 microbial C uptake and turnover, which differs by substrate quality, is, therefore, an important
507 consideration in understanding the influence of microbial physiology on the accumulation of
508 microbial-derived inputs to SOC.

509 *4.3 Hypothesis 3: Microbial-associated compounds are more abundant in ORG*

510 We assessed relationships between SOC chemistry, particularly microbial-associated
511 compounds such as lipids and proteins, microbial physiology and agricultural management.
512 Proteins are a major constituent of microbial intracellular materials, and our evidence shows
513 protein enrichment in ORG, probably from adsorption of proteins on clay surfaces (Kleber et al.,
514 2007). Microbial inputs likely account for the higher relative abundance of protein and lipid
515 concentrations we observed in ORG, yet given the difficulty in resolving the origin of specific
516 SOC molecules, enhanced stabilization of unprocessed plant inputs in ORG is also possible.
517 However, edaphic properties that influence stabilization of plant derived-C are similar or
518 identical between treatments, and greater cultivation in ORG would likely reduce stabilization of
519 plant inputs (Grandy and Robertson, 2007). While legume cover crops are relatively high in
520 proteins, they decompose quickly (Kuo et al., 1997), especially in N-limited organic systems,
521 and polysaccharides, plant-derived aromatics and lignin were not greater in ORG.

522 Providing further evidence that SOC accumulation in ORG is microbial rather than plant-
523 derived, the most dominant plant-extracted lipids were generally similar or lower in ORG
524 compared to CT soils (Fig. 4). Our low recovery of commonly used microbial biomarkers, such
525 as short chain fatty acids (<C16) (Otto et al., 2005), may be because microbial lipid
526 concentrations tend to be higher in the ester-bound lipid pool (Kögel-Knabner, 2000), not
527 obtained by our solvent extraction. However, recovery of the fungal biomarkers squalene and α-
528 linolenic (C18:3) acid (Frostegård and Bååth, 1996; Toh et al., 2001; Amir et al., 2008), not

detected in the plant tissue lipids, were greater in ORG. The apparent lack of preferential plant lipid accumulation in ORG, the greater concentrations of fungal biomarkers, and overall higher lipid and protein abundance in ORG soil are characteristics of SOC chemistry with greater relative inputs from microbial necromass (Grandy and Neff, 2008) and are typical in the most stable SOC pools (Grandy et al., 2007; Bol et al., 2009; Kleber et al., 2011; Tamura and Tharayil, 2014). Moreover, our correlations demonstrate important relationships between microbial physiology, biomass production, labile soil compounds and SOC accumulation.

5.4 Synthesis and Conclusions

Prior studies have reported the same paradox we do here—namely that SOC concentrations do not always appear to be related to soil C inputs or the degree of soil disturbance (Campbell et al., 1991; Drinkwater et al., 1998; Gill et al., 2002; Gregorich et al., 2001). Changes in soil aggregation and preferential retention of root inputs (Dabney et al., 2007; Grandy and Robertson, 2007; Kätterer et al., 2011) have been speculated as potential explanations for this finding. However, at KBS, aggregate turnover is likely faster in ORG given that it has more intensive tillage, and we saw no difference in aggregate size distribution between ORG and CT (SI Fig. 2). Preferential root-C retention in ORG at KBS is a possibility, but our lipid quantification results do not provide strong evidence for this as a significant mechanism. Instead, we examined a more robust mechanism based on emerging theories of SOM formation, where changes in microbial physiology and microbial inputs explain SOC accumulation in ORG.

Differences in fertilization, pesticide applications, or other management factors may explain variations in microbial physiology between ORG and CT (Lee and Schmidt, 2014), but we believe the most parsimonious explanation is the use of cover crops, especially N-fixing leguminous species that increase plant input quality, diversity and input frequency. Cover crops

with a low C:N ratio provide nutrient and energy rich substrates compared to corn and wheat residues, favoring higher community-level CUE and MGR. This additional C source also increases the chemical diversity of available organic matter, potentially facilitating more efficient and rapid microbial growth on crop residues and native SOM via cometabolism (McDaniel et al., 2014). Further, cover crops reduce the duration that microbes experience C-limitation and extend the time microbes are actively growing (Blagodatskaya et al., 2014), thus reducing proportional C losses to cell maintenance (Chapman and Gray, 1986; Sinsabaugh et al., 2013). Our study demonstrates that microbial physiology is directly influenced by agricultural management and is closely linked to SOC cycling and storage. We show that the efficiency and rate at which new C inputs are utilized by soil microbes to build MB and subsequent necromass may be a potential mechanism of SOC accumulation that can counterbalance limitations in input quantity. Such a mechanism would provide the foundation for new approaches in SOC management that are less dependent on C input quantity and instead are aimed at influencing microbial physiology.

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Tables

Table 1. Soil C (SOC) concentrations and total plant inputs in conventional (CT) and organic (ORG) treatments. Total ANPP is the mean annual net primary productivity for each crop rotation (soybean, wheat, and corn) and above and below ground clover cover crop biomass estimates. Conventional and Organic total ANPP are significantly different within a crop rotation if letters are different ($p < 0.05$).

	SOC[§]	ANPP[¥]					
	mg C g^{-1}	g dry biomass yr^{-1}					
Conventional	8.12[a]	Soybean	458	Wheat	640	Maize	1509
		Total	458[a]	Total	640[a]	Total	1509[a]
Organic	10.25[b]	Soybean	413	Wheat	447	Maize	1103
				Clover[‡]	212	Clover	236
		Total	413[a]	Total	659[a]	Total	1339[b]

[¥]Data is from http://lter.kbs.msu.edu/datatables. The ANPP is a 3-yr mean of the crop plus weed biomass for a specific crop rotation (accessed April 2015). For example, ANPP for soybean is the mean for the three most recent years 2006, 2009, 2012; [§]values are from current study; [‡]Clover inputs are sum of above ground and below ground, where below ground root inputs are estimated based on clover shoot: root ratio of 2.5.

875 Table 2. Microbial biomass C (MBC), total PLFA biomarkers, total lipid concentrations, and
876 lipid and N-compound relative abundances between conventional (CT) and organic (ORG)
877 management.
878

	MBC	Total PLFA	Total extractable lipids‡	Lipids¥	N-compounds¥
	µg MBC g C^{-1}	µg MBC g C^{-1}	mg lipids g C^{-1}	% relative abundance	
Conventional	1.37	0.13	1.83	4.14	32.6
Organic	2.32	0.25	2.78	5.72	37.64
P value	<0.05	<0.01	<0.05	<0.1	<0.05

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880 ‡ As quantified by GC-MS/FID; ¥ As determined by py-GC/MS.
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894 **Figure Captions**

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896 **Figure 1.** Microbial carbon use efficiency (CUE) (a) and growth rate (MGR) (b) between
897 conventional (CT) and organic (ORG) treatments for different sampling dates. Significant
898 differences ($p < 0.05$) between treatments within dates are designated with an (*).

899 **Figure 2.** Non-metric multidimensional scaling (NMDS) ordination of total microbial
900 community composition as measured by PLFA analysis for conventional (CT) and organic
901 (ORG) management (a) and the metabolically active microbial community as determined by ^{13}C
902 incorporation into PLFAs (b) for CT (open symbols) and ORG (close symbols) and glucose
903 (circles) and phenol (diamonds). Table insets are Pearson correlation *r* values with ordination
904 axes. Biomarker G+ is the sum of gram positive bacteria; G- is the sum of gram negative
905 bacteria; AMF is arbuscular mycorrhizal fungi (16:1ω5c), and Actino is actinomycetes (10ME
906 18:0). Panel (a) MRPP effect size (*A*) =0.091, *T* = -2.89 (p =0.1); Stress = 11, Monte Carlo p
907 =0.046. Panel (b) MRPP by agriculture management: *A*= 0.0052, *T*= -0.17 (p = 0.283); MRPP by
908 substrate: *A* =0.0418, p <0.0001, *T*= -14; Stress =7.96, Monte Carlo p =0.043.

909 **Figure 3.** The percent recovery of total ^{13}C glucose in microbial biomass carbon (MBC) (a), bulk
910 soil (b), the clay + silt macroaggregate C pool and the % recovery of total ^{13}C phenol into MBC
911 (d), bulk soil carbon (e), and the clay + silt macroaggregate C pool (f). Significance between
912 conventional (CT) and organic (ORG) at $p < 0.05$ is denoted by (*) and $p < 0.1$ by (¥). Error bars
913 are standard errors ($n = 6$).

914 **Figure 4.** The relative abundances of soil (a) and plant (b) lipids. Corn lipids are the sum of corn
915 stalk and root tissue, and clover lipids are the sum of clover leaf, stem and root tissue. Soil lipids
916 shown are those that represent >1% relative abundance in corn tissue. Plant extracted lipids

unique to roots are indicated by (®). Significant differences between conventional (CT) and organic (ORG) soil lipids are denoted by (*), $p < 0.05$. Soil lipids $n = 6$, se; Plant lipids $n = 1$.

Figure 5. Regressions of microbial physiology (CUE), soil chemistry and SOC pools. Panel (e) is the % ^{13}C phenol retained in bulk soil and macroaggregates 12 mo after substrate additions and (f) is the response of % ^{13}C phenol retained in bulk soil after 12 mo to the % recovered in microbial biomass C one mo after substrate additions. Plant-derived lipid relative abundances are α-linoleic acid and microbial-derived lipid relative abundances are the sum of squalene and α-linolenic acid.

Figure 1

Figure 1.

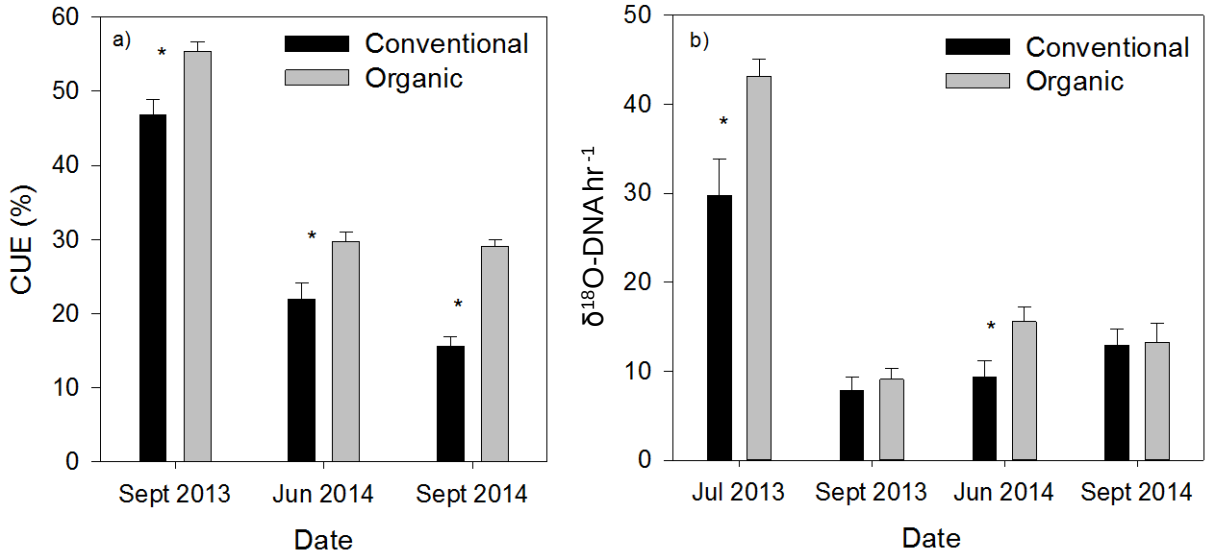

Figure 2

Figure 2.

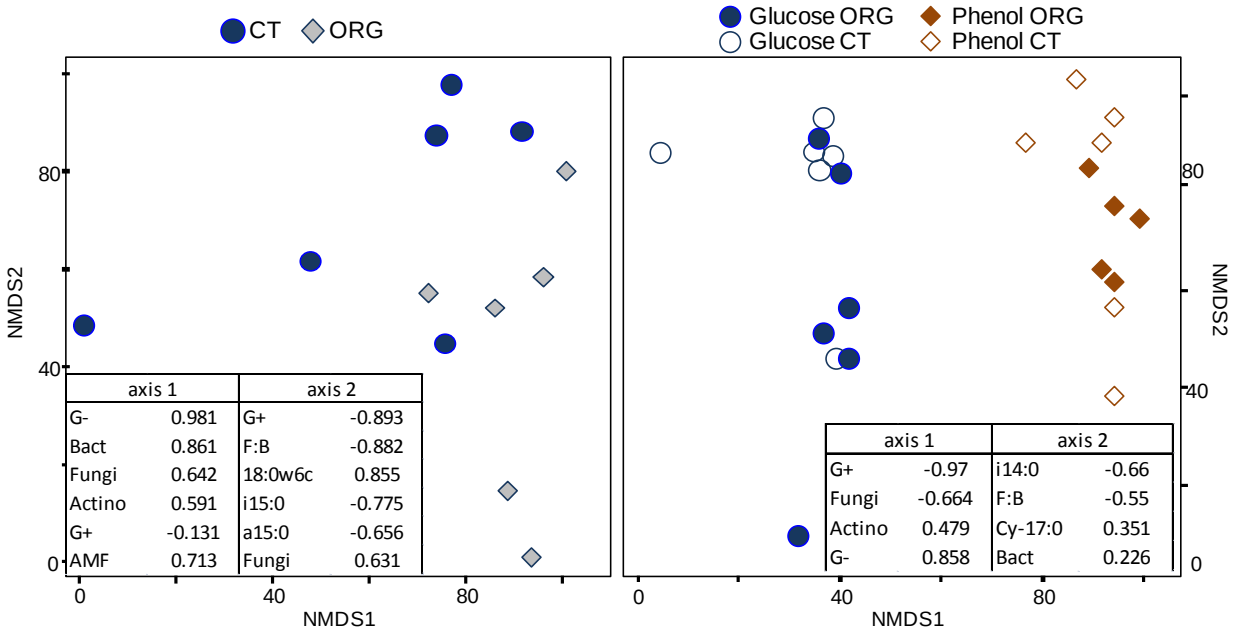

Figure 3

Figure 3

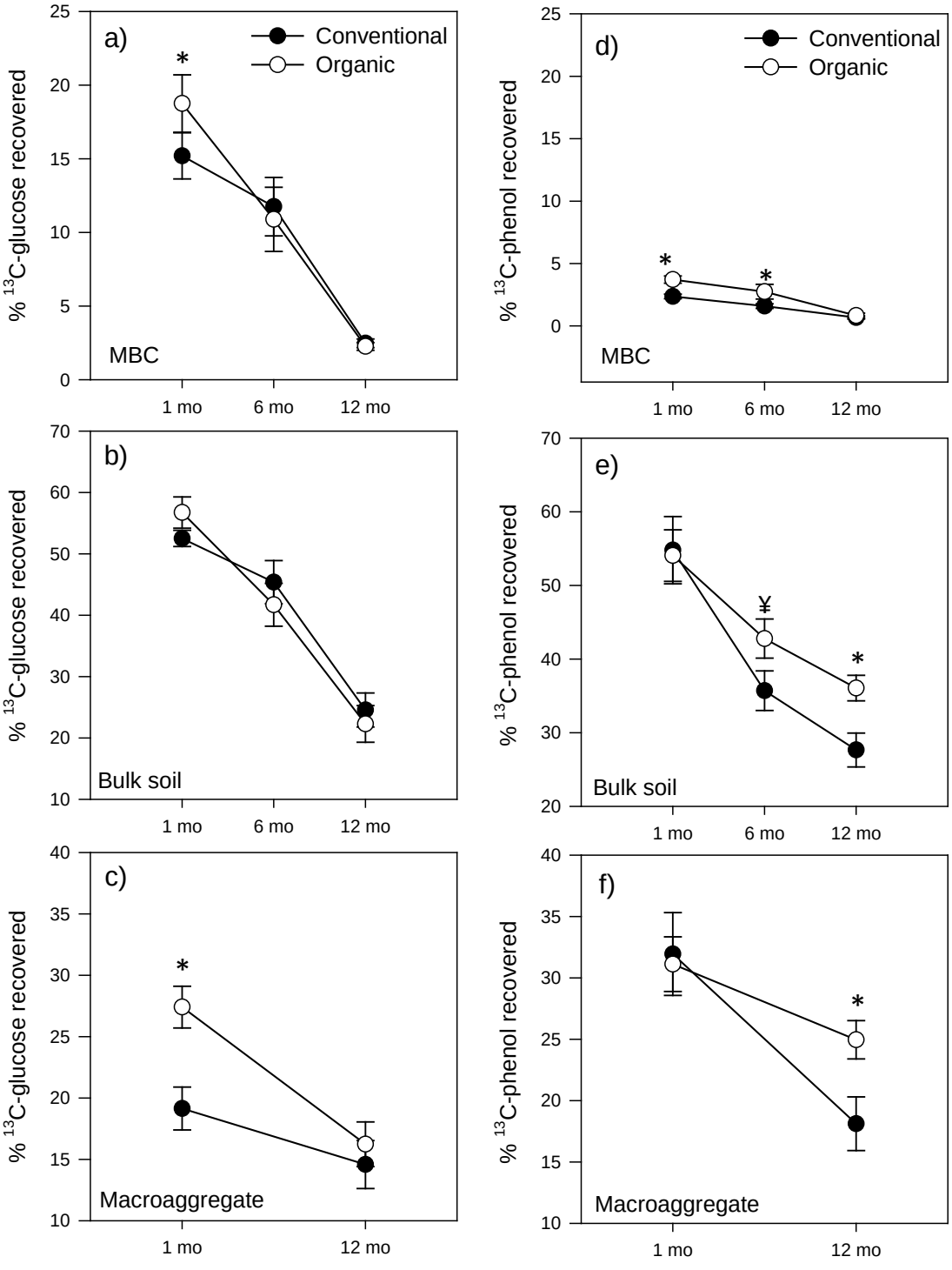

Figure 4

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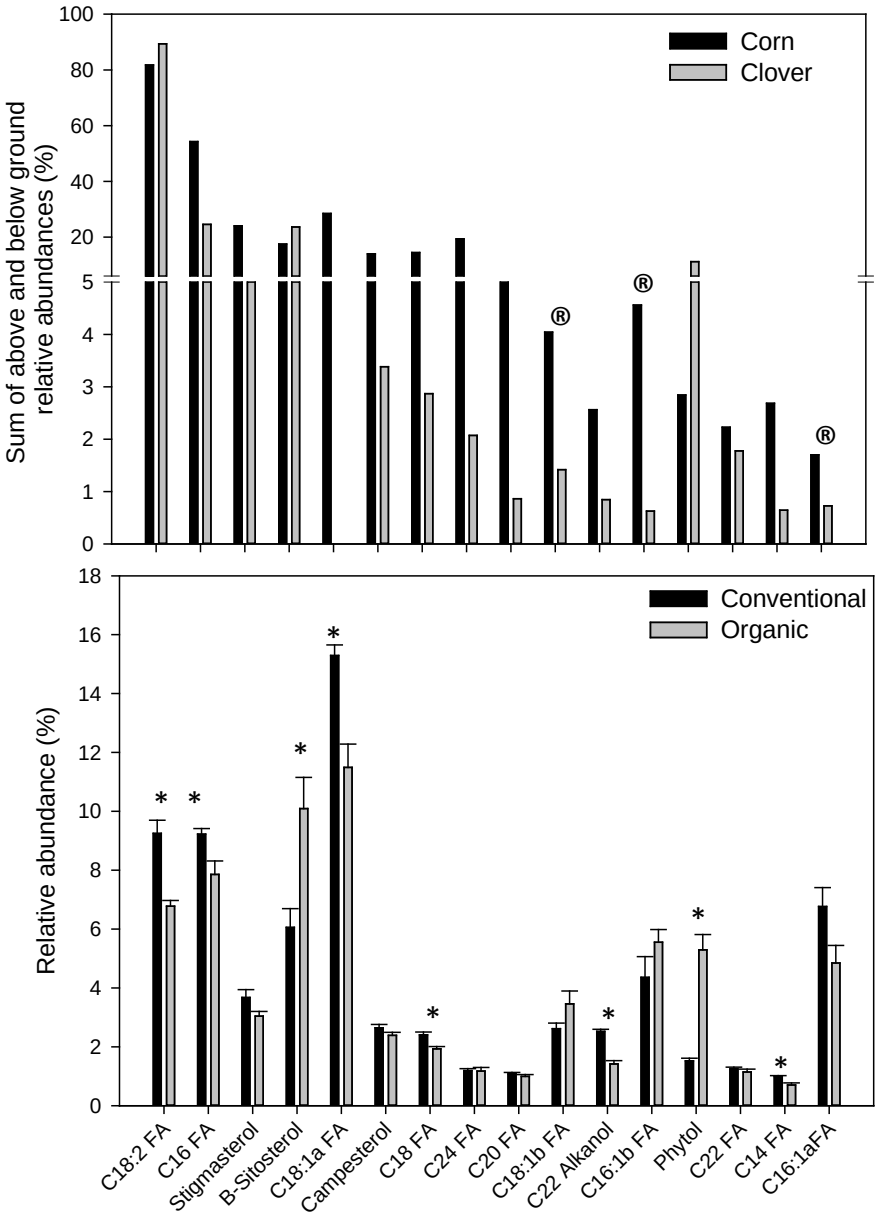

Figure 5

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